Food Research International 150 (2021) 110783

Contents lists available at ScienceDirect

Food Research International

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

Heterogeneity in single-cell outgrowth of *Listeria monocytogenes* in half Fraser enrichment broth is affected by strain variability and physiological state

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

Food Microbiology, Wageningen University & Research, Bornse Weilanden 9, 6700 AA Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Listeria monocytogenes
Pathogen detection
ISO 11290–1:2017
Enrichment
Single-cell heterogeneity
Fluorescence-activated cell sorting

ABSTRACT

The behaviour of pathogens at the single-cell level can be highly variable and can thus affect the detection efficacy of enrichment-based detection methods. The outgrowth of single cells of three *Listeria monocytogenes* strains was monitored after fluorescence-activated single-cell sorting in non-selective brain heart infusion (BHI) broth and selective half Fraser enrichment broth (HFB) to quantify outgrowth heterogeneity and its effect on the detection probability. Single-cell heterogeneity was higher in HFB compared to non-selective BHI and heterogeneity increased further when cells were heat-stressed. The increase in heterogeneity was also strain-dependent because the fast-recovering strain Scott A showed less outgrowth heterogeneity than the slower-recovering strains EGDe and H7962. Modelling of the outgrowth kinetics during the primary enrichment demonstrated that starting at low cell concentrations could fail detection of *L. monocytogenes* at least partly due to cell heterogeneity. This highlights that it is important to take single-cell heterogeneity into account when optimizing enrichment formulations and procedures when *L. monocytogenes* contamination levels are low.

1. Introduction

Listeria monocytogenes is ubiquitous in nature and can be introduced to food processing environments where it may contaminate products. *L. monocytogenes* infections – called listeriosis – have a high mortality rate of 20–30% in the susceptible population (young, old, pregnant, and immunocompromised) (Swaminathan & Gerner-Smidt, 2007). Certain food products are routinely tested for the presence of *L. monocytogenes* to verify the effectiveness of a food safety management system. These detection procedures are based on an enrichment step where the pathogen recovers from the stressful conditions during food processing before growing out to detectable levels. Enrichments for *L. monocytogenes* are standardized in the ISO 11290–1:2017 enrichment protocol (International Organization for Standardization, 2017). This protocol consists of two enrichment steps to repair cell damage and selectively increase *L. monocytogenes* cell concentrations to allow subsequent isolation and detection. The enrichment method has to be able to detect very low levels of *L. monocytogenes* in these products as the European Union Food Law states that the absence of *L. monocytogenes* in five samples of 25 g has to be guaranteed in ready-to-eat food products that can facilitate growth after the production process (European Commission, 2005). In positive-tested food products, the contamination level can often be low with *L. monocytogenes* contamination levels in ready-to-eat foods more often containing between 10 and 1000 CFU/g than more than 1000 CFU/g (Koskar, Kramarenko, Meremäe, Kuningas, Sõgel, Mäesaar, Anton, Lillenberg, & Roasto, 2019). The contamination level is also lower than 100 CFU/g in 97.5% of positive beef and poultry samples (Awaisheh, 2010). The low starting concentrations can be problematic as the outgrowth of single cells can be heterogeneous. Interestingly, it has been shown that growth initiation at the single-cell level is highly heterogeneous due to variability in lag duration (Koutsoumanis, 2008). The heterogeneity in growth initiation is increased even more when cells are recovering from (sub-lethal) injury due to prior food processing (Guillier, Pardon, & Augustin, 2005; Koutsoumanis & Sofos, 2005). Stresses such as freezing, heating, and exposure to low pH, which *L. monocytogenes* may encounter during food production can therefore have a profound effect on single-cell lag durations (Dupont & Augustin, 2009). Also, injured cells can become more sensitive to the

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

https://doi.org/10.1016/j.foodres.2021.110783
Received 14 July 2021; Received in revised form 29 September 2021; Accepted 20 October 2021
Available online 29 October 2021

selective compounds in the enrichment medium, which can retard their outgrowth potential. Furthermore, when starting cell concentrations are low, the differences in single-cell outgrowth potential can lead to failure to reach the necessary detection threshold for detection. Such cases would lead to false-negative results where the presence of *L. monocytogenes* is not detected and thereby posing a risk for food safety. Currently, limited data are available on the influence of single-cell heterogeneity on the outgrowth potential during enrichment with the ISO 11290–1 protocol other than the study by Dupont and Augustin (2009), in which they studied the effect of pre-exposure to stress on lag durations during primary enrichment. All aspects considered, it is conceivable that single-cell heterogeneity in outgrowth efficacy is a significant determinant of the detection rate of *L. monocytogenes* enrichments. Single-cell heterogeneity in growth performance can be effectively studied using flow cytometry combined with sorting of individual cells into multi-well plates and subsequent assessment of their outgrowth kinetics. We previously reported that the variability in *L. monocytogenes* strains during recovery can play an important role in detection at population level (Bannenberg, Abee, Zwietering, & den Besten, 2020). Therefore, the aim of this study was to quantify the impact of outgrowth heterogeneity of *L. monocytogenes* at single-cell level during primary enrichment following the ISO 11290–1 protocol in half Fraser broth using a selection of slow, medium, and fast-recovering strains of *L. monocytogenes*.

