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Not just variability and uncertainty; the relevance of chance for the survival of microbial cells to stress

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ABSTRACT

Background: The definition of realistic models for microbial risk assessment (MRA) requires the inclusion of variability as it is part of the microbial response to stress. Most studies are based on the hypothesis that the variation in the microbial response observed under laboratory conditions is due only to two sources: variability (e.g. differences between cells) and uncertainty (e.g. experimental error); disregarding the impact of chance. In this study, we perform a critical review of this hypothesis, evidencing it may be unrealistic because chance can be more relevant than variability and uncertainty in some scenarios.
Scope and approach: The impact of variability, uncertainty and chance for microbial survival is revised. Chance is identified as a possible relevant factor, different to variability and uncertainty because it is an inherent part of the system that is not associated with any biological mechanism. We derive probability distributions describing the impact of chance on microbial survival based on mechanistic hypotheses. These models are used to simulate inactivation experiments using a Monte Carlo algorithm.
Key findings and conclusions: Our analytical and numerical results demonstrate the relevance of chance for microbial survival and, more generally, for MRA. When the probability of one cell surviving the treatment is low, chance becomes more relevant than variability or uncertainty. Chance can also introduce non-linearities in the survivor curves (fanning and tailing) that are usually associated with uncertainty and/or variability. Therefore, chance is a relevant factor that should be considered in MRA besides variability and uncertainty.

1. Introduction

Human beings inhabit a varying world. Individual humans vary in physical aspects such as sex, weight or blood sugar levels. Moreover, the environment they live in is stochastic; e.g. temperature and humidity of the air vary within days, between days and between seasons. Microorganisms also inhabit a world that is not constant. Individual microbial cells have differences in their genome that make them respond differently to the same environmental condition (Den Besten et al., 2018; Koutsoumanis & Aspridou, 2017). Furthermore, the physicochemical properties of their environment also vary, affecting the microbial response. This effect is not just instantaneous but can have a lasting impact on the bacterial response. For instance, there is evidence that incubation conditions can affect thermal resistance of bacteria (Crespo Tapia et al., 2020). This makes estimating the risk associated with a foodborne disease using Quantitative Microbial Risk Assessment (QMRA) quite challenging, as risk models must consider the fluctuations at human and microbial scale. At the human scale, QMRA models should account for fluctuations in the logistic parameters of the food chain (e.g. storage temperatures or transport times), as well as differences between consumers (e.g. different sensitivities to a disease or different serving sizes). At the microbial scale, QMRA models should consider how differences in the genome of microbial cells and in their physiological state affect their response to the environmental condition they may encounter during the farm-to-fork chain of the product (e.g. ability to grow or survive stress).

In the context of QMRA, these fluctuations due to differences between members of the population (microbial cells, humans, retailers, refrigerators …) are categorized as "variability" (Schendel et al., 2018). On top of variability, QMRA studies are strongly affected by deviations associated with the use of partial or imperfect information (Schendel et al., 2018). These sources are usually categorized as "uncertainty". An

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example of uncertainty for QMRA is the use of predictive models with strongly empirical components. Due to the complexity of the system described, these models are built based on strong simplifications (e.g. log-linear kinetics) that may not describe the actual relationship between the variables or may omit environmental parameters relevant to the microbial response. This introduces an uncertainty in the model, usually called "model uncertainty". Furthermore, these models usually have parameters that must be estimated based on experimental data gathered under controlled laboratory conditions. Because every data point gathered under laboratory conditions is affected by experimental error (understood not just as "human error" but including other empirical limitations), models built based on empirical data are affected by an additional source of uncertainty (data uncertainty). Moreover, model parameters cannot be known with absolute precision, introducing an additional source of uncertainty: "parameter uncertainty". There will always be certain factors or conditions not exactly known and in principle in using models there is always some smaller or bigger extrapolation to other situations, either in time, location, conditions, etc. Note that, unlike variability, uncertainty is not part of the system and can be reduced by gathering further and/or better information (Nauta, 2000). For instance, model uncertainty can, in some cases, be reduced by using higher order reaction kinetics (Peleg & Cole, 1998) or by introducing new explanatory variables to describe the impact of additional environmental factors (Martinez-Rios et al., 2020). Experimental data will thus be affected by both variability and uncertainty. In this article, we use the word "variation" to refer to the magnitude of the fluctuations in an experiment; i.e. including any relevant sources of variability and uncertainty. In order to derive more realistic models, the different sources of variation relevant for QMRA must be understood and described using the most adequate probability distributions.

During the last decades, there has been a strong interest on how to include variability in QMRA studies, separating its contribution from the one of uncertainty (Thompson, 2002; Vose, 2008). Its practical interest is reflected in the efforts made by regulatory agencies to assess the relevance of variability and uncertainty in QMRA studies during the last years (EFSA Scientific Committee et al., 2018; Schendel et al., 2018). This is also relevant for food industries, as a description of variation is crucial for an effective control of microbial risks. This problem has been tackled from different angles. Several studies have extended classical models from predictive microbiology defining probability distributions for the model parameters to describe uncertainty and/or variability to simulate their impact on observations (Aspridou & Koutsoumanis, 2015; Garcés-Vega & Marks, 2014; Garre, Egea, et al., 2019; Koyama et al., 2019). Other studies have applied statistical analysis to quantify the different sources of variation based on an experimental dataset (Aryani et al., 2015; Clemente-Carazo et al., 2020; Garre et al., 2020; Jaloustre et al., 2012; Nunes Silva et al., 2020). Following a more fundamental approach, other studies have isolated cells within a population with extreme phenotypes (e.g. high stress resistance) and used modern molecular techniques to analyze the cause of their extreme behaviour (Maury et al., 2019; Metselaar et al., 2013). In spite of all these research efforts, the inclusion of variability in QMRA studies is currently an active research topic with many open questions.

