International Journal of Food Microbiology 337 (2021) 108914

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Variability in lag duration of *Listeria monocytogenes* strains in half Fraser enrichment broth after stress affects the detection efficacy using the ISO 11290-1 method

Jasper W. Bannenberg, Tjakko Abee, Marcel H. Zwietering, Heidy M.W. den Besten *

Wageningen University & Research, Food Microbiology, P.O. Box 17, 6700 AA Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Listeria monocytogenes
Half Fraser broth
Enrichment
ISO 11290-1:2017
Detection

ABSTRACT

A collection of 23 *Listeria monocytogenes* strains of clinical and food origin was tested for their ability to recover and grow out in half Fraser enrichment broth following the ISO 11290-1:2017 protocol. Recovery of sub-lethally heat-injured cells in half Fraser broth was compared to reference cells with no stress pre-treatment. The enrichments were followed over time by plate counts and the growth parameters were estimated with the 3-phase model which described the data best. The reference cells without stress pre-treatment showed a short lag duration, which ranged from 1.4 to 2.7 h. However, significant variation in the ability to recover after 60 °C heat stress was observed among the tested strains and resulted in a lag duration from 4.7 to 15.8 h. A subset of strains was also exposed to low-temperature acid stress, and the lag duration showed to be also stress dependent. Scenario analyses and Monte Carlo simulations were carried out using the growth parameters obtained in the enrichments. This demonstrated that when starting with one cell, the detection threshold for efficient transfer of at least one cell to the secondary enrichment step, i.e. 2 $\log_{10}$ CFU/ml, was not reached by 11 of 23 strains tested (48%) after exposure to 60 °C heat stress. Increasing the incubation time from 24 to 26 h and the transfer volume from 0.1 to 1.0 ml can increase the average probability to transfer at least one cell to the secondary enrichment step from 79.9% to 99.0%. When optimizing enrichment procedures, it is crucial to take strain variability into account as this can have a significant impact on the detection efficacy.

1. Introduction

The presence of *Listeria monocytogenes* in (growth supporting) food products is a risk factor for food safety because of the severity of illness that can be caused in vulnerable individuals combined with its ability to grow at refrigeration temperatures. The food safety risk is likely to increase with the rising popularity of ready-to-eat products where no heating step is applied before consumption (Lianou and Sofos, 2007). The European Union laid down a criterion for absence testing in five samples of 25-g portions of ready-to-eat food that can support growth of *L. monocytogenes* after the production stage (European Commission, 2005). For this purpose, standardized microbiological procedures and guidelines have been established so that governments and the food industry can routinely test food samples for the presence of *L. monocytogenes*. In the European Union the analytical reference method is the ISO 11290-1 enrichment protocol for the detection and enumeration of *L. monocytogenes* (International Organization for Standardization, 2017). Testing for pathogen presence is established by culture-based standardized enrichments to allow recovery and an increase in the initial low concentrations of pathogen followed by detection with culturing dependent- or molecular methods. Notably, enrichment media composition should be designed such, that conditions are optimal to support damage repair and growth initiation of potential sublethally injured cells, while at the same time suppressing the growth of competing background microbiota (Dailey et al., 2014; Dailey et al., 2015; Ottesen et al., 2016; Zitz et al., 2011). These factors complicate the enrichment steps that are necessary to amplify the pathogen concentration to higher levels in order to support adequate detection.

The current ISO 11290-1:2017 protocol for the enrichment of *L. monocytogenes* from food products (International Organization for Standardization, 2017) consists of a 24-h enrichment in half Fraser broth followed by a secondary enrichment in full Fraser broth for 24 h with streaking on selective ALOA-plates and another selective medium of choice for 48 h after both enrichment steps. Afterwards, suspect colonies

* Corresponding author.
E-mail address: heidy.denbesten@wur.nl (H.M.W. den Besten).

https://doi.org/10.1016/j.ijfoodmicro.2020.108914
Received 18 May 2020; Received in revised form 5 October 2020; Accepted 6 October 2020
Available online 20 October 2020
0168-1605/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

have to be tested with confirmation reactions. The first enrichment step has to facilitate the recovery of sublethally injured cells that can be present in the product. Hence, the primary enrichment medium contains only half the concentrations of the selective compounds acriflavine and nalidixic acid (International Organization for Standardization, 2017). This is followed by the secondary enrichment with full-strength Fraser broth containing two-fold higher concentrations of selective compounds. These culture-based methods are time-consuming with detection taking up to 5 days while there is still the possibility of *L. monocytogenes* cells not growing out and giving false-negative results (Gnanou Besse et al., 2016). Furthermore, there can be large differences among different strains of *L. monocytogenes* that can complicate detection. For example, it has been shown that there is a strain bias during selective enrichment of *L. monocytogenes* strains from ham-slices (Zilelidou et al., 2016a). This strain bias has been shown for different enrichment methods (Bruhn et al., 2005; Gorski et al., 2006; Zilelidou et al., 2016b), but none quantified or prioritized the importance of strain variability and variability introduced when experiments are independently reproduced. Therefore, the objective of this research was to quantify the strain variability of *L. monocytogenes* in recovery following the current ISO 11290-1:2017 enrichment protocol. For this, the recovery of 23 outbreak-related strains of *L. monocytogenes* was assessed after heat stress treatment and after acid stress treatment at low temperature. Also, all experiments were independently reproduced in order to quantify and compare the effects of biological diversity and strain diversity on detection efficacy.

2. Material and methods

2.1. Bacterial strains and growth conditions

The set of 23 strains of *L. monocytogenes* from different isolation sources and serotypes (Table S1) was kept at −80 °C in brain heart infusion (BHI) broth (Becton Dickinson Difco) supplemented with 30% glycerol (Fluka). Cultures were made by inoculating 10 ml of BHI broth with a single colony from a BHI agar plate (1.5% agar, Oxoid) obtained from −80 °C freezer stocks. Cultures were grown statically at 30 °C for 16 h to obtain stationary phase cultures. These cultures were subsequently diluted 1:1000 in fresh BHI broth and incubated at 30 °C for 16 h to obtain a standardized working culture for use in further experiments.

2.2. Stress treatment of cells

Working cultures of all strains were stress-treated to reduce the viable counts with one $\log_{10}$ reduction. The D_{60}-values of the strains previously published by Aryani et al. (2015a) were used to determine the heat treatment time for each of the strains. Working cultures were diluted 1:100 in 50 ml BHI broth pre-heated at 60 °C in a water bath (Julabo SW23) for the time of one D_{60}-value reduction (Table S1). Afterwards, the cultures were quickly cooled on ice for 15 s and decimally diluted in Peptone Physiological Salt (PPS) solution (Tritium Microbiology) to obtain an initial concentration of approximately 2 $\log_{10}$ CFU/ml in the enrichment experiments.