2. Material and methods

2.1. Bacterial strains and growth conditions

Three strains of *L. monocytogenes* from different isolation sources and serotypes were kept at −80 °C in brain heart infusion (BHI) broth (Becton, Dickinson and company, USA) supplemented with 30% glycerol (Fluka, USA). The strains Scott A, EGDe, and H7962 were chosen based on their difference in lag time in half Fraser broth (HFB) after being heat-treated at 60 °C (Bannenberg et al., 2020), with Scott A, EGDe, and H7962 having short, medium, and long lag phases in HFB respectively. Colonies were obtained from −80 °C freezer stocks that were plated out on BHI agar (BHI supplemented with 1.5% agar, Oxoid, UK). From this, cultures were made by inoculating 10 ml of BHI broth with a single colony. Cultures were grown at 30 °C with 180 rpm shaking for 16 h to obtain stationary phase cultures. These cultures were subsequently diluted 1:1,000 in fresh BHI broth and incubated statically at 30 °C for 16 h to obtain a standardized working culture of around 9.5 $\log_{10}$ CFU/ml for use in further experiments.

2.2. Heat treatment of cells

Working cultures of all strains were heat-treated to reduce the viable counts by one $\log_{10}$ CFU/ml. The D_{60}-values of the strains previously published by Aryani et al. (Aryani, Den Besten, Hazeleger, & Zwietering, 2015) 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, Germany) for one D_{60}-value reduction (0.6 min for strain Scott A, 1.6 min for strain EGDe, and 1.2 min for strain H7962). Afterward, the cultures were quickly cooled on ice for 15 s to bring the temperature back to around 35 °C for use in follow-up experiments.

2.3. Fluorescence staining

Reference cells (cultures that did not receive any stress treatment) and heat-treated cells were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, USA). This kit contains the membrane-permeable SYTO 9 dye, which stains all cells green, and the membrane-impermeable propidium iodide (PI) dye, which shows a bright red fluorescence signal upon binding to nucleic acids after entering cells with a permeabilized membrane. Staining of *L. monocytogenes* cells was needed for single-cell sorting to discriminate the small cell size of this bacterium from the background noise. To exclude dye interference on the outgrowth kinetics, SYTO 9 and PI were diluted ten times in DMSO to obtain stock solutions with dye concentrations of 334 μM for SYTO 9 and 2 mM for PI. These concentrations did not affect the growth as measured in additional test experiments using optical density measurements (data not shown). For the staining procedure, 1 ml of cell culture was serially diluted in peptone physiological salt (Tritium Microbiology, Netherlands) until a concentration of around 7 $\log_{10}$ CFU/ml, after which the dyes were added (final concentrations were 500 nM for SYTO 9 and 3 μM for PI) and cultures were incubated at room temperature in the dark for 15 min before use in the flow cytometer.

2.4. Flow cytometry and single-cell sorting

Flow cytometry experiments and cell sorting was done with the FACSAria III cell sorter (BD Biosciences, USA) using a 488 nm laser following the protocol of Warda, Tempelaars, Abee, and Nierop Groot (2016). Briefly, the calibration was done with Cytometer Setup & Tracking beads and Accudrop beads (BD Biosciences, USA). Cells of *L. monocytogenes* were differentiated from electronic background noise using a combination of forward scatter (FSC), side scatter (SSC), propidium iodide red fluorescence (561 nm laser, 600LP with 610/20 nm filter in Texas Red channel), and green Syto 9 (488 nm laser, 502LP with 530/30 nm filter in FITC channel). The gating strategy was based on previous experience in sorting *L. monocytogenes* single cells, and before sorting, this gating strategy was established by collecting 50,000 events to exclude doublet cells. The exclusion of doublet cells was verified by microscopy. Events were randomly sorted from the FITC green fluorescence channel to ensure the sorting of all cells. Wells of 384-wells plates (Greiner Bio-One, Austria) were filled with 50 μl of either BHI or HFB and a sorted single-cell was added to each well. As a control, PI-positive cells were sorted in a range of concentrations (1, 10, 100, 1000, 10,000 cells/well), and only late growth was observed at 10,000 cells/well indicating that most PI-positive cells were indeed too damaged to efficiently grow out. The sorting was done mostly aseptically with the plate enclosed during sorting with the cell sorter. Blank wells were taken along as controls, and no growth was measured in these wells. The plates were briefly centrifuged before optical density measurement to remove air bubbles. Two independent reproductions were done on separate days with a total of 192 single-cell events per combination of strain, medium, and condition (a total of 2304 recorded events).

2.5. Quantifying single-cell outgrowth

Single-cell outgrowth in BHI broth and HFB was measured in a SpectraMax 384 plus (Molecular Devices, USA) set at 30 °C and at an optical density wavelength of 600 nm (OD_{600}). HFB was chosen as a selective broth following the ISO 11290–1:2017 procedure, and BHI was chosen as a non-selective reference medium. Optical density measurements were taken every 5 min for 48 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 0.25, which was chosen because at higher optical densities the HFB will blacken due to esculin hydrolysis, causing a rapid increase in the readings. Wells that did not reach the specified OD_{600} increase of 0.25 within 48 h were plated to verify whether there was outgrowth below the detection limit of the optical density measurements. For this, 10 μl of these wells was spot-plated on BHI agar plates and incubated at 30 °C for 48 h. When colony growth was observed, this indicated that the inoculated cell in the well had grown out but did not reach the concentration needed to cause an optical-density increase within the 48 h timeframe. The mean, the standard deviation, and the coefficient of variation (CV, i.e. ratio of the standard deviation to the mean) of the TTR data were

calculated for each strain (Scott A, EGDe, and H7962), medium (BHI and HFB), and condition (reference cells and heat-treated cells). All data analyses were done in R (R Core Team, 2020).