All these studies considered that variation could be associated with the combination of two factors: variability and uncertainty. Therefore, the impact of pure chance was not explicitly considered. In this article we define **chance** as variation that is not linked to any biological property nor can be reduced by gathering additional data. An alternative term to "chance" with a similar meaning in some contexts would be "stochastic noise". However, because "stochastic" is commonly used as a synonym of random (e.g. "stochastic/random variables" or "stochastic/random processes"), we believe it could be mistaken with the umbrella term "variation". Moreover, we consider the term "chance" to be more familiar to scientists with a background in microbiology (e.g. this term is used by Keller and Taylor (2008) or Travisano et al. (1995)). Hence, we will use the term "chance" throughout this study to refer to variation that cannot be reduced by gathering additional/better information and cannot be associated with inherent factors of the system. As a final note regarding notation, "probability" will be used to refer to probability theory as a field, or to refer to the formal definition of a probability function (a map that assigns a value between 0 and 1 to each event in the event space).

Several fundamental studies have pointed out the possible relevance of chance for microbial survival. Nevertheless, their conclusions have not been transferred to applied studies (e.g. shelf life estimation or QMRA). It has been shown that genetically identical cells can present different phenotypes in identical environments (Elowitz et al., 2002; Munsky et al., 2012; Xia et al., 2014), even arguing that noise in gene expression can be an evolutionary advantage (Viney & Reece, 2013). On a molecular level, it has been proposed that the distinct behaviour of genetically identical cells can be due to gene networks with bistable dynamics, being able to act as a toggle switch (Balázsi et al., 2011; Gardner et al., 2000). Therefore, chance is different to uncertainty, as it cannot be reduced by gathering more and/or better information. Chance does not adhere to the definition of variability either. Variability is the result of "real differences between the members of a population" (Schendel et al., 2018), but chance is not the result of any biological or physical mechanism that is different within the population. In this article we illustrate the potential relevance of chance for QMRA using microbial inactivation as a case study. In section 2, we propose a thought experiment ("a device with which one performs an intentional, structured process of intellectual deliberation in order to speculate, within a specifiable problem domain, about potential consequences [or antecedents] for a designated antecedent [or consequent]" (Yeates, 2004)), that illustrates the importance of chance for the variation in the number of cells surviving a treatment. Then, in section 3, we propose stochastic models to describe the contribution of chance to the variation of the microbial count, combining it with other sources of variation (variation in the initial count and sampling error due to serial dilution). For this step we use probability distributions commonly used in various fields including QMRA (Poisson and binomial) because their simple hypotheses are the most suitable for the system we aim to simulate. These models are applied in sections 4 to 6 to illustrate the relevance of chance for the interpretation of thermal inactivation studies.

2. A thought experiment about variability, uncertainty and chance

Let us imagine a microbial cell whose genomic information is perfectly known; every gene has been sequenced and identified without error and is perfectly annotated. Moreover, every aspect related to its physiological state (e.g. mRNA) is perfectly described. For brevity, we will call this ideal microbial cell AGATA. Now, suppose that an inactivation treatment is applied with enough intensity to potentially inactive the microbial cell. For this thought experiment, we will assume that AGATA is exposed to heat stress, disregarding any medium effect that may be relevant for microbial inactivation (heat transfer, interaction cell-medium …). The question is: does AGATA survive the treatment? Considering the currently available scientific knowledge, we cannot answer this question unmistakably. Although there is some knowledge related to the genes involved in heat resistance (Hill et al., 2002; Richter et al., 2010; Smelt & Brul, 2014), the presence or absence of these genes does not ensure whether a microbial cell will survive a given stress. A similar statement can be made about the influence of the physiological state of the cell. Hence, at best, we can define a probability that AGATA will survive the treatment.

Now, we extend this thought experiment by considering an inactivation treatment to be applied to twelve identical AGATA bacterial cells, divided in three groups of four cells. For this exercise, it can be assumed that each copy of AGATA has a 50% chance of surviving the stress (note that this is an arbitrary number that does not affect the conclusions). Therefore, it is expected that half of the microbial cells (two cells per

group) survive the treatment. However, because every cell is identical, there is no preference about which copy of AGATA should survive. Furthermore, not every group will result in a number of survivors equal to the expected one. Considering that the inactivation of each cell is independent, their survival can be considered as a Bernoulli trial (independent random experiments with two possible outcomes), and the number of survivors follows a binomial distribution (Box et al., 2005). Hence, in this case, there is a probability of 0.0625 of 0 survivors, 0.25 of 1 survivor, 0.375 of 2 survivors, 0.25 of 3 survivors and 0.0625 of 4 survivors. Therefore, in spite of the population being homogeneous and the lack of knowledge gaps, this system shows variation in the number of survivors to the treatment purely based on chance.

