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PROTECT

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Abstract

Fundamental microbial research is conducted at three levels, i.e., the macroscopic, the mesoscopic and the microscopic level. Macroscopic models can accurately predict population dynamics under non-stressing conditions in liquid food model systems and are preferred for process control, monitoring and optimization, as they have a rather simple structure. They are classified as primary models, describing microbial responses, secondary models, which describe the kinetic parameters of the primary models in relation to the changes of intrinsic and extrinsic parameters and tertiary models which turn the primary and secondary models into software applications. However, these models fail when applied to more realistic conditions, e.g., to real food products.

Mesoscopic modelling can be seen as a top-down approach in which macroscopic models are extended with microscopic elements to account for differences in cell behavior. The mesoscopic level studies small populations, part of the population like subpopulations or colonies in structured environments. To completely unravel mechanisms underlying the specific microbial response to, e.g., stressing environments or environmental gradients, information is collected at a microscopic level, i.e., a cellular or even intracellular level. The microscopic modelling approach considers biomass units or individual cells as the basic modelling unit. In this bottom-up approach, population or subpopulation dynamics emerge from the behavior of and interactions between these units.

Another example of unravelling the underlying mechanisms and incorporating intracellular information is the use of metabolic network models. The metabolic network-based modelling approach will ultimately link microscopic level information, enclosed in the metabolic network, with macroscopic level models which – in the end – are mechanistically inspired but still rather simple to use in practice.

An alternative approach takes into consideration uncertainty and variability and estimates the probability of microbial responses, which is important especially when conducting a risk assessment analysis. This is the so-called ‘Stochastic modelling approach’, which considers all possible circumstances with their associated probabilities to quantitative assess the probability that a food is unsafe.

To assess the impact of climate change on food safety, uncertainty driven by the lack of scientific data and unknowns related to this complex issue, should be taken into account. Due to the complexity of both microbial dynamics and climate change, a multidisciplinary research approach is the most suitable.

Introduction

Predictive microbial modelling provides a good quality in the final product and consumer safety. Predictive models are a powerful tool, which can be used for industry, food safety authorities, and education policies to predict the shelf life and safety of food processing options reflect microbial behaviour in foods (Stavropoulou and Bezirtzoglou, 2019). Predictive microbial modelling gives an exhaustive monitoring of the physicochemical factors that could affect the development of microbes (such as temperature and pH) as well as knowledge of the biological characteristics of the target microorganisms in foods in predicted conditions (Pérez-Rodríguez and Valero, 2013).

In predictive microbial modelling, mathematical models at a macroscopic level are used, to predict the growth, inactivation or survival of microorganisms in foods. They are constructed in such a way that the relationships between the inputs and outputs of a given system are developed. These are grouped on two main categories: intrinsic and extrinsic factors. Intrinsic factors comprise the physicochemical properties of the food itself like pH, a_w, and redox potential. Extrinsic factors are noted as all environmental factors responsible for limiting the microbial growth, such as temperature, relative humidity, and gaseous atmosphere (Ross et al., 2000; Stavropoulou and Bezirtzoglou, 2019). The input data are then statistically fitted to equations that can be used to predict the microbial growth, inactivation or survival under other conditions. Proper data collection and analysis are essential for the successful application of predictive modelling (Mermelstein, 2018; Valdramidis, 2016). An example is the use of kinetic models, which can predict the concentration levels of a given microbial strain, and thus the onset of the imminent risk could also be determined. These responses are calculated based on the rates of growth or death response (Pérez-Rodríguez et al., 2006).

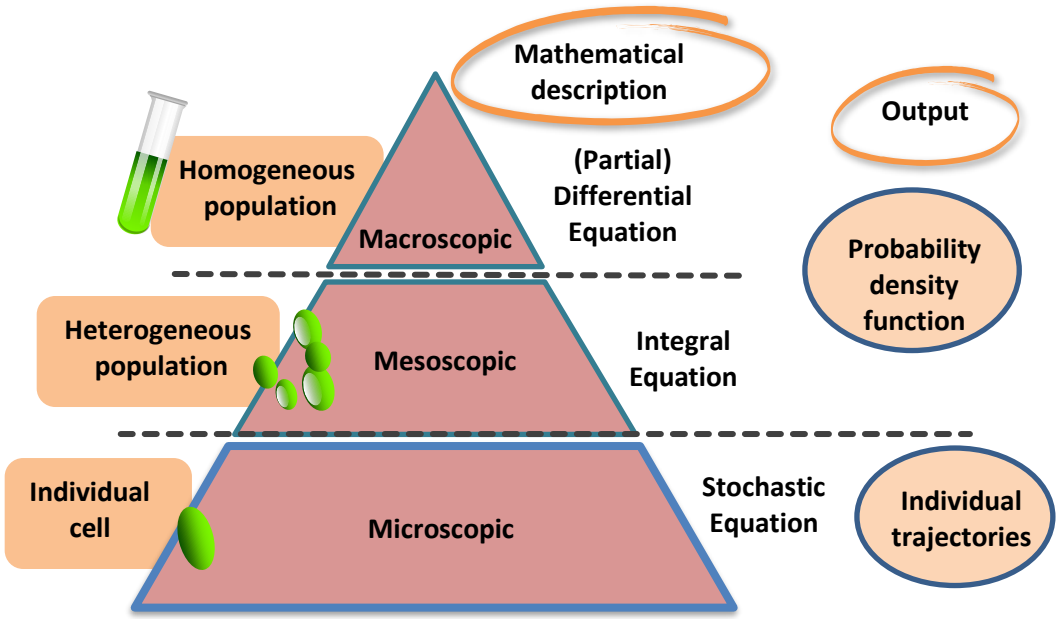

Figure 1: Description of different approaches to multiscale modelling.