2.6. Determining the growth rate in wells

The maximum specific growth rate (μ_{max}) in the wells was determined for reference cells and heat-treated cells for each of the three strains in both BHI broth and HFB. The μ_{max} was estimated using the two-fold dilution method as described by Biesta-Peters, Reij, Joosten, Gorris, and Zwietering (2010). Briefly, reference cells and heat-treated cells were added to 96-wells plates with a starting concentration of around 3 $\log_{10}$ CFU/ml in both BHI broth and HFB. Cells were two-fold diluted in subsequent wells up to the lowest concentration of around 1.8 $\log_{10}$ CFU/ml and the optical density was measured at 600 nm using a SpectraMax 384 plus, set at 30 °C for 48 h. The μ_{max} was then estimated as the negative inverse slope of the TTR plotted against the natural logarithm of the cell concentration. The average μ_{max} for each strain and medium was estimated from the mean of three biologically independent reproductions.

2.7. Modelling of the detection probability

To estimate the probability that single-cell outgrowth will lead to a false-negative enrichment outcome, the μ_{max} in HFB and the single-cell lag time were used to estimate the outgrowth kinetics of single cells. To estimate the single-cell lag phase, the cell density was determined when the optical density increased by 0.25, i.e. the threshold that corresponds to the TTR (**Suppl. Fig. S1A**). This allowed determining the minimum TTR taking into account the μ_{max} and assuming no lag phase for a single cell to grow out in the well to reach the optical density threshold. The difference between the observed TTR and the minimum TTR corresponded to the lag time of the individual cell. This lag phase estimation and the μ_{max} of each of the strains was used to simulate the kinetics at lower concentration assuming 1 cell in 250 ml of enrichment, resulting in $\log N_0$ of −2.4 $\log_{10}$ CFU/ml. The concentration that needs to be reached after 24 h to allow subsequent detection (the detection concentration) is around 2 $\log_{10}$ CFU/ml (Augustin et al., 2016) (**Suppl. Fig. S1B**). This detection concentration was used to determine whether the individual cell would be detected after 24 h of enrichment, starting at an initial concentration of −2.4 $\log_{10}$ CFU/ml.

3. Results and discussion

3.1. Single-cell outgrowth kinetics

The single-cell outgrowth performance was determined for three strains of *L. monocytogenes* in HFB and non-selective BHI (Fig. 1). When comparing the outgrowth in BHI and HFB, it is clear that outgrowth in BHI is faster than in HFB. It has been shown before that the μ_{max} in HFB is lower than in a non-selective medium such as BHI (Cornu, Kalmokoff, & Flandrois, 2002). Moreover, outgrowth in BHI seemed to be more homogeneous than the outgrowth in HFB (Fig. 1), which suggests that outgrowth medium can impact the heterogeneity. Also, heat-treated cells with a 60 °C heat stress history (Fig. 1**B/D/F**) showed an increased outgrowth time when compared to reference cells of the same strain (Fig. 1**A/C/E**), most probably caused by an increased lag duration to recover from the sub-lethal injury induced by the heating treatment. Additionally, the three strains of *L. monocytogenes* showed a clear difference in outgrowth kinetics, which is in line with earlier research