As reviewed in the introduction, variation in QMRA is usually based on two concepts: variability and uncertainty. Variability is related to natural variation due to biological factors or other aspects of the system (Schendel et al., 2018). In this thought experiment an identical, homogeneous stress has been applied directly to groups of 4 identical microbial cells (i.e. with the same stress resistance). Therefore, there is no variability in this system. On the other hand, uncertainty refers (in the context of predictive microbiology) to the use of imperfect information, and can potentially be reduced by gathering additional and/or better data. However, we have hypothesized that the genetics and physiological state of AGATA are known perfectly and that the stress is applied directly to it. Hence, no further knowledge can be gathered to reduce uncertainty. It is certain that the probability of the survival of one cell is exactly 50%. It is true that, by the central limit theorem, the average of several repetitions of the experiments will have lower variation as the number of experiments is increased. Nevertheless, for each individual experiment, the probability of having 2 survivors when the treatment is applied to four copies of AGATA will always be 0.375. Therefore, this variation is not related to any inherent biological differences (variability), nor can it be reduced by gathering additional data (uncertainty). It is associated with a different source of variation independent of biological factors and that cannot be reduced: chance.

These results are in-line with the ones of various fundamental studies that identified chance as a relevant factor for the response of bacterial cells (Elowith et al., 2002; Munsky et al., 2012; Viney & Reece, 2013; Xia et al., 2014). It could be argued that chance is actually an uncertainty component because the variation in cell survival is due to some biological mechanism that is not yet known, so further knowledge would reduce this variation. However, even if this information was available, the mechanisms that are ultimately responsible for microbial inactivation are related to chemical processes that are inherently stochastic. On a molecular level, microbial inactivation can be due to, among others, damage of the cell membrane or the denaturation of key proteins (Smelt & Brul, 2014). Because these molecules (genes, mRNA, proteins) have low copy numbers within an individual cell (Paulsson, 2004; Yu et al., 2006), and also other molecules in the cell (see annex), their bimolecular reactions should be described as stochastic processes (Bressloff, 2017). Therefore, even with novel measurement devices and additional fundamental understanding, microbial survival will depend on the result of stochastic processes. In other words, the survival of individual cells will always depend, to some extent at least, on chance.

3. Definition of probabilistic models for the variation in the number of survivors and stochastic simulations

3.1. A stochastic model for chance

If the initial size of a microbial population under an inactivation treatment was to be represented by the discrete, positive quantity N_0 (i.e. with no variability). Assuming that the stress resistance of the members of the population is homogeneous (i.e. there is no variability due to any biological effect) each individual cell has exactly the same probability of surviving the stress, defined by the real number $p \in [0, 1]$. Let the discrete random variable N_f describe the number of survivors at the end of the inactivation treatment. Under the assumption that the inactivation of each cell is an independent Bernoulli experiment, N_f conditional on N_0 follows a binomial distribution of size N_0 and probability p (Equation (1)) (Nauta, 2001; Vose, 2008); a distribution that is commonly used to describe stochastic processes because it is based on very simple hypotheses. Consequently, the expected value of N_f is given by $E[N_f] = N_0 \cdot p$ and its variance by $Var[N_f] = N_0 \cdot p \cdot (1 - p)$.

$$N_f | N_0 \sim Binom(N_0, p) \tag{1}$$

Equation (1) can be generalized to describe the number of survivors at different time points of the treatment by defining the magnitude p as a function of time $p(t)$. Then, the number of survivors at time t ($N(t)$) conditional on N_0 follows a binomial distribution of size N_0 and probability $p(t)$, as shown in Equation (2).

$$N(t) | N_0 \sim Binom(N_0, p(t)) \tag{2}$$

We can propose functions for $p(t)$ based on models typically used in predictive microbiology (Perez-Rodriguez & Valero, 2012). It is common to describe microbial inactivation under isothermal conditions as an exponential decay process according to Equation (3). In this equation, the microbial count has been written as N_d to emphasize that this variable is deterministic not stochastic. In this model, the treatment time required to reduce 90% of the microbial population is given by the D-value (D).

$$N_d(t) = N_0 10^{\frac{-t}{D}} \tag{3}$$

We can equalize the expected value of $N(t)$ ($E[N(t)] = N_0 \cdot p(t)$) to the right hand side of Equation (3) to obtain a function for $p(t)$ that is equivalent to the log-linear model (Equation (4)).

$$p(t) = 10^{\frac{-t}{D}} \tag{4}$$

Note that by combining Equations (4) and (2), we obtain a model for $N(t)$ where the expected value of $N(t)$ equals $N_d(t)$. However, whereas N_d is a discrete constant, $N(t)$ is a discrete stochastic variable whose probability distribution is defined by the binomial distribution according to Equation (2) (e.g. $Var[N(t)] = N_0 \cdot p(t) \cdot (1 - p(t))$).

Although the log-linear model is commonly used to describe microbial inactivation under isothermal conditions, several studies have shown that survivor curves commonly deviate from log-linearity (Peleg & Cole, 1998; van Boekel, 2002). For that reason, it is common to introduce a deviation from log-linearity in the microbial response based on the Weibull distribution of the individual heat resistance as shown in Equation (5), where β is a curvature parameter and δ is the treatment time required to cause the first ten-fold reduction of the microbial count (Mafart et al., 2002).

$$N_d(t) = N_0 10^{-\left(\frac{t}{\delta}\right)^{\beta}} \tag{5}$$

Following the same approach as for the linear model, we can use a function for $p(t)$, so the expected value of $N(t)$ equals the value of N_d defined in Equation (5). A similar approach was followed by Santos et al. (2020). As already mentioned before, unlike $N_d(t)$, $N(t)$ is a discrete, stochastic variable whose probability distribution is defined by the combination of Equations (6) and (2).

