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Heterogeneity of single cell inactivation: Assessment of the individual cell time to death and implications in population behavior

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ABSTRACT

A direct microscopic time-lapse method, using appropriate staining for cell viability in a confocal scanning laser microscope, was used for the direct assessment of *Salmonella* Agona individual cell inactivation in small two-dimensional colonies exposed to osmotic stress. Individual cell inactivation times were fitted to a variety of continuous distributions using @Risk software. The best fitted distribution (LogLogistic) was further used to predict the inactivation of *Salmonella* populations of various initial levels using Monte Carlo simulation. The simulation results showed that the variability in inactivation kinetics is negligible for concentrations down to 100 cells and the population behavior can be described with a deterministic model. As the concentration decreases below 100 cells, however, the variability increases significantly indicating that the traditional *D*-value used in deterministic first order kinetic models is not valid. At a second stage, single cell behavior was monitored in larger three dimensional colonies. The results showed that colony size can affect the inactivation pattern. The effect of colony size on microbial inactivation was confirmed with validation experiments which showed a higher inactivation rate for populations consisting of single cells or small colonies compared to those consisting of cells organized in larger colonies.

1. Introduction

Traditionally, the efficacy of processing or disinfection procedures is calculated based on the microbial mortality kinetics as reflected on the microorganism survival or inactivation curve. These curves are created based on experimental data of the number of surviving organisms which are plotted against the exposure time to the lethal agent (at a constant intensity). Since the studies-cornerstones of Ball and Olson (1957), Bigelow (1921), Bigelow and Esty (1920), Chick (1910), Esty and Meyer (1922) and Madsen and Nyman (1907) introducing the famous log-linear curve for the death of food related microorganisms, several studies assessing thermal or non-thermal microbial inactivation reported deviations from log linearity proposing that inactivation kinetics should be described by sigmoidal or by non-linear equations having three different phases, i.e. shoulder, exponential and tail (Casolari, 1988; Cerf, 1977; Geeraerd et al., 2000; Mafart et al., 2002; Peleg and Cole, 1998; Xiong et al., 1999). Despite the plethora of available primary models for the description of various shapes of inactivation curves including both linear (with or without shoulder/tail) and sigmoidal curves, there is no satisfactory unifying explanation for the observed variability in inactivation kinetics and the underlying biological mechanisms (Corradini et al., 2010). Several explanations have been proposed for the existence of shoulders or tails with two main approaches dominating, i.e. the mechanistic (Geeraerd et al., 2000) and the vitalistic (Peleg, 2000). According to the vitalistic approach, each microbial cell is characterized by its own resistance and dies at a specific moment, so the inactivation curve is considered as the cumulative distribution of lethal events reflecting the stress resistances of the population (Peleg and Cole, 1998).

Against this alternative vitalistic perspective, most of primary inactivation models are developed in a deterministic manner considering microorganisms' populations as a whole, ignoring major sources of variability that affect microbial responses and, especially, heterogeneity in the resistance of individual cells to a lethal stress (Casolari, 1988).

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Among the major sources of variability, biological variability which is associated with the microorganism of interest and can be referring to variations between strains of a given species or the cell to cell variation from a given species is of great significance (Aspridou and Koutsoumanis, 2015; Membré et al., 2006) and an important component of the latter source is the heterogeneity in the individual cell behavioral response (i.e. growth, survival or inactivation) (Aspridou and Koutsoumanis, 2015). The majority of microbial studies are conducted with high initial populations, where variability is masked and the system seems to behave deterministically (Aspridou and Koutsoumanis, 2015). In the field of predictive food microbiology, and after the establishment of Risk Analysis as the basis of food safety management, the importance of bacterial behavioral individuality was identified and several studies took it into consideration when developing mathematical models for the description of planktonic (Pin and Baranyi, 2006) or colonial (Koutsoumanis and Lianou, 2013) microbial growth. The acknowledgement of single cell heterogeneity came together with the technological and methodological advances driven by microscopy and image analysis (Elfwing et al., 2004; Koutsoumanis and Lianou, 2013; Siegal-Gaskins and Crosson, 2008; Wakamoto et al., 2005; Wang et al., 2010). Single cell division times' (Elfwing et al., 2004; Koutsoumanis and Lianou, 2013; Kutalik et al., 2005; Pin and Baranyi, 2006), growth boundaries' (Aguirre and Koutsoumanis, 2016; Koutsoumanis, 2008) and growth kinetics' (Koutsoumanis and Lianou, 2013; Pin and Baranyi, 2006) heterogeneity have been observed and were found to be variability sources in microbial growth. In contrast to microbial growth, very limited information is available regarding the impact of individual cell heterogeneity on microbial inactivation population dynamics.

In an earlier work of ours (Aspridou and Koutsoumanis, 2015), individual cell heterogeneity as variability source in microbial inactivation was assessed and characterized by applying a statistical modeling approach based on the probability distribution of individual cells inactivation times. However, the variability of individual cell inactivation times was evaluated indirectly, using the cumulative data from the inactivation curve from large bacterial population, resulting to a less accurate description of the distribution shape and, especially, of the tailing part. This right hand part of the distribution is really decisive for the variability in the behavior as well as for the population inactivation dynamics, since referring to the longest individual cell inactivation times, and a more accurate description of the individual cell inactivation times' distribution is required. As a result, the above approach provides less accuracy in assessing the information required for a risk-based safety approach (i.e. the probability of having at least 1 cell with a longer inactivation period than a particular treatment period).