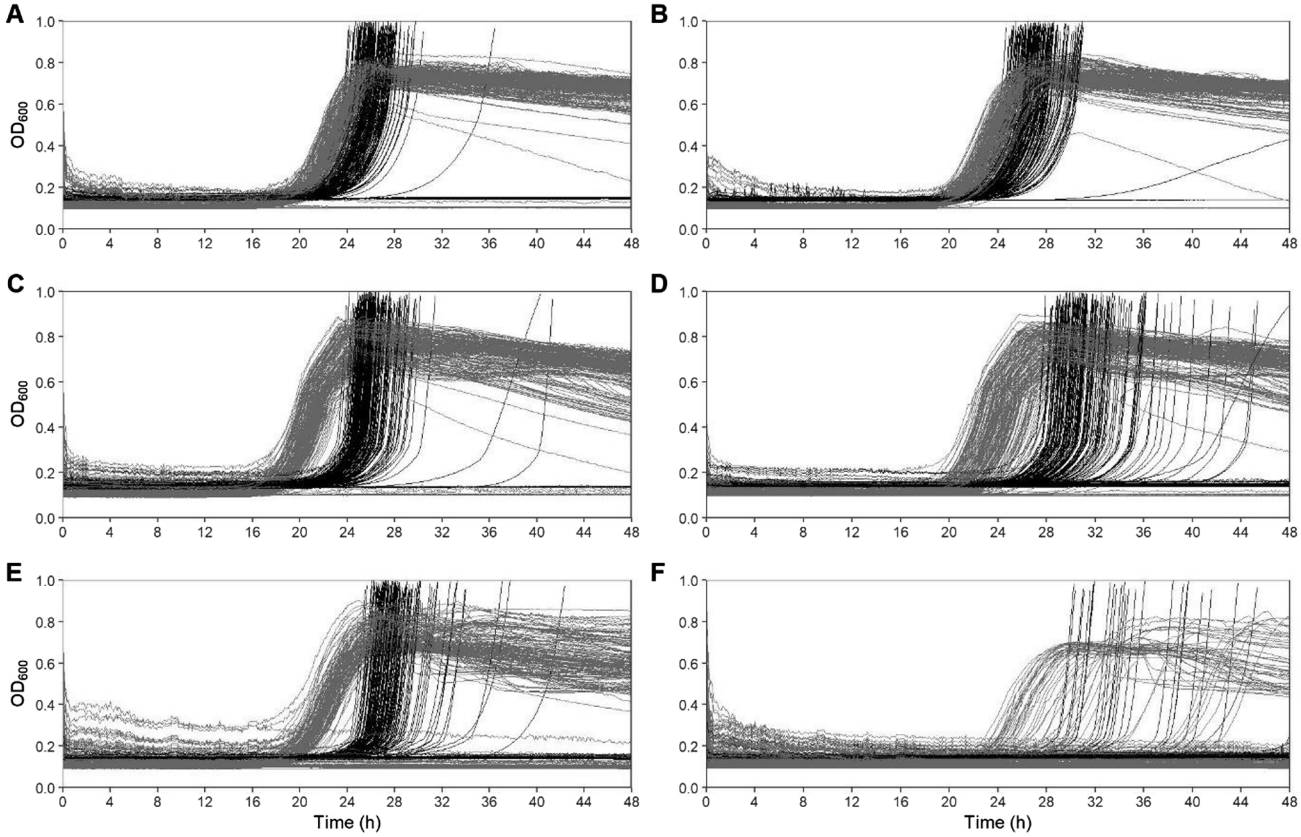

Fig. 1. The outgrowth of single cells of *L. monocytogenes* as measured by optical density at 600 nm in BHI (grey lines) and half Fraser enrichment broth (black lines). The outgrowth in all wells is shown for (A) reference cells of strain Scott A, (B) heat-treated cells of strain Scott A, (C) reference cells of strain EGDe, (D) heat-treated cells of strain EGDe, (E) reference cells of strain H7962 and (F) heat-treated cells of strain H7962. Cells grown in HFB reach much higher optical densities due to the blackening of the medium by the hydrolysis of esculin at higher cell concentrations.

where outgrowth kinetics of reference cells and heat-stressed cells were quantified at the population level for 23 strains (Bannenberg et al., 2020). Here, a fast (Scott A), average (EGDe), and slow recovering strain (H7962) was chosen in order to compare the outgrowth capacity at single-cell level. Indeed, the outgrowth kinetics of the individual cells of strain Scott A were faster than those of strains EGDe and H7962. Interestingly, also the outgrowth of single cells was more homogenous in the Scott A strain with faster outgrowth. Clearly, single-cell outgrowth heterogeneity was influenced by a combination of effects, including recovery medium, (stress) history, and strain characteristics.

3.2. Quantifying heterogeneity in outgrowth

For each well the single-cell outgrowth was quantified as the TTR to reach an optical density increase of 0.25. The distribution of all single-cell outgrowth events is shown in the density histograms of Fig. 2. Irrespective of strain and/or history, the distribution of TTR values mostly resembled a lognormal-distribution.

3.2.1. Effect of recovery medium on heterogeneity

First of all, the choice of recovery medium had an impact on the single-cell outgrowth in both reference cells and heat-treated cells. For reference cells, the mean TTR in non-selective BHI was 2.7 h shorter than in selective HFB for strain Scott A, 5.1 h shorter for EGDe, and 5.3 h shorter for H7962 (Table 1). After heat stress, the difference in mean TTR between BHI and HFB increased to 2.9 h for Scott A, 7.1 h for EGDe, and 6.0 h for H7962. Recovery in HFB did not only increase in mean TTR and subsequent standard deviation, but also had an increased coefficient of variation (Table 1). The higher coefficient of variation in HFB indicates a relative increase in variability in single-cell outgrowth. This observation was tested by comparing the variances with Levene's test of the TTR values. This confirmed that HFB significantly increases the variability in single-cell outgrowth compared to BHI ($p < 0.001$).

A possible factor that could explain the difference in single-cell variability is the presence of antibiotics in HFB. The selective compounds in HFB are acriflavine which inhibits RNA-synthesis (Meyer, Probst, & Keller, 1972), nalidixic acid that can inhibit both RNA- and DNA-synthesis (Crumplin & Smith, 1975), and lithium chloride that can compete with divalent cations in susceptible microorganisms (Mendonca & Knabel, 1994). Next to an increased lag duration and reduced growth rates in HFB due to these selective compounds (Beumer, Te Giffel, Anthonie, & Cox, 1996), membrane damage or increased permeability caused by heat stress history may facilitate easier entry of

Table 1
Effect of medium and heat-stress treatment on the heterogeneity of single-cell outgrowth. For each strain in BHI and HFB and for reference cells and heat-stressed cells, the number of wells where outgrowth was observed within 48 h is shown. The mean, standard deviation (sd), and coefficient of variation (CV) in the TTR are also given.