Multiscale modelling is performed on different scales (Figure 1). These scales are specified by macroscopic, mesoscopic and microscopic. In the first approach, the population is considered as homogeneous, and the modelling is carried out based on the lumped properties of the cell wall and pores. The other is the mesoscopic modelling approach where the population is considered as heterogeneous, and the complex cellular structure is represented by the geometric model (Burger et al., 2018). Lastly, the microscopic approach which focuses on an individual cell (Burger et al., 2018; Rahman et al., 2016).

1 Macroscopic approach

There are several different data collection methods previously reported to collect data at macroscopic scale. The most common are the colony forming unit, optical density, flow cytometry, and the direct microscopy or impedance measurements.

There are three classifications of model types in predictive microbiology: i) primary models describing microbial responses such as population growth, survival or death in relation to time. ii) secondary models describe the kinetic parameters of the primary models in relation to the changes of intrinsic and extrinsic parameters such as temperature, pH, and water activity. iii) tertiary models which turn the primary and secondary models into software applications such as ComBase (Cummins et al., 2016; Pérez-Rodríguez, 2013).

The first type of macroscopic modelling is the **kinetics models based on colony-forming unit counts**. There are various primary models which have been reported in the literature the past years. Hereunder, a summary is provided of those that have been most widely used.

Generalised form of microbial responses

The most general structure which expresses the microbial behaviour as a function of time can be defined as follows (Bernaerts et al., 2004; Van Impe et al., 2005)

$$\frac{dN_i(t)}{dt} = \mu\left(N_i(t), < env(t) >, < phys(t) >, < P(t) >, < S(t) >, < N_j(t) >, ...\right) \cdot N_i(t) \qquad (1)$$

In this equation, $N_i(t)$ [CFU/mL] represents the cell density of species i at time t[h], and μ [1/h] is the overall evolution rate. Growth is sustained when μ is positive; however, when μ is negative, cells inactivate. The magnitude of the evolution rate is mainly determined by (i) the microbial environment, i.e., the physicochemical properties $<env(t)>$, (ii) the physiological state of the cells $<phys(t)>$, (iii) the concentration of the metabolic products $<P(t)>$, (iv) the availability of the substrate $<S(t)>$, and (v) interactions with other species $<Nj(t)>$.

1.1 Growth models

Several models have been used in the past to describe microbial growth kinetics. One of them, the Gompertz model, was reporting having some limitations in relation to the lack of biologically interpretable parameters. A modified version was introduced, by the Baranyi and Roberts (1994) by adding a model block to the logistic equation, which enables the description of the lag phase (Baranyi and Roberts, 1995). The main advantage of this structure is that it can be used in a dynamic environment, in combination with a secondary model. It is assumed that during the lag phase bacteria synthesise a hypothetical unknown substrate *Q*, which is critical for growth. Once cells have adapted to the new environment, they grow exponentially until the growth medium is depleted. Equation 2 defines the model in two differential equations:

$$\frac{dN(t)}{dt} = \frac{Q(t)}{Q(t)+1} \cdot \mu_{\max} \cdot \left(1 - \frac{N(t)}{N_{\max}}\right) \cdot N(t)$$

$$\frac{dQ(t)}{dt} = \mu_{\max} \cdot Q(t)$$

$$\lambda = \frac{\ln(1 + \frac{1}{Q_0})}{\mu_{\max}} \qquad (2)$$

with *N(t)* [CFU/mL] the number of cells at time *t*, *Q(t)* a measure of the physiological state of the cells, μ_{max} the maximum specific growth rate [1/h] and N_{max} [CFU/mL] the maximum cell density.

Three-phase linear growth model

This model describes the lag phase, the exponential growth phase and the stationary phase (Buchanan et al., 1997). This is static model, and therefore implementation under dynamic conditions is limited.

Lag phase: for $t \le t_{lag}$, $N_t = N_0$

Exponential growth phase: for $t_{lag} < t < t_{\max}$, $N_t = N_0 + \mu\left(t - t_{lag}\right)$

Stationary phase: for $t > t_{\max}$, $N_t = N_{\max}$ (3)

with N_t the log of the population density at time *t* [log CFU/mL], N_0 is the log of the initial population density [log CFU/mL], N_{max} the log of the maximum population density [log

CFU/mL], t the time, t_{lag} the time when the lag time ends, t_{max} the time when the maximum population density is reached, and μ the maximum growth rate [log CFU/mL/h]. The lag is divided into two phases: (i) a period to adapt to the new environment (t_a) and (ii) the time to generate energy to produce biological components needed for cell replication (t_m).

1.2 Inactivation models

The most frequently used primary inactivation model is the log-linear model proposed by (Chick, 1908), assuming that inactivation follows a first-order kinetic. A disadvantage of it is that the inactivation process is more complicated and the log-linear model is not valid.

$$\frac{dN(t)}{dt} = -k \cdot N(t) \tag{4}$$

with $N(t)$ [CFU/mL] the microbial cell density at time t, and k [1/min] the specific inactivation rate.

Non-log-linear modelling structures, like the model of (Geeraerd et al., 2000), describing inactivation during mild heat treatment but also implemented under several other conditions results in microbial survival or death.

$$\frac{dN(t)}{dt} = k_{\max} \cdot \frac{1}{1+C_c} \cdot \left(1 - \frac{N_{res}}{N(t)}\right) \cdot N(t)$$

$$\frac{dC_c(t)}{dt} = -k_{\max} \cdot C_c(t) \tag{5}$$

with $N(t)$ [CFU/mL] the number of cells at time t, N_{res} [CFU/mL] the residual cell density, $C_c(t)$ [-] a measure of the physiological state of the cells and k_{max} the maximum specific inactivation rate [1/min].