To determine the pH for the low-temperature acid stress treatments, the working cultures were stressed in acidified BHI broth at 10 °C for 24 h and samples were taken to determine the viable counts. The BHI broth was acidified with 2.5 M HCl until the desired pH value was reached (MeterLab PHM240 pH/ION meter). For each strain, the pH value that gave one $\log_{10}$ reduction after 24 h based on duplicate experiments was chosen for low-temperature acid stress treatment at 10 °C. This low temperature was chosen to simulate the temperature in the cold food chain. Acid stressed cells were subsequently decimally diluted in PPS to obtain an initial concentration of approximately 2 $\log_{10}$ CFU/ml in the enrichment experiments. The working cultures were afterwards incubated for 24 h at 10 °C in plain BHI for subsequent use in enrichments.

2.3. Enrichment kinetics in half Fraser broth

Enrichments were carried out in half Fraser enrichment broth, which was made by supplementing Fraser broth base (Oxoid) with half Fraser supplement (Oxoid). Irrespective of their pre-treatment, all enrichments were started with an initial inoculum concentration of 2 $\log_{10}$ CFU/ml. For the reference cells, the working cultures (reaching approximately 9 $\log_{10}$ CFU/ml) were decimally diluted in PPS until a concentration of approximately 3 $\log_{10}$ CFU/ml. This culture was diluted again 1:10 in 45 ml half Fraser enrichment broth in 150 ml Schott flasks resulting in an initial inoculum concentration of 2 $\log_{10}$ CFU/ml. After addition of the cells to the enrichment broth (timepoint 0), samples were taken at 2-h intervals for 10 h and at 24 h to investigate kinetics at 30 °C according to the ISO 11290-1:2017. Samples were spread plated on BHI agar plates and incubated at 30 °C for 24 h before counting. In order to measure the concentration at time-points 14 and 16 h, a parallel enrichment was started later in the day and samples were taken the next morning. Three independent biological reproductions were carried out for reference cells and two for stressed cells, and experiments took place on different days.

2.4. Model fitting and statistics

Growth of *L. monocytogenes* strains during primary enrichment for both the reference cells and stressed cells was modelled with the 3-phase model (Buchanan et al., 1997), the modified Gompertz model (Zwietering et al., 1990) and the Baranyi model (Baranyi and Roberts, 1994).

2.4.1. Three-phase linear model

Lag phase:

$$\text{For } t \le \lambda \quad \log_{10}N_t = \log_{10}N_0 \tag{1}$$

Exponential growth phase:

$$\text{For } \lambda < t < t_{max} \quad \log_{10}N_t = \log_{10}N_0 + \frac{\mu}{ln(10)}(t-\lambda) \tag{2}$$

Stationary phase:

$$\text{For } t \ge t_{max} \quad \log_{10}N_t = \log_{10}N_{max} \tag{3}$$

With $\log_{10} N_t$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10} N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10} N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{max} the time when stationary phase is reached (h) and μ the maximum specific growth rate (1/h).

2.4.2. Modified Gompertz model

$$log_{10}\,N(t) = log_{10}\,N_0 + (log_{10}N_{max} - log_{10}N_0)\cdot exp\left(-exp\left[\frac{\frac{\mu}{ln(10)}\cdot e}{log_{10}N_{max} - log_{10}N_0}\cdot(\lambda - t) + 1\right]\right) \tag{4}$$

With $\log_{10}$ $N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10}$ N_0 the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10}$ N_{max} the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{max} the time when stationary phase is reached (h) and μ the maximum specific growth rate (1/h).

2.4.3. Baranyi model

$$log_{10}\,N(t) = log_{10}\,N_0 + \frac{\mu}{ln(10)}\cdot A(t) - \frac{1}{ln(10)}\cdot ln\left[1 + \frac{exp[\mu\cdot A(t)] - 1}{10^{[log_{10}\,N(max) - log_{10}N_0]}}\right] \tag{5}$$

$$A(t) = t + \frac{1}{\mu} \cdot ln\left[exp(-\mu \cdot t) + exp(-\mu \cdot t_{lag}) - exp(-\mu \cdot t - \mu \cdot t_{lag})\right] \quad (6)$$

With $\log_{10} N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10} N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10} N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{max} the time when stationary phase is reached (h) and μ the maximum specific growth rate (1/h).

Best estimates for the model parameters $\log_{10} N_0$, μ and λ were obtained by the least squares regression analysis using Microsoft Excel's Solver add-in. For each reproduction the significance of the parameter fitting was determined by calculating the 95% confidence interval by estimating the standard error using the SolverAid add-in for Microsoft Excel. The model fitting performance of the models was compared according to den Besten et al. (2006), where the mean square error of the model describes the fitting performance as follows:

$$MSE_{Model} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{n}\left(\log_{10}N^{i}_{observed} - \log_{10}N^{i}_{fitted}\right)^2}{n-p} \quad (7)$$

where the mean square error of the model (MSE_{Model}) is calculated as the residual sum of squares (RSS) divided by the degrees of freedom (DF). The RSS is the sum of the squared difference between the observed cell concentration $\log_{10} N_{observed}$ (log CFU/ml) and the fitted values $\log_{10} N_{fitted}$ ($\log_{10}$ CFU/ml) for each model. DF is the number of data points n minus the number of model parameters p.

The MSE_{Model} was calculated for each model for all 23 strains and all conditions. The model that had the lowest MSE_{Model} in most cases was deemed to most adequately describe the data.

2.5. Quantifying biological and strain variabilities

The biological and strain variabilities of the lag phases for reference cells, low-temperature acid stressed cells and 60 °C heat stressed cells was calculated according to the protocol of Aryani et al. (2015b). Here biological variability is defined as the variability between the independent reproductions and strain variability is defined as the variability between the strains in their recovery from stress history.

Biological variability:

$$MSE_{Biological} = \frac{RSS}{DF} = \frac{\sum_{S=1}^{j}\sum_{R=1}^{i}(\lambda_{SR} - \lambda_S)^2}{n-p} \quad (8)$$

where the mean square error is calculated from the residual sum of squares divided by the degrees of freedom. The RSS is the sum of squared differences between λ_{SR} and λ_S, where λ_{SR} is the lag duration (h) obtained after enrichment for each reproduction for a certain strain (for reference cells $i = 3$ reproductions and for heat stress $i = 2$ reproductions) and λ_S is the average lag duration (h) from independent enrichments for each strain (for acid stress $j = 5$ and for heat stress $j = 23$). DF is the number of data points per condition (for reference cells 3 * 23 and for heat stress 2 * 23) minus the number of parameters ($p = 1$ * 23). For low-temperature acid stress this was calculated with five strains and two reproductions.