Strain	Medium	Condition	Outgrown wells	mean TTR	sd TTR	CV TTR
Scott A	BHI	Reference	178/192	22.3	0.72	0.032
Scott A	HFB	Reference	180/192	25.0	1.29	0.052
Scott A	BHI	Heat stress	187/192	23.2	0.81	0.035
Scott A	HFB	Heat stress	190/192	26.1	1.97	0.076
EGDe	BHI	Reference	175/192	20.6	0.73	0.036
EGDe	HFB	Reference	189/192	25.7	1.79	0.070
EGDe	BHI	Heat stress	174/192	24.0	1.90	0.079
EGDe	HFB	Heat stress	153/192	31.1	3.18	0.102
H7962	BHI	Reference	145/192	22.1	0.75	0.034
H7962	HFB	Reference	144/192	27.4	2.09	0.076
H7962	BHI	Heat stress	41/192	28.5	4.88	0.171
H7962	HFB	Heat stress	25/192	34.5	4.23	0.123

Note: only the wells are summarized where outgrowth was observed within 48 h.

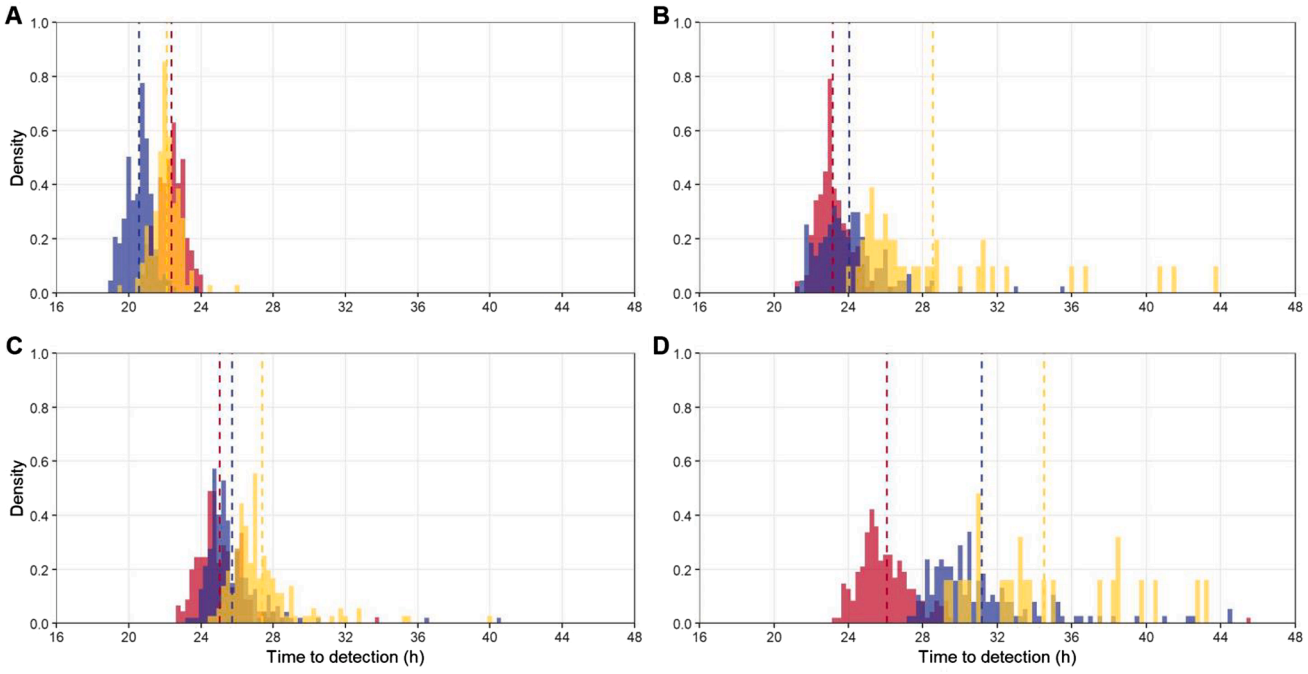

Fig. 2. The probability distribution of the time-to-reach for single cells of *L. monocytogenes* in both BHI and HFB for reference cells and heat-treated cells. Time for outgrowth was determined as the time to reach an increase in OD_{600} of 0.25 with the single-cell outgrowth of reference cells in BHI (A) and in HFB (C), and single-cell outgrowth of heat-treated cells (one $\log_{10}$ CFU/ml reduction) in BHI (B) and in HFB (D). Strain *L. monocytogenes* Scott A is in red, strain EGDe in blue and strain H7962 in yellow. The dotted lines indicate the mean outgrowth for each strain and wells that did not reach the TTR in 48 h were not plotted. The histogram bin size is 15 min and the total area of the histogram is normalized to one for better comparison despite differences in the number of outgrown wells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selective compounds into the cells. This can explain the increase in heterogeneity as cell-to-cell fluctuations in a selective medium often lead to higher heterogeneity than in a non-selective medium such as in BHI.