$$p(t) = 10^{-\left(\frac{t}{\delta}\right)^{\beta}} \tag{6}$$

3.2. A stochastic model for chance and the variation in N_0

The stochastic model for $N(t)$ in Equation (2) can be extended to include the variation in the initial microbial count. For that, instead of defining N_0 as a positive, discrete constant, it can be defined as a discrete random variable (n_0). It is common to disregard any overdispersion (e.g. due to cell clustering) and consider that the initial microbial count

follows a Poisson distribution with expected value N_0; i.e. $n_0 \sim Pois(N_0)$ (Koyama et al., 2016). It can be demonstrated that for any two discrete random variables X and Y, if $X \sim Pois(\lambda)$ and $Y|(X = k) \sim Binom(k, p)$, then $Y \sim Pois(\lambda \cdot p)$. Substituting in Equation (2), a model that combines chance and the variation in the initial count can be constructed as shown in Equation (7). Note that, in this equation, $p(t)$ is defined according to Equation (4) or (6) depending on whether inactivation is log-linear or follows a Weibullian model.

$$N(t) \sim Pois(N_0 \cdot p(t)) \tag{7}$$

3.3. A stochastic model for chance, the variation in N_0 and the error of the serial dilutions

An additional source of uncertainty that contributes to the variation of the observed microbial count is the sampling error associated with the methodologies used, e.g. the serial dilution method used to determine the microbial count (Duarte et al., 2015; Garre, Egea, et al., 2019). Because the microbial count can vary in several orders of magnitude during an inactivation treatment, its surviving cells are diluted before plating through serial dilutions. Then, the number of survivors at time t is estimated based on the number of cells C counted on a plate of dilution d ($C(t, d)$) according to Equation (8), where f is the dilution factor (0.1 for decimal dilutions) and d is the number of decimal dilutions (e.g. 1 for the first decimal reduction).

$$N(t) = C(t, d) \cdot f^{-d} \tag{8}$$

According to the analysis by Garre, Egea, et al. (2019), the number of microbial cells in a plate after d decimal dilutions conditional to the number of survivors $N(t)$ follows a binomial distribution with parameters $n = N(t)$ and p_f^d. Using the same arguments as before and considering that (when the variation in the initial microbial count is considered) $N(t)$ follows a Poisson distribution, $C(t, d)$ also follows a Poisson distribution (Equation (9)) with expected value $\lambda = N_0 \cdot p(t) \cdot f^d$.

$$C(t, d) \sim Pois\left(N_0 \cdot p(t) \cdot f^d\right) \tag{9}$$

3.4. Computer implementation

All the calculations required for the analysis have been implemented in R version 3.5.3 (R Core Team, 2016). The probability distributions have been calculated analytically, except for the model including chance, variation in N_0 and the uncertainty of the dilutions (Equation (9)), which have been estimated using Monte Carlo simulations. Furthermore, to illustrate the impact of the different sources of variation on empirical observations, these have been simulated by uncertainty propagation using Monte Carlo simulations (Garre, Peñalver-Soto, et al., 2019). The convergence of the numerical algorithm was checked repeating the calculations for different values of the internal seed, without observing any difference in the results. The R code is openly available in the GitHub page of the first author (https://github.com/albgarre/chance-model). For simplicity, all the calculations have been done considering an experimental volume of 1 mL. Therefore from now on the number of cells is given as CFU, and this should be considered the number of cells in 1 ml.

4. The relevance of chance on the number of survivors in an inactivation experiment

Fig. 1 depicts the effect of chance (Equation (2)), and both chance and the variation in N_0 (Equation (7)) on the variation of the microbial count for a log-linear inactivation treatment with an (expected) initial count of 6 log CFU and a D-value of 5 min at a given temperature. In both cases, for short treatment times ($t < 20$ min), the expected microbial count is relatively high and the probability mass function is similar to the probability density function of a normal distribution. However, as the expected microbial count becomes smaller, the probability mass function gets more skewed, deviating from normality. This is expected, as the normal distribution is only a good approximation of the Poisson distribution when the expected value ($N_0 \cdot p$) is large (100 CFU can be defined as an approximate threshold).

As illustrated in Fig. 1, the impact of the variation of the initial count in the distribution of the number of survivors is only marginal compared to the one of chance. In all the simulations, the probability mass functions calculated with or without the variation of the initial count are practically identical. The low relevance of the variation of the initial