The objective of the present study was to directly assess and characterize individual cell inactivation behavior as well as to evaluate single cell death as a source of variability in population inactivation dynamics. For this, a direct microscopic time lapse method was developed, using appropriate staining for cell viability, which can provide useful quantitative data of the actual inactivation times of the cells in a population and can be the basis of stochastic inactivation models. An additional goal was to examine if external parameters related to cell micro-community such as colony size can affect individual cell inactivation behavior under given stress conditions. For this, microscopic monitoring and population level experimentations were conducted.

2. Materials and methods

For the direct assessment of individual cell inactivation behavior, microscopic experimentation was conducted as described in detail below. In general terms, single cells of the pathogen were inoculated on the surface of solid laboratory medium and kept under optimum growth conditions till the formation of small two dimensional micro-colonies. Afterwards, the inactivation was initiated by the addition of the inactivation solution (NaCl) containing a fluorescent viability indicator. Individual cell inactivation behavior in small micro-colonies was monitored using time lapse microscopy.

For the study of the effect of the colony size on individual cell inactivation behavior, microscopic monitoring of single cell death in large three dimensional colonies was performed as in the case of small micro-colonies. Additionally, population level experiments were carried out for comparing the inactivation kinetics of a certain population of cells when being immobilized as individual cells or as submerged (preformed) colonies in solid laboratory medium.

2.1. Bacterial strain

The bacterial strain used in this study was a *Salmonella enterica* serotype Agona animal isolate, kindly provided by Dr. Martin Wiedmann (Cornell University, New York, United States of America). Agona serotype is related with several food poisoning outbreaks (EFSA and ECDC, 2018) while is also associated with low moisture food products (Santillana Farakos et al., 2014). Stock cultures of the strain were kept frozen (−70 °C) onto Microbank ™ porous beads (Pro-Lab Diagnostics, Ontario, Canada), while working cultures were stored refrigerated (5 °C) on tryptone soy agar (TSA; Lab M Limited, Lancashire, United Kingdom) slants and were renewed bimonthly.

2.2. Microscopy experiments

The strain was activated by transferring a loopful from TSA slants into 10 ml of tryptone soy broth without dextrose (TSB-G; Lab M Limited) and incubating at 37 °C for 16 h. 200 μl aliquots of the 16-h culture, after two serial decimal dilutions in one-quarter-strength Ringer's solution (Lab M Limited) were surface plated on 10 ml freshly prepared TSA solidified (approximately 1 h) in a 90 mm Petri dish. The inoculated agar was left to dry in a biological safety cabinet for 10 min and, then, a disc (of 10 mm diameter) was removed with a scalpel (with the aid of a preformed metallic disc), inverted, placed in a glass bottom dish (0.17 mm thickness; WillCo Wells BV, Amsterdam, The Netherlands) for microscopic observation. Attention was paid when transferring the agar disc in order not to damage it.

Individual cell growth and, subsequently, inactivation were directly monitored using an inverted confocal laser scanning microscope (TCS – SP5 II; Leica, Heidelberg, Germany) equipped with a SuperZ Galvo stage. The temperature and relative humidity were controlled using Cube 2 and Box for Leica as well as a semi-open stage top incubator for Leica SuperZ Galvo stage rotating insert and condenser.

The formation of micro-colonies originating from individual cells was monitored by Differential Interference Contrast (DIC) mode. The sample was maintained under optimum growth conditions (30 °C, 95% RH) for a total duration of 3–4 h till the formation of 2 dimensional micro-colonies consisting of maximum 100 cells. The same methodology was employed for larger three dimensional colonies but incubation time was longer.

After the formation of micro-colonies of the desired size, 2 ml of the inactivation solution (26% w/w NaCl) with 120 μl Propidium Iodide (PI) solution (1.0 mg/ml in water – filter sterilized, Sigma-Aldrich, Hannover, Germany) were added with care (slowly to avoid flushing of the cells) in order to fully cover the agar disc. Microbial inactivation was monitored using DIC and fluorescence mode simultaneously. Fluorescence of the cells was excited with the 488 nm argon laser line, while samples were observed with a 63x oil immersion objective. Time lapse observations were performed with Live Data Mode option. Images of the selected field of view were acquired every 5 min using Best Focus tool (Contrast based method) with 0.2 μm step size in z axis. The obtained sequences were reviewed and analyzed using a custom MATLAB analysis methodology called *Bacterial Single-Cell Analytics* (BaSCA) (Balomenos et al., 2017). BaSCA segments and tracks cells in a frame-to-frame manner. This novel methodology yields quantitative expression data of cell lineages, which can illustrate dynamic expression profiles and facilitate mathematical and computational modeling.