Strain variability:

$$MSE_{Strain} = \frac{RSS}{DF} = \frac{\sum_{S=1}^{j}\left(\lambda_S - \lambda_{average}\right)^2}{n-p} \quad (9)$$

where λ_S is the average lag duration (h) from three different enrichments for each strain, $\lambda_{average}$ is the average lag duration (h) of all strains for each condition (for reference cells and heat stress $j = 23$ and for acid stress $j = 5$), DF is the number of data points ($n = 23$ for heat and 5 for acid stress) per condition minus the number of parameters ($p = 1$).

An F-test was used to determine statistically significant differences between the mean square error of the biological and strain variability. Data were considered significant with p-values of 0.05 or lower. Furthermore, it was checked that the model choice did not affect the conclusions drawn by calculating the variabilities based on the growth parameter estimates of all three primary models. However, irrespective of the model choice, the significance of the calculated variabilities did not in fact change.

2.6. Modelling the primary enrichment

To model the primary enrichment step in the scenario analysis, a detection threshold after 24 h of 2 $\log_{10}$ CFU/ml was chosen. This concentration was described by Augustin et al. (2016) as the concentration that allows transfer of at least one cell to the secondary enrichment broth with 100% probability.[1] This detection threshold is used for all scenario analyses in this research. For the scenario analysis the starting concentration is one *L. monocytogenes* cell per 25 g food product that is enriched in 225 ml half Fraser broth (−2.4 $\log_{10}$ CFU/ml). The data of the estimated lag durations and maximum specific growth rates of all 23 strains was used to model the growth in the primary enrichment step after stress.

Also, the impact of changes in maximum specific growth rate and lag duration was determined on the ability to reach the 2 $\log_{10}$ CFU/ml detection threshold. For this, Monte Carlo simulations were carried out in Microsoft Excel using the add-in @Risk version 7.5 (Palisade Corporation). The maximum specific growth rates and lag durations were fitted to multiple distributions and the growth parameters were a good fit to the normal-distribution. The normal-distribution with the average and standard deviation was then used for the modelling in @Risk. The probability to transfer at least one cell to the secondary enrichment was determined with the Poisson-distribution in a simulation with 100.000 iterations using Latin Hypercube sampling (Delignette-Muller and Rosso, 2000), together with a Mersenne twister random number generator. The mean probability to transfer at least one cell to the secondary enrichment step was calculated for scenarios with different volumes (0.1 ml and 1 ml) and different incubation times of the primary enrichment (24 h and 26 h).

3. Results

3.1. Recovery duration after 60 °C heat stress is strain dependent

The recovery of 23 strains of *L. monocytogenes* was tested in half Fraser primary enrichment broth as specified in the first step of ISO 11290-1:2017. The lag duration and growth of reference cells and sublethally injured cells after 60 °C heat stress in half Fraser broth was estimated by fitting bacterial growth models to the growth curves. Of the three models, the 3-phase model gave the best fit in 75.7% of the cases, the Gompertz model in 17.3% and the Baranyi model in 7.0% of the cases. Therefore the lag and maximum specific growth rate estimates from the 3-phase model were used in the further analysis of the experimental data. The lag durations for the enrichments in half Fraser broth are displayed in Fig. 1. For the reference condition where no additional stress was applied before enrichment, the strains behaved rather similarly with respect to their lag phase duration. The lag duration of reference condition cells of the 23 tested strains ranged from 1.4 to 2.7 h, with an average lag duration of all 23 strains of 1.9 h (standard deviation of 0.5 h). However, after the 60 °C heat treatment there was a significant increase in lag phase duration, as the heat stressed cells needed more time to recover and also showed larger variability. After heat stress, the lag phase ranged from 4.7 to 15.8 h with the average lag duration of all 23 strains of 10.0 (standard deviation of 2.8 h).

In order to determine a possible correlation between heat resistance and lag duration of individual strains, lag durations were plotted against

[1] Transfer of at least one cell in 0.1 ml of 2 $\log_{10}$ CFU/ml being Poisson(k > 0, 10) is 99.995%

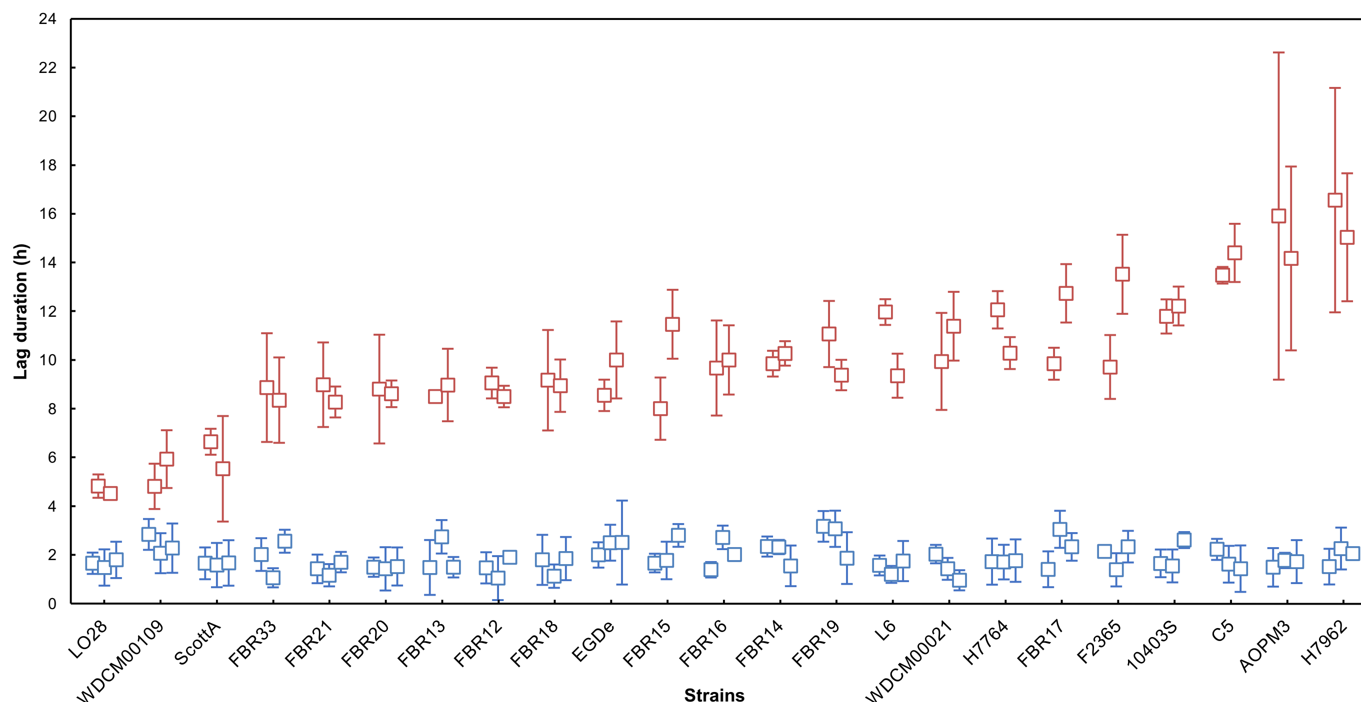

Fig. 1. Lag duration of 23 strains of *L. monocytogenes* in half Fraser enrichment broth with reference cells in blue (with no additional stress pre-treatment applied) and 60 °C heat stress pre-treatment in red (aiming for one D_{60}-value reduction). The 3-phase model was used to fit the growth kinetics and the lag duration was estimated for each biological reproduction. The 95% confidence interval of the fitting was determined for each fitting and displayed as error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