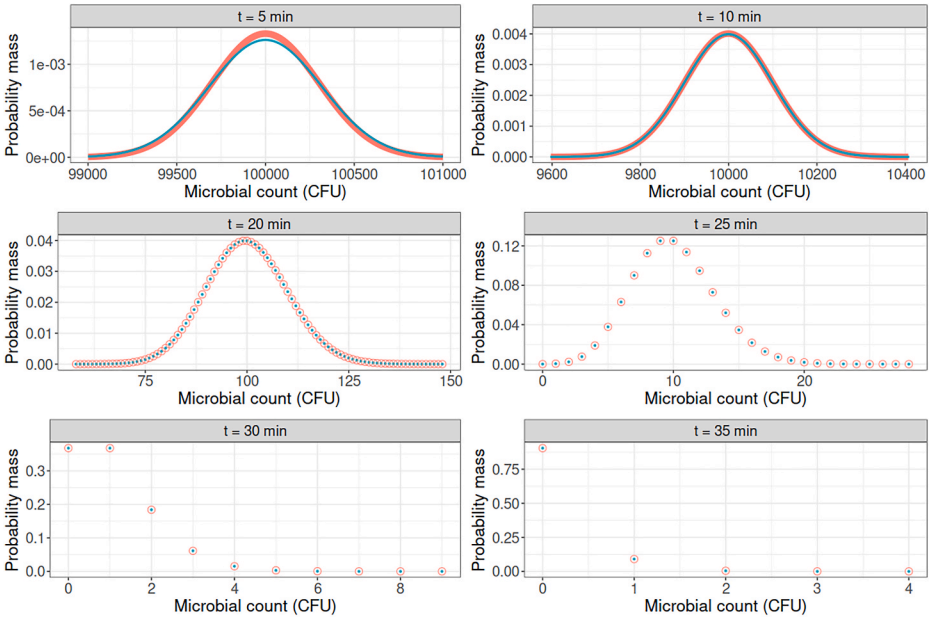

Fig. 1. Probability mass function of the microbial count ($N(t)$) for different time points of a treatment with an initial count of 6 log CFU and a D-value of 5 min. Probabilities have been calculated according to Equation (2) for the case with just chance, and Equation (7) for the case with chance and variation in the initial count. Red lines/points correspond to the calculations for the model with just chance, and blue ones to the ones of the model combining chance and the variation in N_0.

count is evidenced further in Table 1, where the standard error of the microbial count for different heating time points is reported. For treatment times higher than 10 minutes, there is practically no difference in the standard error of $N(t)$ calculated with or without the variation in N_0. The reason for this similarity is that $N(t)$ follows a binomial distribution when only chance is considered, and a Poisson distribution when the effect of the variation of N_0 is added. When the expected value of $N(t)$ is low (<1000 CFU), the probability mass function of both distributions is practically the same (provided they have the same expected value). Consequently, for long treatment times, there is practically no difference between the predictions of both models.

These calculations were done also for inactivation kinetics described by the Weibull model, obtaining similar results (see supplementary material). The reason for this is that the variation of the microbial count is described by a Poisson distribution, whose only parameter is the expected count. As a result, for the same expected count, both the Weibull and log-linear model calculate the same variation.

Regarding the variation associated with the error of the serial dilutions, for high microbial counts, it is more relevant than the variation in N_0 (Table 1). For $t = 0$ min, including this source of variation increases the standard error of the microbial count from 1000 to 99,938 CFU, one order of magnitude lower than the expected value (1,000,000 CFU). At the end of the treatment, however, the error of the serial dilution has a low impact on the total variation. The reason for this is that the magnitude associated with this source of variation is due to the sampling error. In these simulations, unlike in those by Garre, Egea, et al. (2019), we have considered that for the dilution zero the whole experimental volume (1 mL) is plated. Therefore, for this dilution there is no sampling error and, consequently, no contribution to variation.

Another variable that is of high interest for QMRA is the probability of at least one cell surviving the treatment. Because of chance, the probability of at least one microbial cell surviving the treatment is significant even for long treatments (Table 1). For a treatment of 35 min (expected microbial count of 0.1 CFU), there is a 9.5% probability of having one or more survivors; while for a treatment of 40 min (expected count of 0.01 CFU), there is still a 1% probability of at least one survivor. Therefore, chance is of high relevance for the probability of a microbial cell surviving an inactivation treatment.

In order to better illustrate this result, Fig. 2 depicts the variation in the probability of no cell surviving the treatment for a population with homogeneous stress resistance (thick, red line). The calculations were done considering log-linear inactivation (Fig. 2A), and weibullian inactivation with $\beta = 2$ (Fig. 2B) and $\beta = 0.6$ (Fig. 2C). Although the shape of the cumulative distribution function is affected by β, the same conclusions can be drawn in the three cases: chance is of high relevance.

According to Table 1, the variation in the initial microbial count and the error of the serial dilutions have practically no impact on the probability of one cell surviving the inactivation treatment when expected counts are low. This is due to the fact that these sources of variation mostly impact the distribution of the observations for high microbial counts (Fig. 1; Table 1). Hence, they are mostly irrelevant when describing the probability of few cells surviving the treatment. This is further depicted in Fig. 2, where the cumulative distribution function is plotted as a thin, blue line. This line practically overlaps with the function for the case where the variation in N_0 is not considered, illustrating that this factor has practically no influence on the outcome when the expected number of survivors is low.

These analytical results can be used to alleviate the computational burden of Monte Carlo simulations for QMRA. In most cases, the probability of vegetative cells surviving a pasteurization treatment is very