The employed methodology encompassed several steps starting from the application of image denoising techniques to subtract the noise of the input images and an additional preprocessing step so as to overcome the image analysis obstacles that are generated by DIC images, as discussed in Obara et al. (2013). Then, existing micro-colonies were automatically segmented as well as the cells lying into them. For more details about the segmentation methodology, an interested reader can refer to Balomenos et al. (2017). This methodology enabled the extraction of the contours of each cell, the quantification of their attributes and the measurement of PI inside their mask (cell surface) and, correspondingly, the PI coverage percentage (i.e. the percent of a cell's surface covered by PI). In order to measure the fluorescence in each frame, first a measurement of the fluorescence existing in the image's background (i.e. the image having subtracted colonies regions), considering it as uniform, was performed and this was used as the threshold to remove the noise existing in the micro-colonies region and, thus, to get more robust values of PI inside each cell surface. Afterwards, the BaSCA tracking and lineage reconstruction module (Balomenos et al., 2017, 2015) was employed to track cells in time and create the PI coverage cell trajectories. Finally, image data were extracted and stored within a single data structure which was then used for subsequent analysis of individual cell death.

Several preliminary experiments were conducted to check the protocol for cell staining. For more information, see Supplementary Material.

2.3. Plate count experiments

The inactivation kinetics of a certain population of cells when immobilized as individual cells or as submerged colonies in solid laboratory medium were studied. For this, the strain was activated by transferring a loopful from TSA slants into 10 ml of TSB-G and incubating at 37 °C for 18 h. Two types of samples were prepared: colonies and single cells. For the samples with colonial cells, a low initial population of cells (approximately 10 cells) was immobilized in solid laboratory medium and, after incubation under optimum conditions, submerged colonies with a total desired population concentration were formed before the initiation of the inactivation. For the samples with single cells, the desired population concentration was immobilized (as individual cells) in solid laboratory medium and the inactivation was initiated.

2.3.1. Samples with colonial cells

For samples with submerged colonies, the 18 h culture was centrifuged (model PK 120R, Thermo Electron Corporation, Walthman, MA, United States of America) at 6000 rpm for 20 min at 4 °C. The harvested cells were washed with Ringer's solution and, after proper serial decimal dilutions, a 4 ml aliquot containing approximately 400 cells was centrifuged as described previously. The harvested cells of the washed culture were resuspended in 2 ml Ringer's solution and evenly distributed in 20 Petri plates (35 mm). In each plate, 2 ml of TSA (previously prepared and kept at 45 °C) were added and mixed thoroughly. The samples were covered with parafilm to avoid water evaporation and left for 1 h to enhance solidification before being stored under isothermal conditions at 37 °C in high precision incubators (± 0.1 °C). The samples were incubated at this temperature till reaching the total desired population of 10^9 and 10^3 cells with the storage time, based on preliminary experiments, being 22 and 1.5 h, respectively.

2.3.2. Samples with single cells

For samples with single cells, the desired population (10^9 and 10^3 cells) was directly inoculated in the gel and, after solidification, the inactivation was conducted. Sample preparation was similar with the previously described one. More specifically, the 18 h culture was centrifuged as described above and the harvested cells were washed with appropriate amount of Ringer's solution and after serial decimal dilutions, if necessary to obtain the target population level, were centrifuged again. The harvested cells of the washed culture were resuspended in 2 ml Ringer's solution and evenly distributed in 20 Petri plates (35 mm). In each plate, 2 ml of TSA (previously prepared and kept at 45 °C) were added and mixed thoroughly. The samples were covered with parafilm to avoid water evaporation and left for 1 h to enhance solidification before being exposed to the inactivation conditions.

For the inactivation trial, 2 ml of TSB-G supplemented with NaCl (26% w/w) were added in each sample. The plates were covered with parafilm and incubated at 30 °C. The samples were analyzed in appropriate time intervals to obtain effective kinetic analysis of microbial inactivation while the total duration of the trial depended on the inoculum level used. Two independent experiments were conducted for each population level.

At regular time intervals, two plates for each type of sample (single or colonial) were analyzed. For each sample, the inactivation solution was discarded and the gel was transferred to a plastic stomacher bar (Sterile bags for Bag Mixer 400) and a known volume of diluent (Ringer's solution) was added. The sample was firstly homogenized by hand and subsequently by mixing twice for 120 s in a stomacher (BagMixer 400, Interscience, France). For the enumeration of the microorganism, 0.1 ml of appropriate serial decimal dilution of the homogenized gel was surface plated on TSA and incubated at 37 °C for 72 h.

3. Results and discussion

A direct microscopic time lapse method, using appropriate staining for cell viability, was used for the direct assessment and characterization of individual cell inactivation behavior. The growth of *S.* Agona single cells and the formation of micro-colonies (Fig. 1a) as well as the inactivation of single cells was monitored (Fig. 1b). The sequence of frames of the inactivation of single cells of the pathogen with time, transformed to a time lapse video, is quoted (Video 1). Time lapse microscopy is one of the most popular methods for the assessment of single cell behavior and cellular dynamics and when coupled with fluorescent markers can provide valuable information (Koutsoumanis and Aspridou, 2017) regarding, among others, cell viability. Propidium iodide is a fluorescent dye that binds to bacterial nucleic acids and is widely used as viability indicator. Based on membrane integrity, PI is permeant only in cells with compromised cell membrane, enabling the discrimination between dead and alive cells (Berney et al., 2007; Stiefel et al., 2015). In the protocol developed in the present study, the combination of DIC imaging with PI fluorescent staining allows for the identification of each cell in the micro-colony and the monitoring of the PI uptake by individual cells with time.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.fm.2018.12.011.