D_{60}-values after heat stress for all strains tested (Fig. 2). This showed that there was no clear correlation between heat resistance and lag duration during primary enrichment. The lag duration was also measured with cells after extended exposure time to 60 °C resulting in a 3 $\log_{10}$ reduction (data not shown). Data obtained with the selected strains showed that the lag duration was not significantly different from that of cells after a one $\log_{10}$ heat stress-induced reduction, indicating that a higher reduction after 60 °C heat stress did not influence the recovery capacity of the smaller and conceivably more severely injured surviving population.

Next to the lag phase, also the maximum specific growth rate was estimated with the 3-phase model for the reference cells and after 60 °C heat stress. The average maximum specific growth rate of all strains in half Fraser broth for the reference condition was 0.67 ± 0.05 1/h and for heat-stressed cells 0.68 ± 0.11 1/h. No significant difference was found among the average maximum specific growth rate during enrichments of reference cells and heat stressed cells ($p = 0.23$), indicating that once cells got out of the lag phase their growth rate was similar.

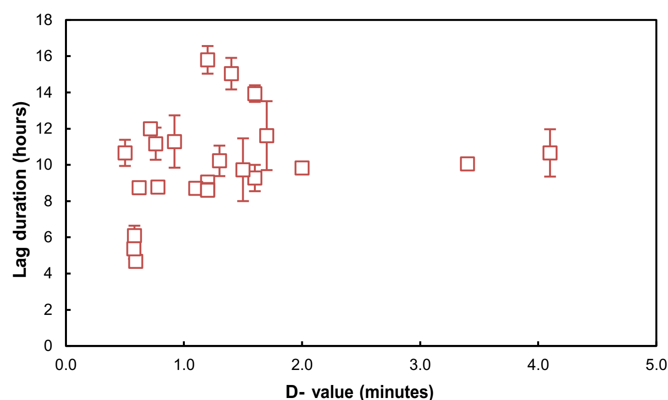

Fig. 2. The average lag duration in half Fraser broth of each of the 23 tested strains of *L. monocytogenes* after 60 °C heat treatment plotted against the D_{60}-value for each strain. This shows that there is no correlation between the D_{60}-value and the subsequent recovery after heat stress. The error bars depict the standard deviation in lag duration.

The strain collection contains strains from different serotypes, and the recovery ability among strains was compared (Supplementary Fig. S1). In reference condition no significant difference among serotypes was observed. Taking heat stress history into account, serotype 4b ($n = 6$) seemed to display the highest lag durations although this was not significant. On the other hand, serotype 1/2c strains ($n = 3$) showed shorter lag durations after heat-stress than the other tested serotypes, though this was neither statistically significant ($p = 0.06$). Furthermore, differences in recovery among lineages of *L. monocytogenes* and/or their origin (food or clinical isolates) were not found.

3.2. Recovery in half Fraser broth is strain and stress dependent

When cells of *L. monocytogenes* were pre-cultured at a lower temperature of 10 °C, there was no significant increase in lag duration observed during primary enrichment when compared to reference cells pre-cultured at 30 °C (data not shown). This led to the hypothesis that a reduction in viable cells was necessary for an increase in lag duration during primary enrichment. Therefore, the recovery after low-temperature (10 °C) acid stress was quantified, in order to test whether the strain recovery is only strain-dependent or also stress-dependent. For this, a fast recovering strain (ScottA), an intermediately recovering strain (EGDe) and a slow recovering strain (H7962) after heat stress together with the two ISO 11290-1 reference strains (WDCM00021 and WDCM00109 (International Organization for Standardization, 2017)) were tested. To be able to quantify the recovery in the same manner as after heat stress, one $\log_{10}$ reduction was also aimed for during the low-temperature acid stress pre-treatment. Because of strain differences in acid resistance, each strain was stressed at a different pH value to achieve one $\log_{10}$ reduction (Supplementary Table S1). The outgrowth in half Fraser broth after low-temperature acid

stress pre-treatment was compared to outgrowth following exposure to heat stress (Fig. 3). Although the strains showed significant differences in lag duration after one $\log_{10}$ reduction following exposure to heat stress, there were no significant differences among the strains after a one $\log_{10}$ reduction by low-temperature acid stress. This is further exemplified by the biological and strain variabilities in lag duration after exposure to stress (Fig. 4). For the reference cells as well as for low-temperature acid stressed cells, the biological variability was comparable to the strain variability. In contrast, although heat stress showed a significant increase in the biological variability between experiments ($p = 1.3 \cdot 10^{-5}$), there was a very significant increase in strain variability ($p = 3.8 \cdot 10^{-15}$). Overall, we saw that stressed *L. monocytogenes* cells showed significant strain variability in outgrowth during half Fraser enrichment.

3.3. Slow recovering strains after 60 °C heat stress can fail to reach the detection threshold

The enrichment kinetics of heat-stressed *L. monocytogenes* strains was used to predict the growth during the primary enrichment step. ISO11290-1:2017 specifies an incubation time of 25 h ± 1 h, which means that after a minimum of 24 h the cells should have reached a concentration that is high enough to allow transfer of at least one cell to the secondary enrichment. The minimal initial concentration at the start of the enrichment is one *L. monocytogenes* cell in 25 g of food that is enriched in 250 ml of half Fraser broth, meaning a minimum starting concentration of −2.4 $\log_{10}$ CFU/ml. After primary enrichment, 0.1 ml of culture is transferred to the secondary enrichment step, so strains need to reach a concentration of 2 $\log_{10}$ CFU/ml in order to allow transfer of at least one cell to the secondary enrichment broth with 100% probability[1] (Augustin et al., 2016). In the scenario analysis the lag duration and maximum specific growth rate was based on the average of the 23 strains, with a variation of two times the standard deviation as a margin. Fig. 5a shows that when starting with the lowest possible food contamination levels (i.e. one cell per 25 g food product), the strains that had the lowest measured lag duration after 60 °C heat stress reach the threshold of 2 $\log_{10}$ CFU/ml within 24 h, also including variation in growth rate. The primary enrichment was also modelled for the average measured lag duration and the strains with the longest lag duration (Fig. 5b/c). Strains with average lag durations after 60 °C heat pre-treatment did not all reach the threshold of 2 $\log_{10}$ CFU/ml within 24 h, and strains with the highest lag duration did not reach this level at all. This corresponds to 11 out of the 23 tested strains not reaching this concentration in the 24 h primary enrichment step (Fig. 6).