Other inactivation models are based on distributions of resistance or sensitivities between individuals in a microbial population like (Mafart et al., 2002)

$$\log N(t) = \log N_0 - \left(\frac{t}{\delta}\right)^p \tag{6}$$

with δ [h] the time for the first log reduction and p the shape parameter. If p<1, the model

corresponds to a concave upward function, when $p>1$ to a concave downward function, and for $p=1$ reduces to an exponential distribution.

For an overview of existing microbial inactivation models references is made to Geeraerd (Geeraerd et al., 2000).

In order to correlate the parameters of the primary models with intrinsic/extrinsic parameters the following secondary models are reported.

1.3 Models for correlating parameters of primary growth models

Zwietering et al. (1992) introduced the concept of dimensionless growth factors, known as the gamma concept:

$$\mu_{\max} = \mu_{opt} \cdot \gamma(T)\gamma(pH)\gamma(a_w) \tag{7}$$

with μ_{opt} the growth rate at optimal conditions, $\gamma(T)$, $\gamma(pH)$ and $\gamma(a_w)$ the relative effects of the environment. The gamma concept assumes that (i) the effect of any factor on the growth rate is described as a fraction of μ_{opt}, using the gamma function normalized between 0 (no growth) and 1 (optimum growth) and (ii) the different influencing factors act independently on the growth rate (Zwietering et al., 1992).

The cardinal parameter models (CPMs) models are related to the gamma equation (7) first introduced by (Rosso et al., 1995). These models embody parameters with biological meaning. Therefore, the models consist of a discrete term for each environmental factor, each with a value between 0 and 1. This way, each term is expressed as the growth rate relative to the growth rate when this environmental factor is optimal, and under optimal conditions $\mu_{max} = \mu_{opt}$. For instance, a CPM developed by Augustin and Carlier (2000) is expressed as:

$$\mu_{\max} = \mu_{opt} \cdot CM_2(T) \cdot CM_2(a_w) \cdot CM_1(pH) \cdot \prod_{i=i}^{n} \gamma(c_i) \cdot \prod_{j=1}^{p} k_j$$

$$CM_n = \begin{cases} 0, & X \le X_{\min} \\ \dfrac{(X - X_{\max})(X - X_{\min})}{(X_{opt} - X_{\min})^{n-z}\left[(X_{opt} - X_{\min})(X - X_{opt}) - (X_{opt} - X_{\max})((n-1)X_{opt} + X_{\min} - nX)\right]}, & X_{\min} \le X \le X_{\max} \\ 0, & X \ge X_{\max} \end{cases} \tag{8}$$

with X the environmental factor, X_{min} and X_{max} the values at the boundary of growth, X_{opt} the value of X for optimal growth, and n a shape parameter (Augustin and Carlier, 2000).

A square root model (Ratkowsky et al., 1982) was developed to explain the growth of microbes at suboptimal temperatures in which growth rate was square-root converted to stabilize variation:

$$\sqrt{\mu_{\max}} = b \cdot (T - T_{\min}) \tag{9}$$

with b a constant and T_{min} the theoretical minimum temperature for growth, being the intercept between the model and the temperature axis.

Polynomial models or response surface models have been constructed to describe linear or nonlinear the microbial responses as a function of the environmental factors. In general, this can be expressed as:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{j \neq 1}^{k} \beta_{jl} X_j X_l + \varepsilon \tag{10}$$

With y the dependent variable (e.g., growth rate), β_0, β_j, β_{jj} and β_{jl} the estimated regression coefficients, X_j and X_l the independent variables or the environmental factors and ε the error term. The lack of biological interpretation of regression coefficients and the possible overfitting, which leads to model experimental error results in a reduction in its utility. As a positive aspect, polynomial models are easy to implement in spreadsheet software (Cummins et al., 2016).

1.4 Models for correlating parameters of primary inactivation models

The Bigelow model is a log-linear and uses a kinetic model to describe the effect of inactivation. The parameters D (decimal reduction time) and z (change of temperature required to obtain a 10-fold change in the D-value) are characterized as necessary:

$$k_{\max} = \frac{\ln 10}{D_{ref}} \cdot \exp\left(\frac{\ln 10}{z} \cdot (T - T_{ref}) \right) \tag{11}$$

with k_{max} [1/min] the inactivation rate, D_{ref} [min] the decimal reduction time at the reference temperature T_{ref} [°C]. The D parameter is often used to describe the heat resistance properties of specific strains (Bigelow, 1921).

The Arrhenius model was originally developed to describe the effect of the temperature on chemical processes. The model assumes a linear relationship between the evolution rate and *1/T*:

$$\ln(\mu) = \mu_0 \cdot \left(\frac{\Delta E_a}{R \cdot T} \right) \tag{12}$$

with μ the inactivation rate, μ_0 a constant, T [K] the absolute temperature, R [J/(mol.K)] the universal gas constant and E_a [J/mol] the activation energy of the system. However, this linear relation is only valid for a limited temperature range, i.e., the normal physiological temperature range.

Another type of macroscopic kinetic models is reported in the literature and are based on the **biomass measurements**. These measurements are optical density (OD) readings, which are related to the biomass of the microbial cells. However, two essential limitations have to be considered: (i) the high detection limit of the technique and (ii) the non-linearity of the responses (Dalgaard & Koutsoumanis, 2001).

Different techniques were used to model growth parameters from data obtained with optical density, but the most effective is the time to detection (TTD) method. The TTD is a final method to estimate the maximum specific growth rate and lag phase duration based on OD data (Cuppers & Smelt, 1993). The TTD can be defined as the time required for a detectable increase in OD, and is related to the growth parameters by the following equation:

$$TTD = \lambda + \frac{1}{\mu_{\max}} \cdot \ln\left(N_{turb} / N_i\right) \tag{13}$$

with *TTD* [h] the detection time, λ [h] the duration of the lag phase, μ_{max} the maximum specific growth rate [1/h], N_{turb} the cell population at detection time [CFU/mL] and N_i the initial inoculum level [CFU/mL].