Since 'viability' is a term difficult to be defined (Davey, 2011) and 'death' is a non-easily detectable and quantifiable state, membrane integrity as a well-accepted criterion for alive cells (Gregori et al., 2001) was selected. In order to 'translate' the time lapse video into quantitative data of individual cells inactivation times, the in-house image analysis pipeline, BaSCA, (Balomenos et al., 2017) was used. With the aid of BaSCA, the percentage of the cell surface that is covered with PI was estimated for a total of 235 cells. The coverage data of each cell with time are presented in Fig. 2. In order to define the % of the cell surface coverage corresponding to cellular death, the PI coverage data P (%) for each single cell with time were fitted to modified Gompertz equation (1) (Zwietering et al., 1990):

$$P_i = P_{max} \exp\left(-\exp\left[\frac{\mu_c e(1)}{P_{max}}(\lambda_c - t) + 1\right]\right) \quad (1)$$

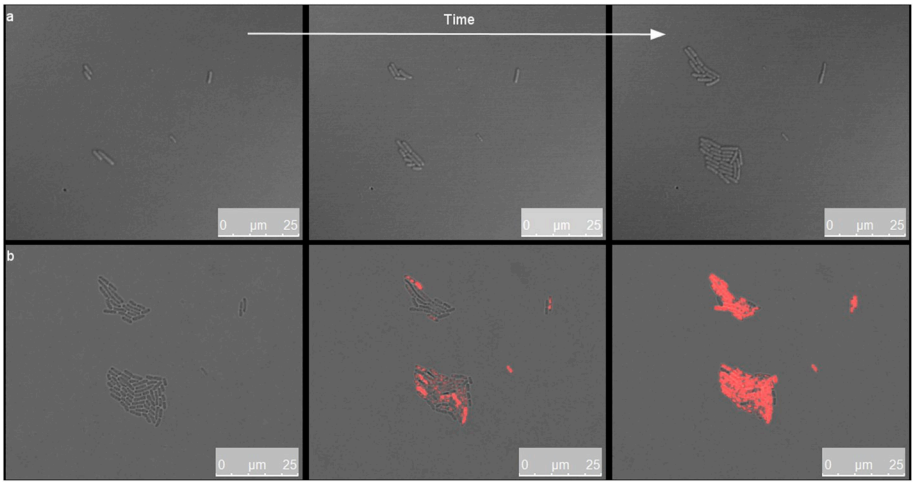

Fig. 1. Confocal micrographs (overlay of Differential Interference Contrast with fluorescence) of *Salmonella enterica* ser. Agona single cells (a) colony formation originating from single cells (on tryptone soy agar at 30 °C) at times 0min, 50min and 90 min and (b) inactivation (on tryptone soy agar disc at 30 °C after the addition of inactivation solution (26% w/w NaCl)) at times 0min, 130 min and 420min. As red are presented the cells with damaged membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

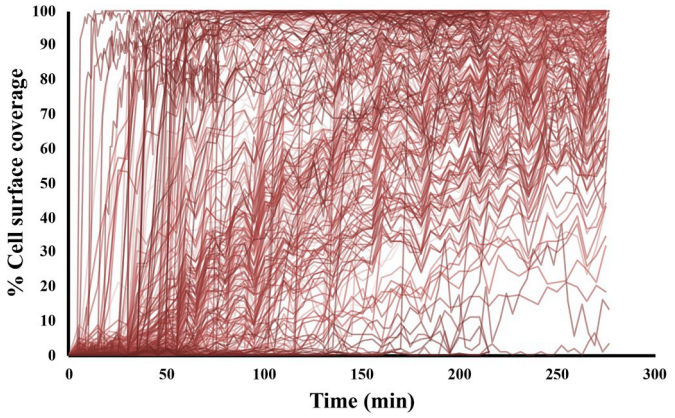

Fig. 2. Evolution with time of a total of 235 *Salmonella enterica* ser. Agona single cells' percentage of surface coverage with Propidium Iodide based on image analysis.

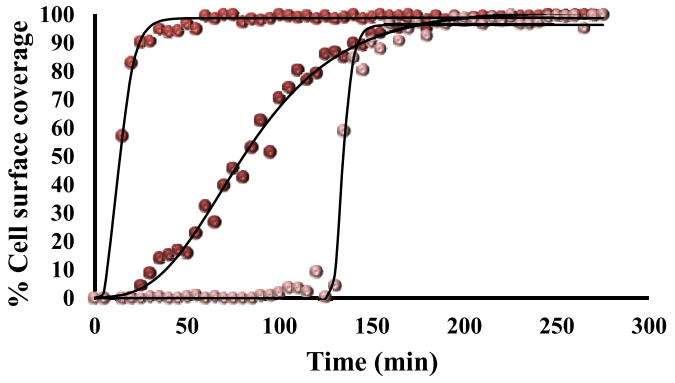

Fig. 3. Surface coverage with Propidium Iodide kinetics of three representative individual cells of *Salmonella enterica* ser. Agona. The line depicts the fitting of the modified Gompertz model to the observed cell surface coverage data as obtained by image analysis (symbols).