To further illustrate this, Monte Carlo simulations were carried out in

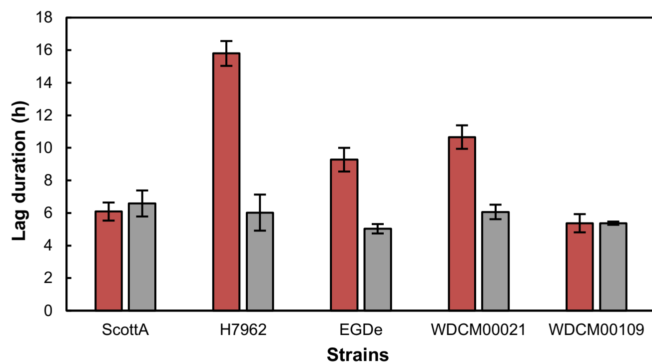

Fig. 3. Comparison of the lag duration after 60 °C heat stress (red) and 10 °C low-temperature acid stress (grey) in half Fraser enrichment broth. Growth kinetics were fitted with the 3-phase model and the average lag duration of two independent reproductions is displayed with the standard deviation as error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

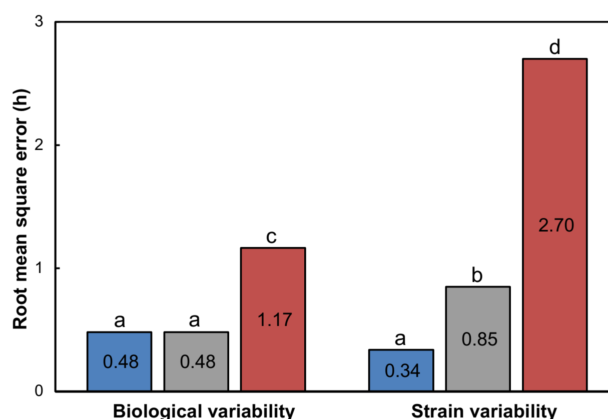

Fig. 4. The biological and strain variabilities calculated as the root mean square error of the lag phases of strains of *L. monocytogenes* for the reference cells (blue, $n = 23$), the 10 °C acid pre-treatment (grey, $n = 5$) and the 60 °C heat stressed cells (red, $n = 23$). Bars with different letters indicate significant differences calculated by *F*-test with a *p*-value lower than 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

@Risk to simulate the probability that the threshold of 2 $\log_{10}$ CFU/ml was reached after 24 h (Supplementary Fig. S2). This showed that when starting with one cell per 25 g food product, 100% of the iterations reached the threshold in the reference cells without prior stress history. However, when *L. monocytogenes* cells have been pre-exposed to heat stress, the detection threshold is reached in only 39.1% of the iterations.

In addition, the probability was calculated that at least a single cell would be transferred to the secondary enrichment step, assuming that transfer of cells in the enrichment medium follows a Poisson-distribution (Table 1). This showed that the average chance to transfer at least one cell of 60 °C heat-stressed *L. monocytogenes* after 24 h of enrichment is 79.8%. This probability increases to 90.8% when enrichment is increased to 26 h and can even reach 99.0% when 1 ml is transferred instead of the 0.1 ml that is specified by the current ISO11290-1:2017 protocol.

4. Discussion

In this study the lag duration of a diverse collection of *L. monocytogenes* strains from different isolation sources and of different serotypes was quantified in half Fraser enrichment broth using non-stressed control cells and sub-lethally acid and heat-damaged cells. This is important as the presence of pathogens in food products can be underestimated because cells sublethally injured during food processing can show extended lag durations, hence minimum cell concentrations are not reached in the 24 h time-span of the primary enrichment specified by the enrichment protocol. It is therefore important to quantify the growth dynamics during enrichments to increase the effectiveness and reliability of the culture-based detection methods for *L. monocytogenes*.

In order to quantify recovery of sublethally injured cells in half Fraser enrichment broth, heat treatment and low-temperature acid stress was used to stress cells. From previous experiments (data not shown) it was observed that a mild stress that does not lead to a reduction in cell concentration, does not affect the subsequent lag duration during enrichment. Therefore, a one $\log_{10}$ inactivation from heat-stress was chosen as a model stress treatment. Heat treatment is a common inactivation technique in food processing, which causes damage by inhibiting intracellular protein- and enzyme activity and by damaging nucleic acids and cell membranes (Wu, 2008). The strains used in this research show a large natural variation in their heat resistance (Aryani et al., 2015a), with a factor 8 difference in D_{60}-value between the least and most resistant strains. Because of this natural

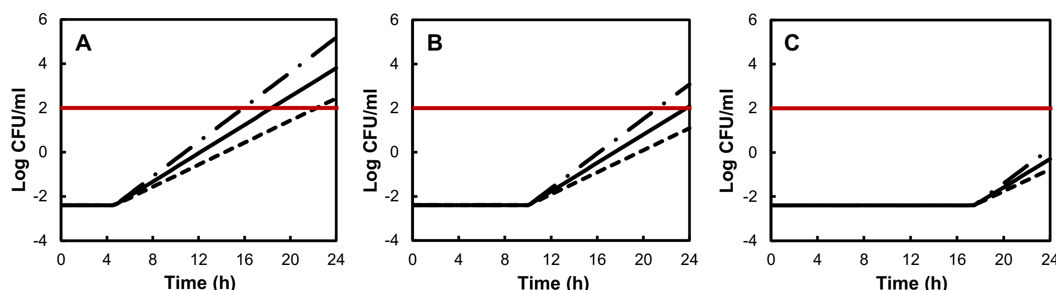

Fig. 5. Scenario analyses of the primary enrichment step in half Fraser broth starting with one CFU of *L. monocytogenes* per 25 g product. A threshold of 2 $\log_{10}$ CFU/ml needs to be reached in the 24 h of enrichment to transfer at least one cell to the secondary enrichment step with 100% probability. The inoculum concentration is one CFU that is enriched in 250 ml half Fraser broth meaning a concentration of −2.4 $\log_{10}$ CFU/ml. Here, (A) is the minimum lag with the average strain growth rate of 0.67 1/h ± two times standard deviation, (B) is the mean lag with the average strain growth rate of 0.67 1/h ± two times standard deviation and (C) is the maximum lag with the average strain growth rate of 0.67 1/h ± two times standard deviation.