Figure 2 represents the TTD method graphically. First of all, a detection limit should be defined under the technical specifications of the spectrophotometer, but moreover to include the environmental conditions as they can influence the growth curve. Secondly, based on a previously determined calibration curve relating ln(OD) (optical density) to ln(N) (plate counts), N_{turb} can be determined. When the detection limit is selected and N_{turb} determined, the time required (TTD) to reach this detection limit is computed for the different inoculum levels (Cummins et al., 2016).

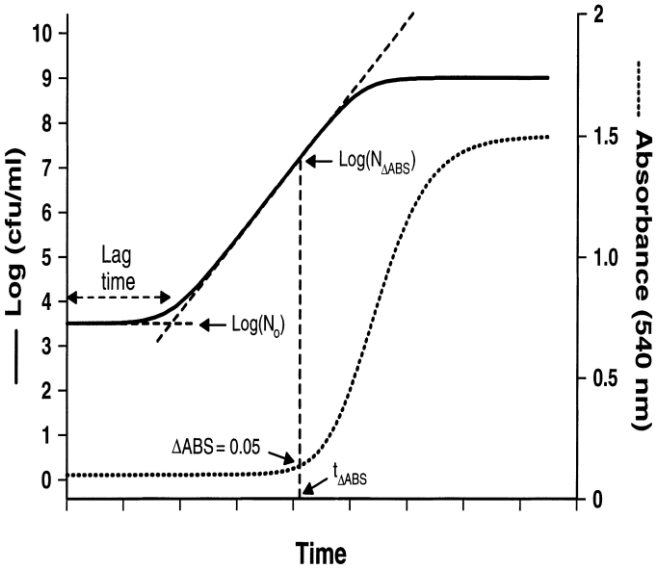

Figure 2: Graphical representation of the TTD method (Dalgaard and Koutsoumanis, 2001).

The third technique of the kinetics models is based on the **flow cytometry measurements**. This technique is based on the measurement of parameters such as the fluorescence of individual cells in a fast-flowing current (Alcon et al., 2004). Related to predictive microbiology and more specifically on a macroscopic level, models based on flow cytometry are rare. For instance, flow cytometry methods were applied to determine the cell viability, damage and death of *Saccharomyces cerevisiae* after heat or ultrasound treatment and the D-value was used to quantify the success of the inactivation treatments (Wordon et al., 2012).

The last technique is based on the **binary responses**. Microbial responses are described by using a logistic type of model when different responses within a population are observed. These models include growth/no growth models, survival/death models, recovery/no recovery models and spoilage/no spoilage models (Valdramidis et al., 2009). These type of models are often applied (i) to assess the microbial stability of a food product, (ii) to design the product or (iii) to design maintenance methods. Logistic type models are based on binary responses, which can be analysed using a broad spectrum of data collection methods, including colony-forming units, optical density, flow cytometry or manual counts using direct microscopy (Cummins et al., 2016).

In ordinary logistic regression models, the use of the logit function enables to predict the probability for a given specific set of conditions, by relating $logit(p) = ln(p / (1-p))$ (with p the probability of the studied event, i.e., growth, survival, recovery or spoilage) to a polynomial expression. A general second-order equation, including three environmental factors, can be expressed as:

$$logit(p) = b_0 + b_1 \cdot x_1 + b_2 \cdot x_2 + b_3 \cdot x_3 + b_4 \cdot x_1^2 + b_5 \cdot x_2^2 + b_6 \cdot x_3^2 + b_7 \cdot x_1 \cdot x_2 + b_8 \cdot x_2 \cdot x_3 + b_9 \cdot x_1 \cdot x_3 \quad (14)$$

with b_i (i=1,… 9) the parameters to be estimated and x_i different environmental factors. In general, the use of the logistic type models is limited, because as a model is only valid for the specific conditions (i.e., environment, microorganism, inoculum) under which constructed.

1.5 Applications of macroscopic models

One team used two models to evaluate the kinetics of *L. monocytogenes* in fishery products, measurements of OD under different atmosphere conditions such as decreased oxygen and aerobic environment was realized. The growth curves were estimated by applying the Baranyi model for the maximum growth rate (μ_{max}) of them. Furthermore, Ratkowsky square root model was used to estimate the effect of storage temperature on μ_{max}. All developed models were validated. The using of developed models were provided safe predictions for *L. monocytogenes* in the fishery products (Bolívar et al., 2018).

Another team (Wedzicha and Roberts, 2006) described a model for the microbial interaction and the death of *Escherichia coli* O157:H7 during the fermentation of green table olives. For this purpose, two different starter cultures and various amounts of glucose and sucrose were used. During fermentation, high amounts of lactic acid were produced under these stressful conditions. In this study, the death of *E. Coli* O157:H7 was evaluated by a differential equation including multiple factors such as pH, the protective effect of the substrate, and protonated lactic acid.

Other authors (Arroyo et al., 2005) for studying the growth of the yeast *Pichia anomala* in olive fermentation, applied four primary models; the modified Gompertz, modified logistic, modified Richards-Stannard, and lastly, the Baranyi-Roberts model. Hence, the maximum specific growth rate (μ_{max}) and lag phase period from the growth curves were defined. Despite the good fit of all models, the modified Gompertz and Richards–Stannard models were shown to be the most proper.