where t (min) is the time, P_t (%) is the percentage of cell surface covered with PI at time t, P_{max} (%) is the asymptotic P_t value at $t \rightarrow +\infty$, μ_c is the slope term of tangent line through the inflection point, and λ_c (min) is the geometrical lag time (t-axis intercept of the tangent through the inflection point). The duration of lag phase was obtained as the individual cell time of inactivation. From the equation, it can be demonstrated that the 6.6% of the cell surface is covered with PI at the time that corresponds to the geometrical lag time (Gougouli and Koutsoumanis, 2012). This low level of coverage is in accordance with the results of the calibration procedure where the percentage of the population reduction (plating method) was related to the fraction of PI covered cells (microscopic method) (Supplementary Material). This fitting approach was adopted since alleviates the noise characterizing the microscopic method. In Fig. 3 the PI surface coverage kinetics of three representative individual *S.* Agona cells are illustrated. As can be seen from the latter curves, the rapid increase in the PI uptake after the time corresponding to the geometrical lag time can be reasonably assumed to be explained by a change of the cellular state (membrane damage) sufficient to cause cell death (Crowley et al., 2016; Davey, 2011). The estimation of λ_c, based on the fitting of the cell surface coverage data to the primary model, for all the 235 cells revealed that the time of inactivation of individual cells is highly heterogeneous. The individual cell time to inactivation was found to range from only a few minutes to approximately 4.5 h with a mean and standard deviation of 57.1 and 40.6 min, respectively, despite the uniformity of the environmental conditions for all the cells. These findings confirm our previous results obtained indirectly from population level experiments through the statistical modeling approach applied (Aspridou and Koutsoumanis, 2015) where it was shown that the individual cell time to death can vary significantly constituting a source of biological variability in population inactivation dynamics. The direct microscopic assessment of single cell time to death enables the study and description of the actual time to inactivation distribution of the population of the pathogen under given stress conditions and improves the accuracy of stochastic modeling in microbial inactivation (Koutsoumanis and Aspridou, 2017).

Individual cell inactivation times were fitted to a variety of continuous distributions using @Risk 6.1 for Excel software (Palisade Corporation, Newfield, United States of America). The best fitted distribution (Kolmogorov- Smirnof statistic; 0.0634) was the *LogLogistic* with a location parameter *Gamma*, scale parameter *Beta* and shape parameter *Alpha* equal to 0, 45.957 and 2.8583, respectively (Fig. 4) (Supplementary Material). The LogLogistic distribution was further used to predict the inactivation of *S.* Agona populations of various initial levels N_0 using Monte Carlo simulation, with the number of iterations in each simulation being equal to N_0 and the number of simulations representing the variability in the population inactivation behavior. The microbial inactivation based on this approach can be described as follows (Aspridou and Koutsoumanis, 2015):

$$N_{t_i} = N_0 - Rank(t_i),\ t_i \sim LogLogistic(0, 45.957, 2.8583),\ i = 1,2,3,\ \ldots, N_0 \quad (2)$$

where t_i is a randomly selected value from the *LogLogistic* probability distribution of individual cell times of inactivation, N_0 is the population at time 0 and N_{t_i} is the population at time t_i. By repeating the above

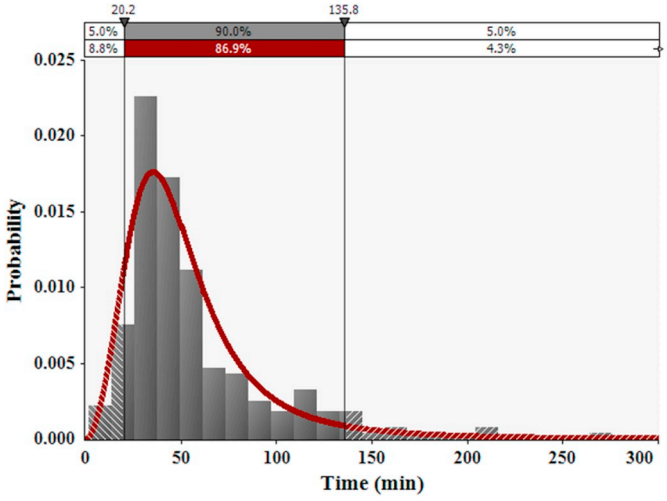

Fig. 4. Probability distribution of *Salmonella enterica* ser. Agona individual cell time of inactivation fitted to LogLogistic distribution.

process and performing several simulations, the variability of the population behavior can be evaluated. The iterations are random values from the distribution of individual cell time to death and the ranking provides the time intervals in which the population is reduced by one cell.

Fig. 5 is illustrating the Monte Carlo simulation results of the inactivation behavior of population initially consisting of 100 and 10,000 cells. For both cases, the variability in the predicted behavior is negligible for bacterial populations higher than 100 cells. At these high concentrations, the population evolution seems to be deterministic even though the underlying law is probabilistic. As the population decreases, however, the variability in the population inactivation behavior increases significantly since individual cell time of death determines the time for the total reduction. This is due to the probabilistic nature of the event that an individual cell with a certain inactivation time is present in the population. Indicatively, the time for total reduction for a population of 100 cells can range from 120 to approximately 550 min (100 simulations) (Fig. 5b) while the %CV of the time for total reduction (based on 1000 simulations) equals 54% supporting that the inactivation behavior of individual cells is a source of variability in the population inactivation behavior.