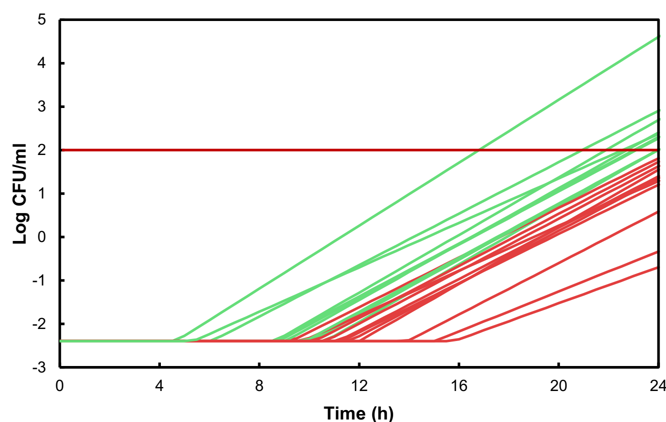

Fig. 6. Spaghetti plot with the growth of all 23 tested strains of *L. monocytogenes* after 60 °C heat stress starting with one CFU per 250 ml of half Fraser broth. Strains that reach the detection threshold of 2 $\log_{10}$ CFU/ml (red horizontal line) in the 24 h of primary enrichment are shown in green, strains in red do not reach this threshold within the allotted time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Exploration of modifications on the current primary enrichment step on the probability to transfer at least one cell to the secondary enrichment medium. Transfer of cells in 0.1 ml of primary enrichment to full Fraser broth is assumed to follow a Poisson-distribution. The simulated mean probability is calculated as the mean of the Poisson probability curve after 100.000 iterations with normal distributions of the lag duration and the maximum specific growth rate as measured from the 60 °C heat stress experiments.

Scenario	Simulated mean probability
24 h enrichment	0.798
26 h enrichment	0.908
24 h enrichment + inoculum to 1 ml	0.965
26 h enrichment + inoculum to 1 ml	0.990

variation in heat resistance, the 60 °C pre-treatment was standardized to one $\log_{10}$ reduction resulting in different heat treatment times for each of the strains. Clearly, stress history is not exclusively delineated by the log reduction at population level affecting the subsequent cell recovery because heat adaptive responses are strain dependent (Lin and Chou, 2004; Skandamis et al., 2008). It is hypothesized that exposure of the strains to the same heat treatment time would have resulted in a higher strain variability in lag phase, because the heat-induced reduction and subsequent repair capacity will significantly vary for each of the strains. The strains also varied with respect to acid stress robustness and a large difference in acid resistance meant that different pH levels were used to standardize the reduction. A glucose-rich medium was used to culture and stress the strains, and strain variability in acid stress adaptive responses might also have contributed to differences in strain recovery mechanisms.

Extended exposure to 60 °C heat stress to obtain 3 $\log_{10}$ reduction in cell concentration did not significantly influence the recovery, indicating that the recovery capacity of the injured population remains comparable. In contrast, Bréand et al. (1997) showed for *L. monocytogenes* that an increase in stress duration at 60 °C causes a rapid increase in lag duration that eventually reaches a steady threshold, but these studies were not carried out in half Fraser broth but in tryptic soy broth.

In the reference condition there was no significant difference between the biological- and strain variabilities. However, significant differences in the ability to recover in half Fraser enrichment broth were found among strains after 60 °C heat stress. Strain variation in recovery suggests that there are differences in repair capacity among strains in the tested conditions. These differences are important to take into account when optimizing the enrichment protocol. Since cells can undergo a multitude of stresses during food processing, we also investigated whether the observed strain difference in recovery after heat stress would also translate to other stresses. After low-temperature acid stress, the lag duration increased compared to reference cells but the strain variability was not significantly different from the biological variability. Thus, for the 5 tested strains no significant inter-strain variation was observed. This is interesting as these tested strains show large differences in recovery from heat stress, which would indicate that recovery in half Fraser broth is not only strain-dependent, but also stress-dependent.

In enrichment media there is a balance needed between an optimal recovery capacity for stressed *L. monocytogenes* cells and the suppression of background microbiota. Despite this, the lag phase duration in full Fraser broth was found to be significantly higher than other enrichment broths after heat stress (Silk et al., 2002). Our results showed that the maximum specific growth rate is similar in reference cells and stressed cells in half Fraser broth. This indicates that in the conditions tested, once cells of *L. monocytogenes* have recovered, they grew at the same maximum specific growth rate irrespective of their history. This corresponds with the work of Guillier and Augustin (2005) where they showed that variability in detection time was mainly explained by variation in lag duration following stress exposure and that there was no correlation found with the maximum specific growth rates.

Furthermore, we showed that there were no significant differences in recovery from heat stress based on isolation source, lineage and serotype. This is in agreement with the results of Lianou et al. (2006) where they characterized the growth of 25 *L. monocytogenes* strains after heat and ambient acid stress in tryptic soy broth. They also found extensive variation in growth and stress resistance among tested strains, but could not correlate this to specific serotypes. It should be noted however, that

the statistical power to elucidate serotype effects in the current study was quite low, due to a relative small number of strains per serotype. Strains of serotype 4b did show the highest lag duration in half Fraser broth, which could indicate that a detection bias against type 4b strains is present. This can have complications for the detection of serotype 4b as this serotype is common among epidemic outbreaks of *L. monocytogenes* (Kathariou, 2002).

A low inoculum concentration of 2 $\log_{10}$ CFU/ml was used to mimic the low concentrations of *L. monocytogenes* that are found in food products, although cell numbers recovered on plates can be even lower in contaminated samples (Chen et al., 2003). The effect of inoculum level on the lag duration has been studied before for *L. monocytogenes*, where the lag duration has been shown to be unaffected by inoculum size in enrichment broth (Duffy et al., 1994) and under optimal conditions in media containing non-inhibitory salt concentrations (Robinson et al., 2001). Other studies (Gnanou Besse et al., 2006; Stephens et al., 1997) showed an extension and a larger variability in lag duration only at starting concentrations of less than 10 CFU/ml. Dupont and Augustin (2009) studied the effect of stress on lag durations of individual cells in half Fraser broth. They stated that injured cells increase in mean and variability of lag and that the physiological state of a cell has a strong impact on its ability to initiate growth. Thus at very low cell concentrations that have to be enriched, the variability in lag duration and the individual cell state becomes increasingly important. Therefore, the performance at the individual cell level in enrichments remains to be further elucidated as this is an important aspect in the optimization of the enrichment protocol.