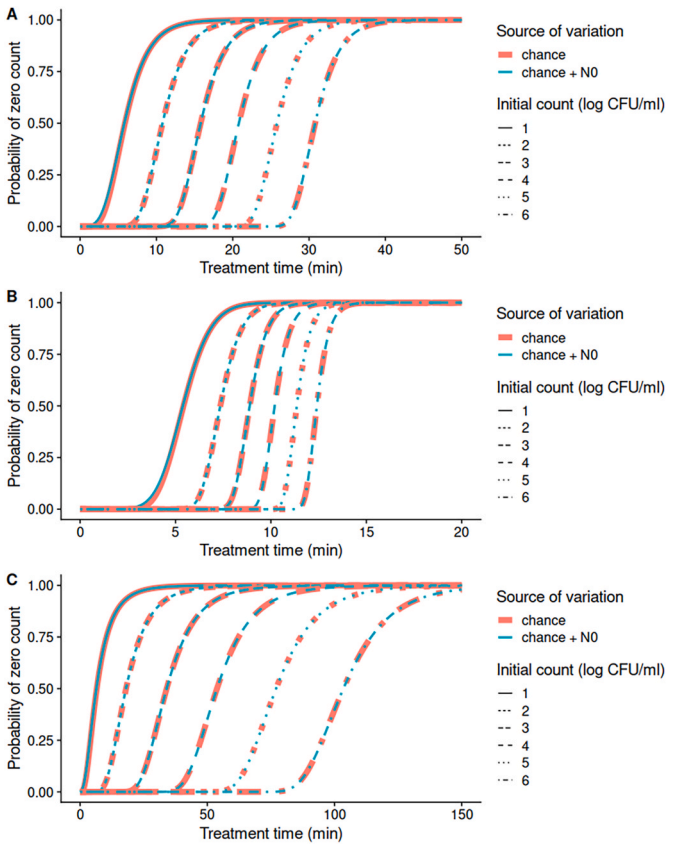

Fig. 2. Probability of no cells surviving inactivation treatments of different durations considering an initial count between 1 and 6 log CFU (linetype). The microbial kinetics are described by a log-linear model with a D-value of 5 min (A), the Weibull model with $\delta = 5$ min and $\beta = 2$ (B), and the Weibull model with $\delta = 5$ min and $\beta = 0.6$ (C). The colour of the lines represents the sources of variation considered in the simulations.

Table 1
Probability of zero count and expected standard error of the observed microbial count for different time points of a heat treatment with an initial count of 6 log CFU and a D-value of 5 min. The values considering variation due to *Chance* and due to *Chance* + N_0 have been calculated analytically (Equations 2 and 7), whereas those for *Chance* + N_0 + *dilution* have been calculated using 5000 Monte Carlo simulations per condition (time point X dilution).

		Probability of zero survivors			Probability of one or more survivor			Standard error of $N(t)$ (CFU)		
Treatment time (min)	Expected count ($N(t)$) (CFU)	Chance	Chance + N_0	Chance + N_0 + dilution	Chance	Chance + N_0	Chance + N_0 + dilution	Chance	Chance + N_0	Chance + N_0 + dilution
0	1000000	0.000	0.000	0.000	1.00	1.00	1.00	0.000	1000	99938
5	100000	0.000	0.000	0.000	1.00	1.00	1.00	300	316	10009
10	10000	0.000	0.000	0.000	1.00	1.00	1.00	99.5	100	998
15	1000	0.000	0.000	0.000	1.00	1.00	1.00	31.6	31.6	100
20	100	0.000	0.000	0.000	1.00	1.00	1.00	10.0	10.0	10.0
25	10	0.000	0.000	0.000	1.00	1.00	1.00	3.16	3.16	3.17
30	1	0.368	0.368	0.367	0.632	0.632	0.633	1.00	1.00	1.00
35	0.1	0.905	0.905	0.904	0.095	0.095	0.096	0.32	0.32	0.32
40	0.01	0.990	0.990	0.990	0.010	0.010	0.010	0.10	0.10	0.10

small (Zwietering et al., 2021). As a result, the microorganism would be absent in the majority of the Monte Carlo simulations. The probabilities of at least one cell surviving the treatment calculated analytically and reported in Table 1 can, therefore, be used to reduce the number of Monte Carlo simulations, limiting the number of calculations to just those with positive samples.

5. The effect of chance on the survivor curve observed in an inactivation treatment

Fig. 3 illustrates the potential effect of chance on the survivor curves observed under laboratory conditions. They show several effects that have been previously associated with variability and/or uncertainty. The simulated observations have a "fanning effect" (increased variation for low microbial counts) that has previously been associated with variability in the resistance of individual cells to the treatment (Abe et al., 2020; Aspridou & Koutsoumanis, 2015; Hiura et al., 2020; Koyama et al., 2019), or to experimental error (Garre, Egea, et al., 2019). However, as demonstrated in this work, the fanning effect can also be a result of chance. Moreover, the observations show an upwards curvature with respect to the expected response. This spurious tailing effect had already been observed empirically and had been attributed to the sampling error of the serial dilution and plating method (Garcés-Vega & Marks, 2014; Garre, Egea, et al., 2019). As shown in this investigation, this effect can also be due to the effect of chance.

The fact that models based on different hypotheses (variability, uncertainty or chance) are able to describe the same response poses a challenge for model validation. In this kind of situation, it is generally advisable to apply the principle of parsimony for model selection (Zwietering, 2009). For the analysis of variation, this could be approached by building several models, each including the contribution of a unique source of variation (biological variability, uncertainty or chance), and selecting the one that best fits the data. In case none of the models is able to describe the data, one could propose a model that combines several sources of variation. However, this approach could lead to spurious conclusions for the case of microbial inactivation. As already argued, empirical limitations can be mitigated but are unavoidable, chance is of high relevance when the probability of survival is low, and plenty of empirical evidence supports the hypothesis that (biological) variability affects the bacterial response to stress. Consequently, the variation observed empirically will be a combination of these three factors. In this case, if a model that only considers variability can describe the variance perfectly, it is very likely this model overpredicts the contribution of variability by assigning to this source the effect of chance and uncertainty.

As an alternative approach, we suggest that this type of study should start with the hypothesis that the total variation is a combination of variability, uncertainty and chance. Then, the goal of the study could be the quantification of the fraction of variation attributable to each source. As shown in this article, for chance and some sources of uncertainty, it is possible to derive the expected variation in the microbial response based on entirely mechanistic hypotheses. This is not the case for variability, which is not yet well understood and thus cannot be implemented in stochastic models using mechanistic hypotheses. Indeed, previous studies following this approach proposed models based on empirical hypotheses (Abe et al., 2020; Aspridou & Koutsoumanis, 2015; Hiura et al., 2020; Koyama et al., 2019). For this reason, we suggest as a starting point a stochastic model that includes the effect of chance and the one of those sources of uncertainty that can be described mechanistically (e.g. dilution error). The variation predicted with the model can then be compared against the one observed empirically. In cases where the observed variation is higher than the theoretical one, it is reasonable to propose new hypotheses (related to uncertainty and/or variability) to explain the variation that cannot be described by the model.