Microbial individuality is an inherent characteristic that is the apparent result of stochasticity in gene expression. Two cells are never at identical state since, often, show a high degree of variability in their cellular components. The fluctuations in the levels and activities of intracellular components, known as “molecular noise”, can lead to different behavior among genetically identical cells in a homogeneous environment (Koutsoumanis and Aspridou, 2017). Studies on bacterial responses to stresses have shown that noise can lead to fitness benefits for individual cells and enhanced adaptability and hardiness of the microbial population as a whole (Viney and Reece, 2013). Only recently, the heterogeneity in the behavioral responses of individual cells attracted the scientific interest since, traditionally, microbial studies were conducted with high microbial concentrations where heterogeneity is masked and ‘averaged’ outputs are obtained. However, heterogeneity exists and it is manifested when dealing with low microbial populations. From a theoretical point of view, the vitalistic concept has introduced years ago (Casolari, 1988; Peleg, 2000) the idea that the inactivation curve is the cumulative distribution of lethality events but only in a few studies a frequency distribution model has been applied to describe bacterial inactivation time (Couvert et al., 2005; Mafart et al., 2002). Nevertheless, these models have been used in a deterministic way which results in less accurate predictions in low bacterial populations since variability has not been taken into account (Membré et al., 2006). It is promising that, recently, more studies deal with the stochastic nature of microbial inactivation (Corradini et al., 2010; Koyama et al., 2017) while several methods are becoming available for ‘live’ observation of cell death (Skommer et al., 2010).

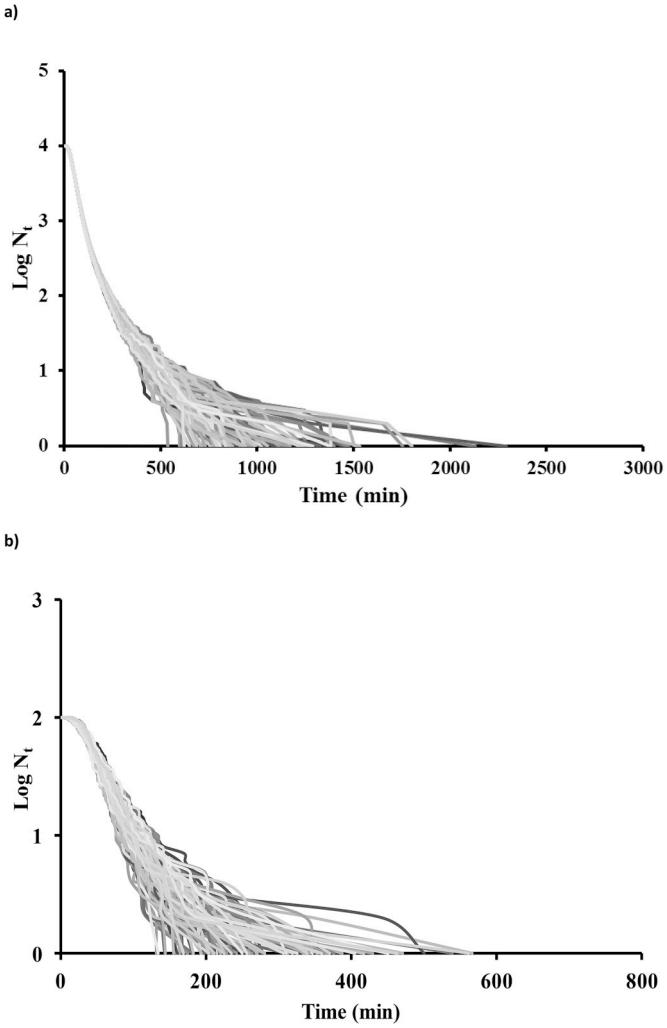

Fig. 5. Monte Carlo simulation results (100 simulations) for the inactivation of *Salmonella enterica* ser. Agona with initial concentration 10,000 cells (a) and 100 cells (b) based on equation (2) and the LogLogistic distribution of individual cell inactivation times.

The variability in microbial responses has practical implications for the control of pathogens. When it comes to food safety, traditionally, decisions about food processing design are taken based on deterministic approaches using point (mean) estimates, which are less accurate (Membré et al., 2006; Ross and McMeekin, 2003), and by adopting the ‘worst case scenario’ which leads to unrealistic estimations with negative impact on food quality. In the risk-based food safety management approach, however, all the major sources of variability affecting microbial responses including the prevalence and initial contamination, processing factors, food characteristic, storage conditions, strain variability and single cell variability are taken into account. The present study contributes towards this approach by describing the latter source of heterogeneity. More specifically, the knowledge of the distribution of individual cell time to death and the evaluation of the population inactivation dynamics enable the quantification of the variability of the number of survivors when applying a certain lethal stress to control microbial burden and can be used for decision making. By adopting such an approach, food industry can have answers about the probability of having one surviving cell after the application of a treatment for a

Table 1
Probability of having one cell with longer inactivation time than the duration of the treatment applied for various initial populations based on Monte Carlo results (500 simulations) using equation (2) and the LogLogistic distribution of individual cell inactivation times.