However, other authors, by studying the inactivation of *Salmonella enterica* serotype Agona, concluded that kinetics models are valid only for large populations, as in small populations, the D-value presents a high variability due to the cell heterogeneity of the population. The authors proposed characterization by a probability distribution to quantify the variability in the inactivation of mixed microbial populations (Aspridou and Koutsoumanis, 2015).

The influence of the pulsed light technology (PL) on the kinetics of *Bacillus cereus* spores surviving the treatment was studied. PL seems to effect on the kinetic parameters of the microorganism. The μ_{max} reduced with increasing intensity. A polynomial regression was adjusted between the μ_{max} of the survivors and the final inactivation. As a result, PL treated foods would have more extended shelf-life than those operated by other thermal or irradiation procedures (Aguirre et al., 2015).

In an attempt to estimate the heat resistance of *Escherichia coli* K12 MG1655 during thermal treatments was studied by one team (Valdramidis et al., 2006). A variety of models were used to predict population and to define heating scenarios; the dynamic sigmoidal model of Geeraerd (2000), the Bigelow model, the modified Dabes model, and finally a MatLab software. As a result, microbial heat resistance is extremely perceived at the lowest heating rates considered.

2 Implementing mechanistic knowledge

Traditionally, models are classified into two types based on the information used to construct the model: mechanistic and empirical models. Mechanistic (also reported as deductive or white box) models are based on general laws and understanding the underlying phenomena governing the system. These models are constructed without the need to perform any experiments. These types of models generally don't apply when it comes to predictive microbiology. In turn, empirical models (also reported as inductive or black box) simply describe the observed response and have as starting point the available data. Black box models are only valid in a specific range of operating conditions for which they are designed and are most suited when little a priori process knowledge is available, but large amounts of process data are accessible. When combining both mechanistic and empirical knowledge, which is a common practice in predictive microbiology, the outcome is a so-called grey box model. The more mechanistic the modelling approach is, the more reliable and robust the obtained predictions will be.

As previously mentioned, fundamental microbial research is conducted at three levels, i.e., the macroscopic, the mesoscopic and the microscopic level (Figure 1). Multi-scale models serve as linkage between different spatial levels. At the macroscopic level, the overall microbial population characteristics and behaviour are studied. Macroscopic models can accurately predict population dynamics under non-stressing conditions in liquid food model systems. Macroscopic models are preferred for process control, monitoring and optimization, as they have a rather simple structure, i.e., a limited number of model components and parameters (Baranyi and Roberts, 1994). However, these models fail when applied to more

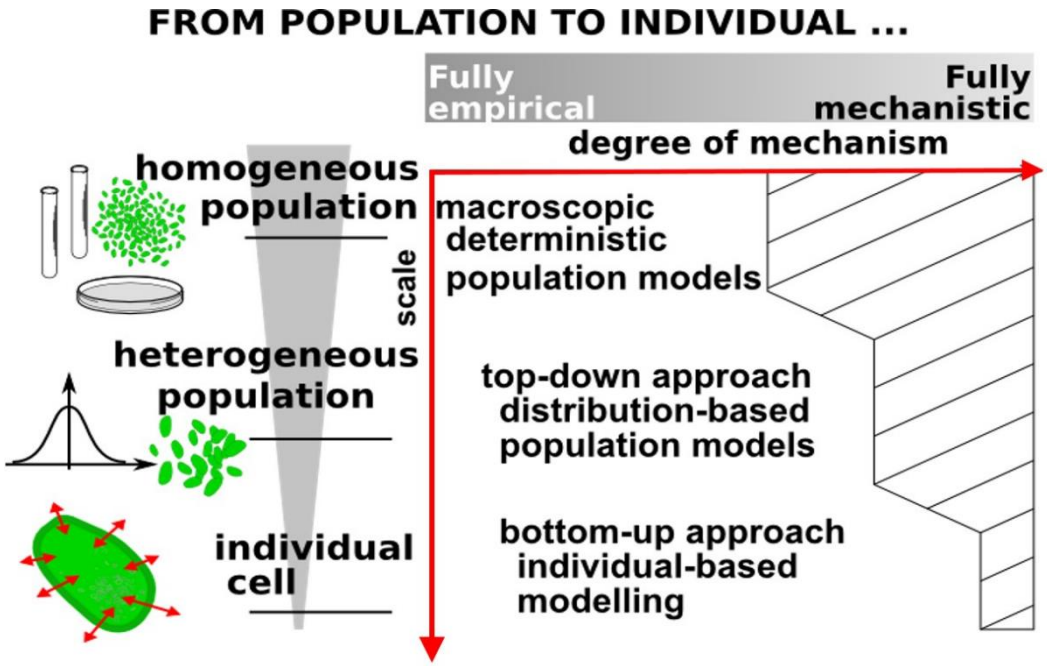

Figure 3: Description of different approaches of multiscale modelling (Smet et al., 2017)

realistic conditions, e.g., to real food products and under more stressing conditions. These models consider rather simple liquid systems, mainly influenced by temperature, pH, water activity, acids and preservatives. More complex elements, like background flora, microbial competition, stress and stress adaptation, and physico-chemical properties of the food structure are rarely considered. This is described as the *completeness error* and is considered as (one of) the largest source of error in predictive microbiology (McMeekin and Ross, 2002). Furthermore, it has been reported that the variability of single cells increases under more stressing conditions (Ratkowsky et al., 1996). In simulations of complex microbial dynamics this variability cannot be ignored, especially when the microbial population is low. Therefore, including mechanistic, physiological and molecular knowledge from new experimental techniques and systems biology models in micro- or mesoscopic models is essential.