3.2.2. Effect of heat-stress history on the heterogeneity

Next to the medium effect on outgrowth, Fig. 2**B** and **D** also show that heat-treatment increased the average TTR and the heterogeneity in both BHI and HFB (Levene's test $p < 0.001$). When comparing reference cells with heat-treated cells in the same medium, there was a significant increase in average TTR, standard deviation, and in the coefficient of variation (Table 1). The addition of a heat-stress history increases the skewness towards the right, meaning that the variability in time-to-reach increases in favour of longer outgrowth. Here, due to differences at the single-cell level, stress history can increase the range of lag durations and thereby complicate adequate detection during enrichment. This corroborates with a previous research in which we found that heat stress can be of significant influence on the subsequent recovery in enrichment broth (Bannenberg et al., 2020). Next to an increase in mean TTR, single-cell outgrowth was also more heterogeneous and this can be of importance during detection. This is in line with earlier research which showed that single-cell growth initiation is also dependent on the stress history (Dupont & Augustin, 2009). In that study, (55 °C) heat treatment was found to be a stress with a significant effect on the single-cell growth initiation in HFB. Furthermore, that study also showed that in injured cells, the single-cell lag times increased in mean and variability. However, in our study single-cells were sorted by flow cytometer and the single-cell behaviour could be compared to the population behaviour during enrichments.

3.2.3. Effect of strain differences on the heterogeneity

Because the selected strains differed in their heat resistance, the D_{60}-value was used to standardize the reduction to one $\log_{10}$ CFU/ml. Even so, the heat treatment affected the tested strains to different extents, with an average increase of 1.0 h for strain Scott A, 4.5 h for strain EGDe, and 6.8 h for strain H7962 after 60 °C heat-stress for both media (Table 1). Arguably, this matches with the previously described recovery behaviour of the strains (Bannenberg et al., 2020) because the fast-recovering strain Scott A has the least heterogeneity, the average-recovering strain EGDe has average heterogeneity and the slow-recovering strain H7962 displays the most heterogeneity. This suggests that stress recovery capacity affects heterogeneity at least to some extent, where stressed strains with faster recovery exhibit less heterogeneity in single-cell outgrowth. When outgrowth heterogeneity is dependent on the degree of cell damage that needs to be repaired and the outgrowth characteristics of the strain, this could contribute to a selection bias during enrichment. Notably, single-cell sorting of heat-treated cells did not always lead to quantifiable outgrowth within 48 h (Table 1). This was especially the case for the slow-recovering strain H7962 after heat treatment. To evaluate whether outgrowth below the optical density detection threshold occurred, the contents of the wells were spot-plated on BHI-agar. This showed that even though some single-cell events did not reach the optical density threshold within 48 h, in 21% of the cases heat-treated cells in HFB still showed colony formation (data not shown). A likely reason is that those single-cells were either too damaged for repair and growth initiation or that they had a considerably increased lag duration. The cell sorting with the flow cytometer might also be a contributing factor, with the conditions during the sorting procedure on already damaged membranes of heated cells giving extra stress on subsequent recovery in the wells. However, cells were sorted using lower flow rates and a sufficiently sized 85 μm nozzle which means that this should have a limited impact on cell integrity. Still, it has been shown previously that the sorting of cells can affect cell resuscitation capabilities, especially for heat-treated cells (Sibanda & Buys, 2017). And even though a 10 times diluted dye concentration was used for sorting, a slightly toxic effect cannot be excluded.

3.3. Estimation of single-cell probability to miss detection

The often harsh environments that food microbes are exposed to can lead to differences in repairability and subsequently in single-cell outgrowth heterogeneity. Especially, outliers with increased lag durations can become problematic when detection of low levels of *L. monocytogenes* is required because differences in outgrowth capacity can lead to failure to detect these cells. To estimate the impact of heterogeneity in single-cell outgrowth on the detection probability, the outgrowth of single cells was modelled. Taking the μ_{max} of each strain into account (**Suppl. Table S1**), the minimum TTR (i.e. assuming no lag phase) for a single cell to grow out in a well to reach the optical density threshold in HFB could thus be estimated for each strain (**Suppl. Fig. S1a**). The difference between the observed TTR and the minimum TTR is assumed to correspond to the lag time of the individual cell. To estimate the possibility that heterogeneity affects the successful detection probability during enrichment, the maximum lag duration was estimated that still allows efficient transfer of cells to the secondary enrichment step. The concentration that needs to be reached after 24 h of enrichment to transfer at least one cell with a Poisson-chance higher than 99.9% is a concentration of 1.8 $\log_{10}$ CFU/ml (Augustin et al., 2016; Bannenberg et al., 2020). Again, considering the μ_{max} of each strain, then the estimated maximum lag phase that still allows for subsequent detection is 11.3 h for strain Scott A, 11.6 h for strain EGDe, and 9.8 h for strain H7962 (**Suppl. Fig. S1b**). Combining this maximum lag duration and the outgrowth in the wells allowed us to set a threshold after which these cells can start to fail the transfer to the secondary enrichment broth and in turn fail detection with the current enrichment protocol. We call this tipping point the 'detection-fail probability' in this context because the probability to fail detection starts to increase after this threshold. This model estimated that the detection-fail probability is reached for sorted single cells with a TTR of higher than 32.9 h for strain Scott A, 30.9 h for strain EGDe, and 33.3 h for strain H7962. The discrete distribution of single-cells that did not reach the detection concentration is shown in Fig. 3 for reference and heat-stressed cells. Here, single-cells that grew out in the red-shaded areas are at risk of not reaching the detection concentration. The detection-fail probability was then estimated as the number of wells that have a TTR longer than the estimated threshold. In this way, single-cells that did not grow out within 48 h were considered to fail to reach the detection concentration as well. For strain Scott A (Fig. 3**A/B**), the estimated detection-fail probability is 0.07 for reference cells and 0.02 for heat-stressed cells. This low probability to not reach the detection concentration corresponds with its fast recovery in HFB as was shown in earlier research (Bannenberg et al., 2020). For strain Scott A the impact on the detection probability is small because even after heat-stress the single-cell heterogeneity in recovery is still within a safe margin for successful detection. On the other hand, the remaining tested strains that already had a longer lag duration after stress history also had higher probabilities to miss the detection concentration. For the average-recovering strain EGDe and the slow-recovering strain H7962, the detection-fail probability increased significantly after 60 °C heat stress. Detection-fail probabilities for strain EGDe (Fig. 3**C/D**) were estimated as 0.03 in reference condition and 0.52 after heat-stress. For H7962 (Fig. 3**E/F**) this increased to 0.27 in reference condition and 0.93 for stressed cells in HFB, where most of the single-cell events did not grow out to detectable levels. Strains that can recover faster and have a lower heterogeneity in outgrowth potential during enrichment have a higher probability to be detected than slow recovering strains. In this case, however, it should be noted that it is a possibility that some single-cells could be too damaged to still facilitate resuscitation and subsequent outgrowth. All in all, variability in single-cell outgrowth in strains of *L. monocytogenes* can lead to failure to detect stressed pathogens in food products.