As an illustrative example, from Fig. 3 and Table 1, it is clear that the model including only chance and the variation in N_0 would not be able to describe all the variation in a typical inactivation experiment, especially at the beginning of the treatment. Therefore, this model could be extended including the dilution error, resulting in the simulations illustrated in Fig. 4. Although this model predicts a higher variance, the standard deviation of the log count is approximately 0.05 log CFU for microbial counts lower than 100 CFU (standard deviation of 1000 Monte Carlo simulations), still smaller than the one usually observed in inactivation experiments (~0.5 log CFU (Garre et al., 2020; Jarvis, 2008)). Therefore, an extension of this model considering other sources of variability or uncertainty could be reasonable. For instance, one could propose a stochastic model for the single cell time to inactivation describing population heterogeneities (recently reviewed by Aspridou and Koutsoumanis (2020)), or including the effect of the plated volume (Garre, Egea, et al., 2019). Nevertheless, this model should be added on top of the model based on chance. Otherwise, the model could overpredict the contribution of variability, assigning to this source of variation the variation due to chance.

6. The relevance of chance the interpretation of inactivation studies

Previous scientific studies analysing the survival of microbial cells to inactivation treatments were based on the hypothesis that variation could be attributed to two sources: inherent (biological) variability and uncertainty (that can be reduced with better information) (Den Besten et al., 2018; Koutsoumanis & Aspridou, 2017). The results from the previous sections have shown that chance can also be at least as relevant as variability and uncertainty for microbial inactivation when the number of survivors is low. Using our analytical equations, we have

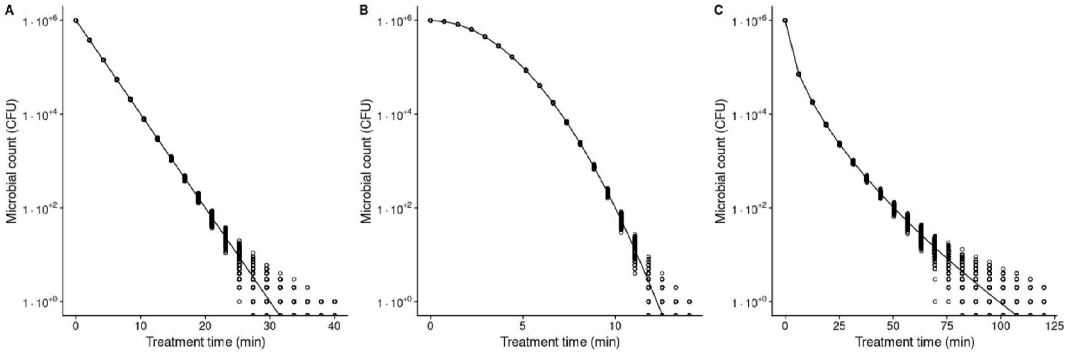

Fig. 3. Simulated microbial count in inactivation treatments considering the variation solely due to chance. The microbial kinetics are described by a log-linear model with a *D*-value of 5 min (A), the Weibull model with $\delta = 5$ min and $\beta = 2$ (B), and the Weibull model with $\delta = 5$ min and $\beta = 0.6$ (C). In every case, the initial count was fixed to 6 log CFU. The solid line represents the expected microbial count according to the deterministic model.

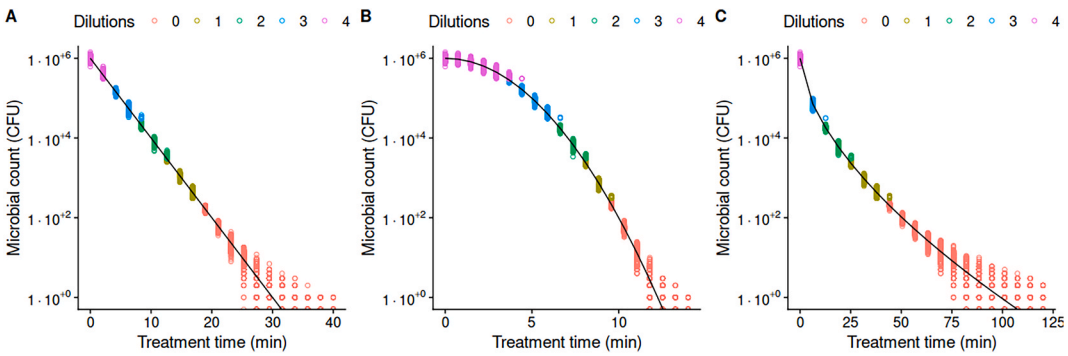

Fig. 4. Simulated microbial count in inactivation treatments considering the variation due to chance, the variation in the initial count (expected value of 6 log CFU), and the sampling error of the serial dilutions. The microbial kinetics are described by a log-linear model with a *D*-value of 5 min (A), the Weibull model with $\delta = 5$ min and $\beta = 2$ (B), and the Weibull model with $\delta = 5$ min and $\beta = 0.6$ (C). The solid line represents the expected microbial count according to the deterministic model. The dots are coloured according to the number of dilutions that were used for plating.

demonstrated the potential importance of chance, especially when the expected microbial count is low (<100 CFU). This result is of high importance for microbial risk assessment, as microorganisms usually have a low probability of surviving inactivation treatments applied in the food industry (Zwietering et al., 2021). Therefore, it raises the need for specific statistical methods to analyze and model the data at low microbial counts (Duarte et al., 2015; Garcés-Vega et al., 2014). Although there are some methods based on hypotheses that deviate from normality (e.g. the Most Probable Number method assumes a Poisson distribution (Alexander, 1965)), these methods usually consider a single probability distribution. In order for these methods to be able to describe the contribution of each source of variation (variability, uncertainty and chance), they must consider more complex statistical methods that include a combination of distributions (Garre et al., 2020). As already discussed in this article, this requires first a realistic identification of the relevant sources of variation (variability, uncertainty and chance), and their implementation as probability distributions that correctly describe the model hypotheses.