Initial Population	Time (min) of stress applied	Probability
100 cells	**1500**	0.004
	1000	0.02
	500	0.1
	300	0.36
	150	0.78
500 cells	**4000**	0.002
	2000	0.012
	1000	0.078
	500	0.44
	300	0.93
1000 cells	**4000**	0.004
	3000	0.008
	2000	0.01
	1000	0.142
	500	0.646
10000 cells	**8000**	0.004
	5000	0.022
	3000	0.066
	2000	0.208
	1000	0.77

specific duration based solely on variability of single cells (Table 1). Of particular applicability is, for example, the case of minimally processed products if there are available data regarding initial contamination. If all the sources of variability along the continuum 'from farm to fork' are taken into account, a final percentage of contaminated products with the pathogen is estimated. Based on this information, food industry can estimate or modify product characteristics or interventions that combine the mildest possible processing while ensuring food safety based on the acceptable level of risk set by Risk managers (Koutsoumanis and Aspridou, 2016). Single cell heterogeneity is not important only for food safety but can find application in other fields where control of pathogens is of great interest, such as decontamination and sterility procedures of surfaces, clinical settings, implants etc. Given the above, it can be understood that the highest possible accuracy in determining the heterogeneity in single cell behavior (along with all the sources of variability) and, in turn, in stochastic inactivation models is of high priority. Direct monitoring of single cell inactivation brings pathogen control one step closer towards this goal while providing also the chance to study external parameters that may affect the distribution of inactivation times such as colony size.

As can be observed from Fig. 1b, dead cells appear randomly in the small two-dimensional micro-colony indicating that their position in the colony does not affect the time of inactivation. Nevertheless, this was not observed in larger three-dimensional colonies (Fig. 6) where a characteristic ring inactivation pattern was observed (Video 2). The images show various z positions in the colony at the same acquisition time (120 min) and the same z position (bottom of the colony) at various acquisition times. It can be suggested that the inactivation times are affected by the position of the cells in the colony since the cells at the periphery are the first to be inactivated while, on the other hand, the cells in the colony center tend to be more protected with longer inactivation times, seeming to belong to another distribution of inactivation times.

In order to study the effect of the colony size on the microbial inactivation behavior, experimentations for comparing the inactivation kinetics of a certain population of cells when being immobilized as individuals or as colonies (formed in the gelifying medium) were conducted. Two different population levels were studied as presented in Fig. 7. Fig. 7a shows a comparison between two populations both consisting of 10^9 cells. In the first population, the cells are organised in colonies (approximately 10 colonies with 10^8 cells each) while the second population consists of 10^9 individual cells. As it is shown in the latter figure, the first population is more resistant with the difference between colonial and single cells, after 50 h, approaching 1.5 logs. These results indicate the protective effect of colony size on the inactivation of the pathogen. Several mechanisms could be responsible for this behavior such as the production of polymeric substances and the reduced diffusion of the inactivation agent, the reduced growth rate in the center of the colony or the stressful conditions of colonial growth compared to planktonic one (Aspridou et al., 2014; Skandamis and Jeanson, 2015) resulting in a reduced inactivation rate during the subsequent exposure to a lethal stress, the adoption of a distinct