2.1 Mesoscopic approach

Mesoscopic modelling can be seen as a top-down approach in which macroscopic models are extended with microscopic elements to account for differences in cell behavior. The mesoscopic level studies small populations, part of the population like subpopulations or colonies in structured environments. Environmental or population heterogeneity is considered as well and differences in the microbial response are observed. In this approach cells – or their dynamics – can no longer be assumed as identical. In McKellar (1997) and Skandamis et al. (2002) typical mesoscopic models have been developed. In the former, a modified model was presented to predict the effect of the temperature on the growth of *L. monocytogenes* (between 5°C and 35°C), while in the latter the effect of low pH, chill temperatures and oil on *Salmonella* and *E. coli* strains in Greek salads have been modelled. The mesoscopic modelling approach focuses on those aspects of the individual cells that are needed to explain the observed population-level characteristics, it does not aim to include detailed knowledge about the biological mechanisms inside the cell. Hence, the individual cells are not regarded as discrete entities, but the population is subdivided in multiple discrete subpopulations. For instance, the population can be subdivided into growing and nongrowing compartments to model lag phases (McKellar, 1997; McMeekin and Ross, 2002) or into heat-sensitive and heat-resistant subpopulations (Van Derlinden et. al., 2009). To completely unravel mechanisms underlying the specific microbial response to, e.g., stressing environments or environmental gradients, information is collected at a microscopic level, i.e., a cellular or even intracellular level. The last decades a lot of research has focused on the intracellular stress response, e.g., the heat shock response of *Escherichia coli* (Arsène, Tomoyasu and Bukau, 2000; Yuk and Marshall, 2003; Chung, Bang and Drake, 2006).

2.2 Microscopic approach

The microscopic modelling approach considers biomass units or individual cells as the basic modelling unit. In this bottom-up approach, population or subpopulation dynamics emerge from the behavior of and interactions between these units. In grid-based or biomass-based models (BbM), the environment is represented as a grid of discrete spatial units, characterized by the local biomass concentration and environmental conditions (e.g., nutrient concentration). A biomass-based model for spatially dependent growth of *Listeria monocytogenes* in heterogeneous systems has been described by Hills and Wright (1994). The two-dimensional environment is subdivided in square patches, where the increase in biomass and viable cell number is dependent on the acidity and the local concentrations of nutrients, oxygen and growth-inhibiting metabolic products. In addition, the influence of temperature, salt concentration and preservatives on biomass growth and cell proliferation is incorporated in the model. A second example of biomass-based modelling in predictive microbiology is the two-dimensional model of Dens and Van Impe (2001) to simulate spatial variability for microbial growth, including the competition between two species in structured foods.

To include direct intercellular interactions, microbial cells need to be described as discrete entities in individual- or agent-based models (IbM/AbM). IbMs describe global dynamics of a system in terms of its composing individuals or agents. In this case, the microbial cell is considered as the basic modeling unit and population dynamics are not implemented explicitly but emerge from processes at the microscopic cellular level (Tack et al., 2015). They are conceived to deal with a high level of detail, including spatial and microbial heterogeneity, randomness and interactions in a straightforward way. However, this high level of complexity can lead to significant computational costs. This burden is often exacerbated by the stochastic elements in these models, necessitating multiple repetitions of the same simulation in a Monte Carlo analysis to give statistical significance to the simulation results. In addition, microscale experimental information about food structures and cellular behaviour is required to construct IbMs. As this knowledge is currently not yet available, assumptions need to be made, introducing possible inaccuracies in the model. A population model can be considered as IbM, if four prerequisites are satisfied. These are: (i) the variability of the cells even of the same characteristics (e.g., age, size), (ii) the considerations of the resources dynamics, (iii) the characterization of the population size should be made in real or natural numbers, (iv) take into account the complexity of the individual's life cycle in the model (Uchmański and Grimm, 1996). If all these prerequisites are not satisfied, then the models can be considered as 'individual oriented', rather than 'individual-based' (Ferrer et al., 2009). Generally, IbMs are an appropriate tool to incorporate (i) mechanistic data from -omics databases at cell and molecular levels, (ii) information about individual biological variability, and (iii) knowledge about interactions between cells and environment.

One of the first microbial IbMs was applied in the BacSim simulator of Kreft et al. (1998) to study colony behavior of *E. coli*. BacSim was implemented as an extension of the Gecko framework (Booth, 1997), an Objective-C software toolkit based on Swarm for the simulation

of ecosystem dynamics. The two-dimensional aerobic environment is split up in a grid of uniform square patches. Glucose concentrations in these discrete spatial units are updated each time step according to a diffusion process. Microbial cells are represented as circular disks and take up glucose from the spatial patch in which their center is positioned. The consumed glucose is used to satisfy maintenance requirements and to generate new biomass. If the growing cell reaches a critical volume for division, the cell is split in two equal daughter cells. However, if the cell volume drops below a critical death value, the cell lyses. Spatial overlap between the microbial cells is prevented by cellular repulsion in the continuous space, implemented by a cell shoving algorithm. BacSim has been replaced by iDynoMiCS, hence it is not further developed or supported.

A second simulator for the study of bacterial cultures, INDISIM (INDividual DIScrete SIMulations), has been developed by Ginovart et al. (2002). The INDISIM model has similar structure to the BacSim schedule. Both models include nutrient diffusion and uptake, bacterial growth, maintenance, cell division and lysis. However, in INDISIM, the spatial coordinates of the microorganisms are integers and cellular movement occurs in a discrete space. INDISIM was originally implemented in Fortran, but has been ported to the user-friendly and widely used NetLogo toolkit in order to make it available for a general scientific community including non-experts in programming or microbiological modelling (Ginovart and Prats, 2012).