Obviously, strains that recover the slowest will pose the highest risk of evading detection in the 24 h of the primary enrichment step by not reaching high enough cell concentrations for transfer to the second enrichment step. By combining all heat stress recovery data, the Monte Carlo simulations suggested that when starting with one cell in an enrichment, the threshold of 2 $\log_{10}$ CFU/ml was not reached after 24 h in 61% of the simulations. Notably, the current ISO-procedure states that primary enrichment broth has to be incubated at 30 °C for 24–26 h. After the minimal 24 h of enrichment, the probability to transfer at least one cell to the secondary enrichment step was calculated to be 79.8%. Incubating 2 h more increases this probability to 90.8%. Because stressed cells with long lag durations may still reach low cell concentrations even after 26 h of incubation, the transfer of only 0.1 ml to the secondary enrichment broth can cause false-negative results. Increasing the transfer volume to 1 ml increases the probability to transfer cells significantly, even up to 99% when also incubated for 26 h. Transfer of an even larger volume is not preferred, since this would also increase the levels of competing microbiota that are transferred, as well as slightly decreasing the antibiotics concentration in full Fraser broth in secondary enrichment.

The detection of *L. monocytogenes* in food products is affected by the effectiveness of the enrichment medium. This can be a problem even in optimized enrichment media because significant differences can be found in the ability to detect especially injured cells (Osborne and Bremer, 2002; Silk et al., 2002). These injured strains can however resuscitate and pose a risk for food safety (Donnelly, 2002). Furthermore there can be large variation among strains in stress resistance and recovery (Cauchon et al., 2017; De Jesús and Whiting, 2003; Francis and O'Beirne, 2005; Lianou et al., 2006; Lundén et al., 2008). Although strain variation in recovery has been shown before for *L. monocytogenes* (De Jesús and Whiting, 2003; Francis and O'Beirne, 2005; Lianou et al., 2006), the current study shows that strain variability can also influence the detection efficacy while using the ISO11290-1:2017 protocol. For detection of pathogens in food, a reduction of the detection time is favourable but with increased lag durations of stressed and sublethally injured cells it comes with the risk of false-negatives.

5. Conclusion

This research shows that strains of *L. monocytogenes* differ significantly in their ability to recover in half Fraser enrichment broth, and that this strain variation should be taken into account when trying to optimize the current enrichment protocol. Our dataset shows that cells with a heat stress history can fail to reach the detection threshold for efficient transfer to the secondary enrichment step. Increasing the incubation time of the primary enrichment from 24 to 26 h, and subsequent transfer volume from 0.1 to 1 ml for the secondary enrichment broth, significantly increases the probability to detect those stressed *L. monocytogenes* strains that have extended lag durations in half Fraser broth.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Elisabetta Saverio and Sam Zarean for the lab work with regards to the low-temperature acid stress experiments. Furthermore, the authors would like to thank all partners in this project (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO and Wageningen Food Safety Research) for their financial and in-kind contributions. This work is part of the research programme "Rapid and reliable enrichment-based detection of foodborne pathogens" with project number 15002, which is partly financed by the Dutch Research Council (NWO).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108914.

References

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015a. Quantifying variability on thermal resistance of *Listeria monocytogenes*. Int. J. Food Microbiol. 193, 130–138. https://doi.org/10.1016/j.ijfoodmicro.2014.10.021.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015b. Quantifying strain variability in modeling growth of *Listeria monocytogenes*. Int. J. Food Microbiol. 208, 19–29. https://doi.org/10.1016/j.ijfoodmicro.2015.05.006.

Augustin, J.C., Kalmokoff, M., Ells, T., Favret, S., Desreumaux, J., Decourseulles Brasseur, E., Gnanou Besse, N., 2016. Modeling the behavior of *Listeria monocytogenes* during enrichment in half Fraser broth; impact of pooling and the duration of enrichment on the detection of *L. monocytogenes* in food. Food Microbiol. 60, 131–136. https://doi.org/10.1016/j.fm.2016.07.004.

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294. https://doi.org/10.1016/0168-1605(94)90157-0.

Bréand, S., Fardel, G., Flandrois, J.P., Rosso, L., Tomassone, R., 1997. A model describing the relationship between lag time and mild temperature increase duration. Int. J. Food Microbiol. 38, 157–167. https://doi.org/10.1016/S0168-1605(97)00093-7.

Bruhn, J.B., Vogel, B.F., Gram, L., 2005. Bias in the *Listeria monocytogenes* enrichment procedure: lineage 2 strains outcompete lineage 1 strains in University of Vermont selective enrichments. Appl. Environ. Microbiol. 71, 961–967. https://doi.org/10.1128/AEM.71.2.961-967.2005.

Buchanan, R.L., Whiting, R.C., Damert, W.C., 1997. A comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiol. 14, 313–326.

Cauchon, K.E., Hitchins, A.D., Smiley, R.D., 2017. Comparison of *Listeria monocytogenes* recoveries from spiked mung bean sprouts by the enrichment methods of three regulatory agencies. Food Microbiol. 66, 40–47. https://doi.org/10.1016/j.fm.2017.03.021.

Chen, Y., Ross, W.H., Scott, V.N., Gombas, D.E., 2003. *Listeria monocytogenes*: low levels equal low risk. J. Food Prot. 66, 570–577. https://doi.org/10.4315/0362-028X-66.4.570.

Dailey, R.C., Martin, K.G., Smiley, R.D., 2014. The effects of competition from non-pathogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered Listeria enrichment broth. Food Microbiol. 44, 173–179. https://doi.org/10.1016/j.fm.2014.05.004.

Dailey, R.C., Welch, L.J., Hitchins, A.D., Smiley, R.D., 2015. Effect of *Listeria seeligeri* or *Listeria welshimeri* on *Listeria monocytogenes* detection in and recovery from buffered Listeria enrichment broth. Food Microbiol. 46, 528–534. https://doi.org/10.1016/j.fm.2014.09.008.

De Jesús, A.J., Whiting, R.C., 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. J. Food Prot. 66, 1611–1617. https://doi.org/10.4315/0362-028X-66.9.1611.

Delignette-Muller, M.L., Rosso, L., 2000. Biological variability and exposure assessment. Int. J. Food Microbiol. 58, 203–212. https://doi.org/10.1016/S0168-1605(00)00274-9.

den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T., Zwietering, M.H., 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Appl. and Environ. Microbiol. 72 (9), 5884–5894. https://doi.org/10.1128/AEM.00780-06.