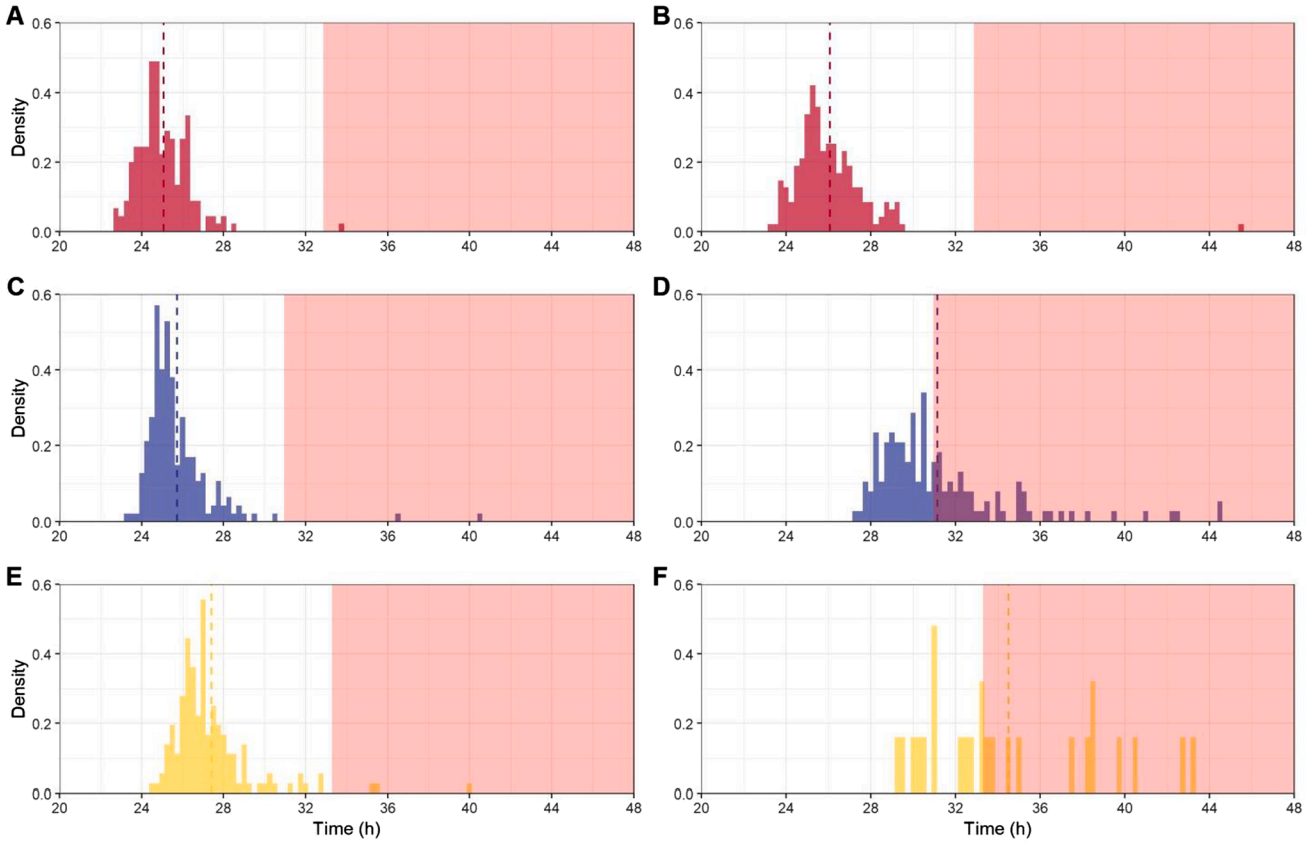

Fig. 3. Heterogeneity of the TTR and the predicted detection-fail probability during enrichment in HFB. For each strain and condition, the maximum TTR has been estimated that could lead to a detection-fail outcome, and TTR values falling outside this detection range are shaded in red and are considered to be at risk of failing detection. The single-cell outgrowth of reference cells of strain Scott A with a detection-fail probability of 0.07 (A), heat-treated cells of strain Scott A with a detection-fail probability of 0.02 (B), reference cells of strain EGDe with a detection-fail probability of 0.03 (C), heat-treated cells of strain EGDe with a detection-fail probability of 0.52 (D), reference cells of strain H7962 with a detection-fail probability of 0.27 (E), and heat-treated cells of strain H7962 with a detection-fail probability of 0.93 (F). Cells that did not grow out within the 48 h time period are not plotted but these events are taken into account for the calculation of the detection probability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