2.3 Metabolic network modelling

The applicability and reliability of existing models under more realistic scenarios can be improved by looking inside the black box, i.e., unravelling the underlying mechanisms and incorporating intracellular information (Brul and Westerhoff, 2007). As more knowledge about the underlying mechanisms of biochemical processes becomes available, new opportunities arise. One such example is the use of metabolic network models to build next generation predictive models. These metabolic networks are a blueprint of the reactions that occur inside the microorganisms during biochemical processes. They can range from small-scale representations of a metabolism containing only the most important reactions, up to genome-scale metabolic reaction networks covering all reactions based on the sequenced genome. For *E. coli* a widely used medium-size network is the core metabolic network described in Orth et al. (2010). This network is made up of a subset of the reactions of a genome-scale metabolic reconstruction of *E. coli*. Reconstructions of this type are also available in different sizes, depending on the amount of information that is contained in the model.

Metabolic flux analysis (MFA) is an excellent tool to gain in-depth insight (i.e., at the intracellular level) on the impact of environmental conditions on (the fluxes in) the cell metabolism and growth dynamics. Relevant (extracellular) process conditions and key metabolic reactions/pathways can be identified, which is valuable information in the development of predictive models for more complex and realistic situations. Exploitation of

MFA as a technique to develop accurate mathematical models in the field of predictive microbiology is a largely unexplored domain. In MFA the extracellular fluxes (uptake and secretion) are measured such that the current phenotype (i.e., the observable characteristics of the microorganism) can be determined. The difficulty in this method is obtaining the measurement data that are required to solve this system. This means the complexity of the network that can be used, depends on how many measurements can be taken on the different extracellular metabolites of the microorganism under study. The more metabolite concentrations or fluxes are measured, the more complex the network becomes. The less possible measurements there are, the less complex the network that can be used to describe the intracellular dynamics is. The optimization problem to be solved in this method is a parameter estimation problem. MFA has, however, one large downside, it is unable to solve fluxes for parallel metabolic pathways, bidirectional reversible reaction and cyclic pathways (Bonarius et al., 1998; Schmidt et al., 1998; Wiechert, 2001).

Metabolic networks generally tend to be underdetermined, i.e., the number of unknown variables/fluxes in the network is significantly smaller than the measured components. For the application of metabolic network models in predictive microbiology, the search space for these models needs to be reduced. A first reduction is obtained by posing a steady-state constraint yielding a subspace of possible solutions. The metabolic network may acquire any flux distribution within the allowable solution space, but points outside this space are denied by constraints. These constraints can be obtained based on (bio)chemical knowledge, such as measured fluxes, reaction capacities and physicochemical properties (Gianchandani, Chavali and Papin, 2010). Additional constraints can be introduced by defining one or more fluxes explicitly by kinetic reactions like Michaelis-Menten, or by defining specific relations between certain fluxes (Haag, Wouwer and Bogaerts, 2005). Generally, a metabolic network with reduced complexity is preferred to obtain a better understanding of the metabolism of a studied microorganism. Such a reduction of the size and complexity of the network can be achieved by the removal of certain unwanted reactions and metabolites. These models can be reduced by using certain reduction tools e.g., NetworkReducer. Only a set of carefully selected key variables, reactions (e.g., branch points) and regulatory interactions, vital to describe the studied dynamics, will be retained to build a structured model within the limits of acceptable complexity. Even though the metabolic network model has been reduced in complexity, most often the model remains underdetermined. To tackle this problem, a Flux Balance Analysis (FBA) approach can be used. Through optimization of an objective function, FBA can identify a single optimal flux distribution that lies on the edge of the allowable solution space. In FBA, intracellular flux distributions are optimized with respect to the objective function. A major challenge is to harmonize the objective function used at macroscopic (population) level with the objective function used at microscopic (cell) level. In FBA the free flux parameters are predicted by defining a cellular objective function, which has to be minimised by modifying the fluxes. So, it is assumed that the cells' behaviour is optimal with respect to a known objective. The fluxes are in this case the control variables. For this method, measurements are not a requirement but can be added as a constraint to the

optimization problem (Vercammen, van Derlinden and Van Impe, 2011). Other constraints that are typically added are irreversibility and capacity constraints. The objective function can be for instance cell growth, negative side product formation or wanted end product (Varma and Palsson, 1994; Ramakrishna *et al.*, 2001; Boyle and Morgan, 2009; Zheng, Hu and Peng, 2009). In FBA, the natural regulations and dynamics of the pathways in the metabolic network are not considered, i.e., the fluxes within the network are predicted while optimizing the objective function. This means that the main assumption of FBA, i.e., the cell has evolved to achieve an optimal behaviour, has an important drawback: the optimal solution may not correspond to the actual flux distribution.

The metabolic network-based modelling approach will ultimately link microscopic level information, enclosed in the metabolic network, with macroscopic level models which – in the end – are mechanistically inspired but still rather simple to use in practice.

3 From deterministic to stochastic modelling approach

As explained in detail in the previous sections, predictive microbiology is the knowledge of microbial responses to environmental conditions, based on the integration of mathematical models into traditional microbiology. The last few decades predictive microbiology has been widely used to evaluate food safety and quality during process, distribution and storage (McMeekin et al., 1993).

Risk analyses marked the beginning of a new era in food safety management (Lammerding, 1997). The introduction of risk analyses in food safety management was a milestone in predictive microbiology history. Codex Alimentarius guideline underlined the importance of taking into consideration variability and uncertainty when conducting a microbial risk assessment (Codex Alimentarius, 1999), especially when conducting a quantitative microbial risk assessment (Thompson, 2002). Hence, moving from a hazard-based approach to a risk-based approach, the use of traditional predictive microbiology models proved to be problematic (Koseki et al., 2011). Traditional predictive microbiology models are based on a deterministic approach. Most of the predictive models for growth, survival or inactivation are deterministic e.g. use points estimates and their outcome is a single value.