Donnelly, C.W., 2002. Detection and isolation of *Listeria monocytogenes* from food samples: implications of sublethal injury. J. AOAC Int. 85, 495–500. https://doi.org/10.1093/jaoac/85.2.495.

Duffy, G., Sheridan, J.J., Buchanan, R.L., McDowell, D.A., Blair, I.S., 1994. The effect of aeration, initial inoculum and meat microflora on the growth kinetics of *Listeria monocytogenes* in selective enrichment broths. Food Microbiol. 11, 429–438. https://doi.org/10.1006/fmic.1994.1048.

Dupont, C., Augustin, J.C., 2009. Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half Fraser broth. Appl. Environ. Microbiol. 75, 3069–3076. https://doi.org/10.1128/AEM.02864-08.

European Commission, 2005. Commission regulation (EC) no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 50, 1–26.

Francis, G.A., O'Beirne, D., 2005. Variation among strains of *Listeria monocytogenes*: differences in survival on packaged vegetables and in response to heat and acid conditions. Food Control 16, 687–694. https://doi.org/10.1016/j.foodcont.2004.06.005.

Gnanou Besse, N., Audinet, N., Barre, L., Cauquil, A., Cornu, M., Colin, P., 2006. Effect of the inoculum size on *Listeria monocytogenes* growth in structured media. Int. J. Food Microbiol. 110, 43–51. https://doi.org/10.1016/j.ijfoodmicro.2006.03.002.

Gnanou Besse, N., Favret, S., Desreumaux, J., Decourseulles Brasseur, E., Kalmokoff, M., 2016. Evaluation of reduction of Fraser incubation by 24 h in the EN ISO 11290-1 standard on detection and diversity of *Listeria* species. Int. J. Food Microbiol. 224, 16–21. https://doi.org/10.1016/j.ijfoodmicro.2016.02.010.

Gorski, L., Flaherty, D., Mandrell, R.E., 2006. Competitive fitness of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed cultures with and without food in the U.S. Food and Drug Administration enrichment protocol. Appl. Environ. Microbiol. 72, 776–783. https://doi.org/10.1128/AEM.72.1.776-783.2006.

Guillier, L., Augustin, J.C., 2005. Influence of stress and growth conditions on individual lag time distributions of *Listeria monocytogenes*. Acta Hortic. 674, 31–37. https://doi.org/10.17660/ActaHortic.2005.674.2.

International Organization for Standardization, 2017. Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. In: Part 1: Detection method, International Standard; ISO 11290-1, 2017. International Organization for Standardization, Geneva.

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65, 1811–1829. https://doi.org/10.4315/0362-028X-65.11.1811.

Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. J. Food Prot. 70, 2172–2198. https://doi.org/10.4315/0362-028X-70.9.2172.

Lianou, A., Stopforth, J.D., Yoon, Y., Wiedmann, M., Sofos, J.N., 2006. Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. J. Food Prot. 69, 2640–2647. https://doi.org/10.4315/0362-028X-69.11.2640.

Lin, Y., Chou, C., 2004. Effect of heat shock on thermal tolerance and susceptibility of *Listeria monocytogenes* to other environmental stresses. Food Microbiol. 21, 605–610. https://doi.org/10.1016/j.fm.2003.10.007.

Lundén, J., Tolvanen, R., Korkeala, H., 2008. Acid and heat tolerance of persistent and nonpersistent *Listeria monocytogenes* food plant strains. Lett. Appl. Microbiol. 46, 276–280. https://doi.org/10.1111/j.1472-765X.2007.02305.x.

Osborne, C.M., Bremer, P.J., 2002. Development of a technique to quantify the effectiveness of enrichment regimes in recovering "stressed" *Listeria* cells. J. Food Prot. 65, 1122–1128. https://doi.org/10.4315/0362-028X-65.7.1122.

Ottesen, A., Ramachandran, P., Reed, E., White, J.R., Hasan, N., Subramanian, P., Ryan, G., Jarvis, K., Grim, C., Daquiqan, N., Hanes, D., Allard, M., Colwell, R., Brown, E., Chen, Y., 2016. Enrichment dynamics of *Listeria monocytogenes* and the associated microbiome from naturally contaminated ice cream linked to a listeriosis outbreak. BMC Microbiol. 16, 275. https://doi.org/10.1186/s12866-016-0894-1.

Robinson, T.P., Aboaba, O.O., Kaloti, A., Ocio, M.J., Baranyi, J., Mackey, B.M., 2001. The effect of inoculum size on the lag phase of *Listeria monocytogenes*. Int. J. Food Microbiol. 70, 163–173. https://doi.org/10.1016/S0168-1605(01)00541-4.

Silk, T.M., Roth, T.M.T., Donnelly, C.W., 2002. Comparison of growth kinetics for healthy and heat-injured *Listeria monocytogenes* in eight enrichment broths. J. Food Prot. 65, 1333–1337. https://doi.org/10.4315/0362-028X-65.8.1333.

Skandamis, P.N., Yoon, Y., Stopforth, J.D., Kendall, P.A., Sofos, J.N., 2008. Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. Food Microbiol. 25, 294–303. https://doi.org/10.1016/j.fm.2007.10.008.

Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M., Humphrey, T.J., 1997. The use of an automated growth analyser to measure recovery times of single heat-injured *Salmonella* cells. J. Appl. Microbiol. 83, 445–455. https://doi.org/10.1046/j.1365-2672.1997.00255.x.

Wu, V.C.H., 2008. A review of microbial injury and recovery methods in food. Food Microbiol. 25, 735–744. https://doi.org/10.1016/j.fm.2008.04.011.

Zilelidou, E., Manthou, E., Skandamis, P., 2016a. Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol. Int. J. Food Microbiol. 235, 60–70. https://doi.org/10.1016/j.ijfoodmicro.2016.07.016.

Zilelidou, E., Karmiri, C.V., Zoumpopoulou, G., Mavrogonatou, E., Kletsas, D., Tsakalidou, E., Papadimitriou, K., Drosinos, E., Skandamis, P., 2016b. *Listeria monocytogenes* strains underrepresented during selective enrichment with an ISO method might dominate during passage through simulated gastric fluid and in vitro infection of Caco-2 cells. Appl. Environ. Microbiol. 82, 6846–6858. https://doi.org/10.1128/AEM.02120-16.

Zitz, U., Zunabovic, M., Domig, K.J., Wilrich, P.T., Kneifel, W., 2011. Reduced detectability of *Listeria monocytogenes* in the presence of Listeria innocua. J. Food Prot. 74, 1282–1287. https://doi.org/10.4315/0362-028X.JFP-11-045.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van''t Riet, K., 1990. Modeling of the bacterial growth curve. Appl. Environ. Microbiol. 56, 1875–1881. https://doi.org/10.1128/aem.56.6.1875-1881.1990.