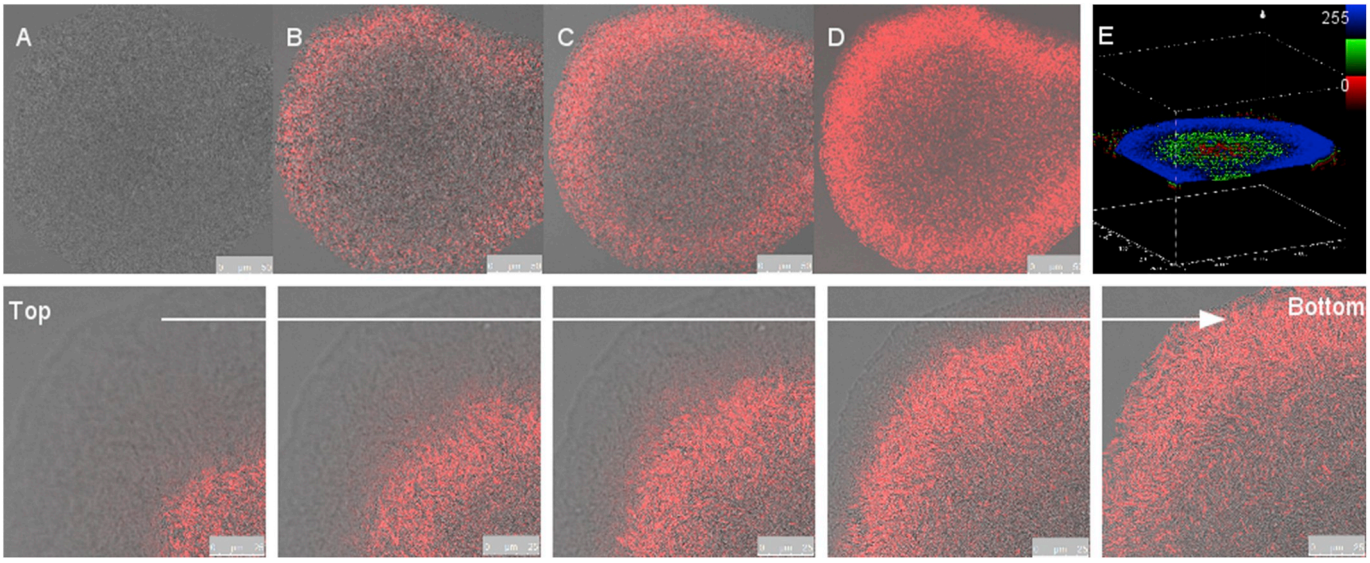

Fig. 6. Confocal micrographs (overlay of Differential Interference Contrast with fluorescence) of *Salmonella enterica* ser. Agona single cells inactivation in three dimensional colonies (on tryptone soy agar disc at 30 °C after the addition of inactivation solution (26%w/w NaCl)). As red are presented the cells with damaged membrane. The images (upper line) show the same z position (bottom of the colony) at various times ((A) 0, (B) 120, (C) 300, (D) 420min) and (E) the spatial distribution of PI intensity (at 420min) and various z positions in the colony at the same time (120 min) (lower line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

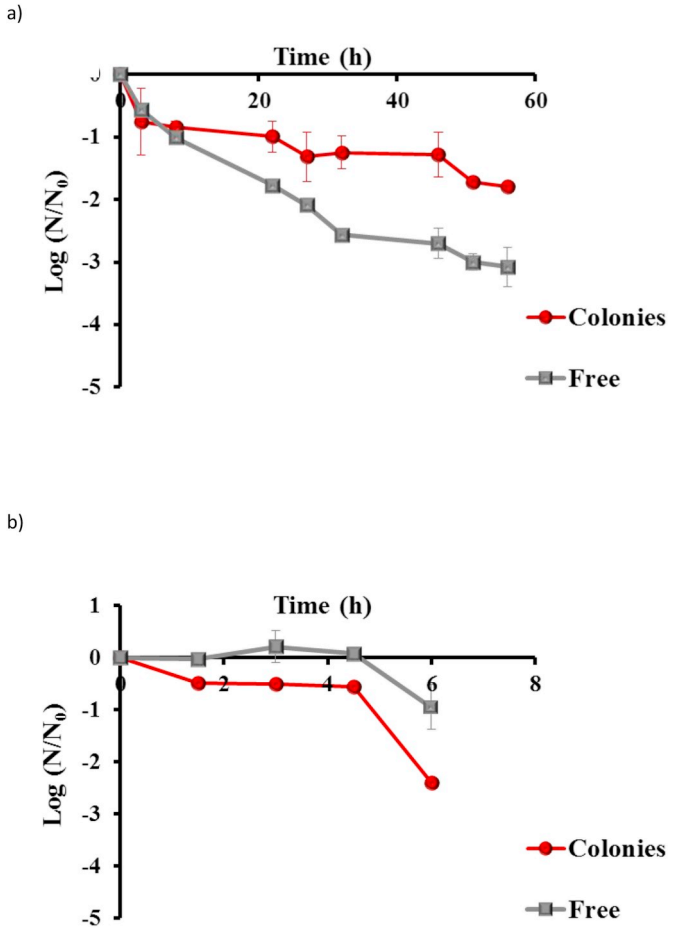

Fig. 7. Mean reduction ± standard deviation (Log (N/N_0) ± sd, n = 4) of *Salmonella enterica* ser. Agona population immobilized in tryptone soy agar (2 ml) as colonies (circles) or as single cells (squares) at 30 °C after the addition of 2 ml inactivation solution (26%w/w NaCl) with a total initial concentration of 10^9 (a) and 10^3 (b) cells.

phenotype as the result of altered gene expression from only a part of the population (Costerton et al., 1999) or even community (Allee) effect (Goswami et al., 2017) and quorum sensing (Gao et al., 2016). Whatever the biological phenomenon behind this increased survival, it seems that the cells organized in large colonies may follow a distribution of inactivation times other than single cells. Further research on the latter observation for other stresses (i.e. thermal, acid etc.) would be of great importance.

For a smaller colony size (10^3 cells), the protective effect was not observed (Fig. 7b). In this case, the organization of the cells in small colonies did not seem to confer protection while, on the contrary, the slightly increased survival of single cells could be attributed to the increased resistance that characterizes non-dividing cells (Costerton et al., 1999). Overall, it can be proposed that colony size, maybe among other parameters also, can affect the inactivation pattern of the pathogen and the distribution of individual cell inactivation times. These findings highlight the importance ot time and environmental conditions before hazard reduction steps. In food processing for example, storage time and temperature that support microbial growth and colony formation should be taken into account, since colony size affects individual cell time to death and, in turn, may affect the effectiveness of processes designed to control microbial load, most importantly when these processes have been designed based on planktonic cells or based only on the population size.

Conclusively, the direct assessment of individual cell inactivation behavior, using time lapse microscopy and appropriate staining for cell viability, showed that single cell time to death is highly heterogeneous and is a source of variability for population inactivation. This microscopic method enables the direct description of the distribution of single cells' times to inactivation, more accurately than indirect methods, and using Monte Carlo simulation a quantitative assessment of the variability in microbial inactivation can be performed. Beyond the scientific interest, the exploration and quantification of single cell variability has the potential to increase the accuracy in risk assessment models as well as to be the basis of stochastic microbial inactivation models for the development and improvement of risk-based designs and management systems.

Conflicts of interest

None conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.011.

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