Another implication of our observations is that, in many situations, it is not possible to draw a clear line separating the contribution of the different sources of variation in risk assessment. For example, it is well known that we cannot know exactly the inactivation parameters of a specific outbreak strain, being uncertainty. Similarly, if we want to describe the general inactivation kinetics of *Listeria monocytogenes* and have to include strain variability, this variability is estimated from data, so it cannot be known exactly. In other words, it is affected by uncertainty. Therefore, drawing a clear line separating between variability and uncertainty may be unattainable in some cases. This observation is especially relevant when QMRA models are implemented using methodologies that require a strict separation between variability and uncertainty (e.g. second-order Monte Carlo) (Pouillot & Delignette-Muller, 2010; Vásquez et al., 2014)

The results of this investigation also impact the interpretation of studies with a more fundamental approach. Several studies have sought for biological markers of stress response, for instance by applying several cycles of inactivation treatments and studying the survivors (Metselaar et al., 2013). The conclusions of this investigation are very relevant for this kind of study. Through a thought experiment, we have demonstrated that identical cells can still show variation in their stress response. This does not imply that biological differences have no influence on the variation of microbial inactivation; there is plenty of scientific evidence suggesting otherwise. However, there will always be a "residual variability" due to chance, independent of biological factors. Therefore, the relevance of chance should be considered in this type of study. Otherwise, scientists may end up searching for biological markers of stress resistance in a population whose variation in survival is only due to chance.

Another point worthy of discussion is that the variation of the microbial count due to uncertainty and chance is unavoidable in experimental observations. In this article we have quantified the influence of chance and uncertainty in the variation of the microbial count. Because this estimation is based on fundamental hypotheses (i.e. not on parameter estimation), the prediction holds as long as the hypotheses are true. These hypotheses would be too conservative for actual experimental conditions (e.g. the stress resistance of the population will never be homogeneous), so they serve as a lower bound for the variation in the experimental observations. The calculation of such a lower bound has interesting implications. It is common for scientists to show concerns when reviewing a dataset with too much variation, suspecting a mistake in the experimental protocol. The existence of a lower bound for the observed variation implies that scientists should also consider with care a dataset where the total variation is smaller than the one defined by the lower bound (Table 1), and this should be considered as an indication of a mistake in the experimental protocol.

7. Conclusions

This article has demonstrated that, besides variability and uncertainty, chance can be very relevant for microbial risk assessment. Using microbial inactivation as a case study, we have derived analytical equations to describe the probability distribution of the microbial count for a homogeneous microbial population (i.e. every cell with identical stress resistance). Moreover, we have expanded this model by including the variation in the initial microbial count and the sampling error associated with the serial dilution counting method. Our analytical and numerical results demonstrate that chance is more relevant than these two other sources of variation for low microbial counts, and that chance is a strong determinant of the probability of single cells to survive a treatment. Therefore, this source of variation should be considered, as well as variability and uncertainty, when analysing the variation in the microbial response in risk assessment studies.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2021.10.033.

Annex I: The number of H^+ ions in a bacterial cell

The regulation of H^+ intracellularly is essential for bacterial cells, as this molecule is involved in many biochemical reactions required for cell growth and survival and the free H^+ concentration in a bacterial cell is rather constant at neutral internal pH. However, the abundance of free H^+ in a typical bacterial cell is extremely small. As an example, *Listeria monocytogenes* is a small (0.5–2 μm x 0.5 μm) organism (Beaufourt et al., 2014). If we consider the organism to be a cylinder of 1 μm (length) x 0.5 μm (diameter), its volume will be

$$V = \pi \times (0.25)^2 \times 1 = 0.2\mu m^3 = 2{\cdot}10^{-19} m^3 = 2{\cdot}10^{-16} liter$$

At pH 7, the number of H^+ ions in a liter is (considering the contact of Avogadro $N_A = 6{\cdot}10^{23}$ (number of particles/mol) and $[H^+] = 10^{-pH}$ (ions/mol)

$$n_{H+} = 10^{-7} \times \left(6{\cdot}10^{23}\right) = 6{\cdot}10^{16} \ ions/_{liter}$$

Then, the typical number of H^+ ions in a bacterial cells is

$$N_{H^+} = 6{\cdot}10^{16} \times 2{\cdot}10^{-16} = 12 \ ions/_{cell}$$

For smaller cells, the number of H^+ ions will be even smaller. For instance, a cell of 0.4 μm length and 0.2 μm diameter has a volume of 1.3×10^{-17} liter. At a pH of 7 then the number of H^+ molecules in a cell would be 1.3×10^{-24} moles, and this would be 0.75 molecules only. This would mean that at every moment there is a probability of 0.75 at any moment that there would be a free H^+ ions in the cell present.

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