Strain differences, history, and single-cell heterogeneity affect the successful detection of *L. monocytogenes* using the ISO 11290–1 enrichment-based detection method. The recovery of stressed cells of *L. monocytogenes* depends not only on strain differences in population behaviour, but the recovery at single-cell level can also vary drastically within a specific strain. Heterogeneity in outgrowth also seems to be higher in strains with larger lag durations, indicating that for strains that already have a hard time recovering in HFB this effect is enlarged at single-cell level. Furthermore, kinetic modelling of the primary enrichment step showed that the beforementioned factors can contribute to false-negative detection outcomes, highlighting the importance to consider this when optimizing the ISO 11290–1 enrichment protocol for the detection of *L. monocytogenes*.

CRediT authorship contribution statement

Jasper W. Bannenberg: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Marcel H. Tempelaars:** Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Marcel H. Zwietering:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Tjakko Abee:** Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Supervision. **Heidy M.W. den Besten:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgments

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 program “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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110783.

References

Aryani, D. C., den Besten, H. M. W., Hazeleger, W. C., & Zwietering, M. H. (2015). Quantifying variability on thermal resistance of *Listeria monocytogenes*. *International journal of food microbiology, 193*, 130–138.

Augustin, J.-C., Kalmokoff, M., Ells, T., Favret, S., Desreumaux, J., Brasseur, E. D., & Besse, N. G. (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 Microbiology, 60*, 131–136.

Awaisheh, S. (2010). Incidence and contamination level of *Listeria monocytogenes* and other *Listeria* spp. in ready-to-eat meat products in Jordan. *Journal of food protection, 73*(3), 535–540.

Bannenberg, J. W., Abee, T., Zwietering, M. H., & den Besten. (2020). 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. *International Journal of Food Microbiology*, 108914.

Beumer, R. R., te Giffel, M. C., Anthonie, S. V. R., & Cox, L. J. (1996). The effect of acriflavine and nalidixic acid on the growth of *Listeria* spp. in enrichment media. *Food Microbiology, 13*(2), 137–148.

Biesta-Peters, E. G., Reij, M. W., Joosten, H., Gorris, L. G. M., & Zwietering, M. H. (2010). Comparison of two optical-density-based methods and a plate count method for estimation of growth parameters of *Bacillus cereus*. *Applied and environmental microbiology, 76*(5), 1399–1405.

Cornu, M., Kalmokoff, M., & Flandrois, J.-P. (2002). Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. *International journal of food microbiology, 73*(2–3), 261–274.

Crumplin, G. C., & Smith, J. T. (1975). Nalidixic acid: An antibacterial paradox. *Antimicrobial agents and chemotherapy, 8*(3), 251–261.

Dupont, C., & Augustin, J.-C. (2009). Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half Fraser broth. *Applied and environmental microbiology, 75*(10), 3069–3076.

Warda, A. K., Tempelaars, M. H., Abee, T., & Nierop Groot, M. N. (2016). Recovery of heat treated *Bacillus cereus* spores is affected by matrix composition and factors with putative functions in damage repair. *Frontiers in microbiology, 7*, 1096.

European Commission. (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union, 50*, 1-26.

Guillier, L., Pardon, P., & Augustin, J.-C. (2005). Influence of stress on individual lag time distributions of *Listeria monocytogenes*. *Applied and environmental microbiology, 71*(6), 2940–2948.

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

Koskar, J., Kramarenko, T., Meremäe, K., Kuningas, M., Sõgel, J., Mäesaar, M., … Roasto, M. (2019). Prevalence and numbers of *Listeria monocytogenes* in various ready-to-eat foods over a 5-year period in Estonia. *Journal of food protection, 82*(4), 597–604.

Koutsoumanis, K. (2008). A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. *International journal of food microbiology, 128*(1), 116–121.

Koutsoumanis, K. P., & Sofos, J. N. (2005). Effect of inoculum size on the combined temperature, pH and aw limits for growth of *Listeria monocytogenes*. *International journal of food microbiology, 104*(1), 83–91.

Mendonca, A. F., & Knabel, S. J. (1994). A novel strictly anaerobic recovery and enrichment system incorporating lithium for detection of heat-injured *Listeria monocytogenes* in pasteurized milk containing background microflora. *Applied and environmental microbiology, 60*(11), 4001–4008.

Meyer, R. R., Probst, G. S., & Keller, S. J. (1972). RNA synthesis by isolated mammalian mitochondria and nuclei: Effects of ethidium bromide and acriflavin. *Archives of biochemistry and biophysics, 148*(2), 425–430.

R Core Team. (2020). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Sibanda, T., & Buys, E. M. (2017). Resuscitation and growth kinetics of sub-lethally injured *Listeria monocytogenes* strains following fluorescence activated cell sorting (FACS). *Food Research International, 100*, 150–158.

Swaminathan, B., & Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and infection, 9*(10), 1236–1243.