Hence, an alternative approach, which could take into consideration uncertainty and variability and estimate the probability of microbial responses, was identified as an urgent need (Nicolaï and Van Impe, 1996), especially when conducting a risk assessment (Nauta, 2002). The so-called ‘Stochastic modelling approach’ was proposed in order to tackle this issue. Stochastic modelling can take into account all possible circumstances with their associated probabilities to quantitative assess the probability that a food is unsafe. Once the mathematical model is developed and the variability of the input factors is estimated through a distribution, propagation method such as Monte Carlo simulation can be implemented to consider the distribution of possible outcomes for different values of the input factors (Couvert et al., 2010; Koutsoumanis and Angelidis, 2007; Ross et al., 2009).

Variability in microbial behaviour can occur due to different reasons. The last decade several stochastic predictive models have been developed to integrate and quantify the numerous sources of variability (Augustin et al., 2011; Delignette-Muller et al., 2006; Membré et al., 2005; Koutsoumanis et al., 2010; Pouillot et al., 2003). One of the major sources of variability is related to genetic biological variability. Biological variability includes both the heterogenicity in individual cell behaviour, known as cell variability (Figure 4B), as well as the inherent differences in microbial behaviour among identically treated strains of the same species, known as strain variability (Figure 4A). Stochastic modelling approaches used to describe strain and cell variability of foodborne pathogens bacteria are presented in the following paragraphs.

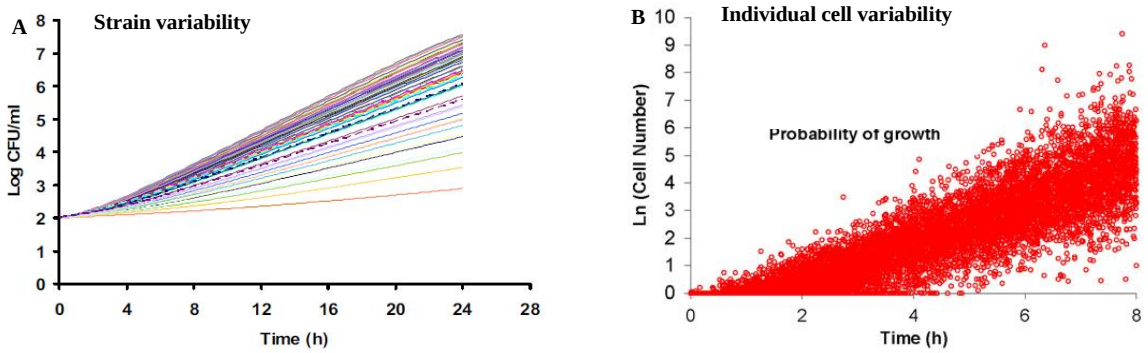

Figure 4: Different sources of biological v variability influencing microbial responses. (A) Strain variability of 60 Salmonella enterica *strains adapted from Lianou and Koutsoumanis, 2011 (B) Individual cell variability of 220 individual cells of* Salmonella enterica *serotype Typhimurium adapted from Koutsoumanis and Lianou, 2013.*

3.1 Model strain behaviour

In various research studies, secondary models' parameters have been described by using probability distribution to assess the effect of the environment on strain variability (Couvert et al., 2010; Delignette-Muller et al., 2006; Delignette-Muller and Rosso, 2000; Koutsoumanis et al., 2010; Lianou and Koutsoumanis, 2011a; Pouillot et al., 2003).Variability in microbial growth among different strains of a single bacteria species of several foodborne pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enteritica* and *Staphylococcus aureus*, has been observed in different studies (Coleman et al., 2003; Dengremont and Membré, 1995; Fehlhaber and Kruger, 1998; Lianou et al., 2006). Moreover, stochastic modelling approach which can take into consideration strain variability has been described mainly during the last decades (Couvert et al., 2010b; Delignette-Muller et al., 2006; Delignette-Muller and Rosso, 2000; Koutsoumanis et al., 2010; Membré et al., 2005; Nauta and Dufrenne, 1999; Pouillot et al., 2003).

Delignette-Muller and Rosso developed a stochastic model to illustrate the effect of strain variability of *Bacillus cereus* in pasteurised milk on the result of the outcome of the exposure assessment and its accuracy (Delignette-Muller et al., 2006b). Koutsoumanis and colleagues have also point out the influence of strain variability on the growth of *Listeria monocytogenes*

during distribution, retail and domestic storage of pasteurised milk (Koutsoumanis et al., 2010). According to Membré and colleagues, variations in growth rates have been reported for different strains of *Listeria monocytogenes, Salmonella, Escherichia coli, Clostridium perfringens* and *Bacillus cereus* (Membré et al., 2005). In addition, Nauta and Dufrenne reported that there is a significant difference between the minimum growth temperature of the 75 different strains of *Escherichia coli* O155:H7 (Nauta and Dufrenne, 1999). Lianou and Koutsoumanis developed a model to assess the growth kinetics and predict the maximum specific growth rate of 60 *Salmonella enterica* strains, by using the cumulative probability distributions of the value of pH_{min}, pH_{opt} and aw_{min} as inputs to the developed model (Lianou and Koutsoumanis, 2011b). The latest study demonstrates that strain variability of foodborne pathogens is a major issue in food safety and therefore, stochastic modelling approaches incorporating strain variability can improve the accuracy in food safety management in general

3.1 Model singe cell behaviour

After the development of quantitative microbial risk assessment, single cell behaviour was identified as an important source of variability (Voysey and Brown, 2000). Numerous stochastic models have been developed to assess the impact of individual cell heterogenicity on growth, survival, duration of lag and inactivation, both for vegetative cells and spores.

The biological heterogenicity of individual cell division, lag time and growth has been widely studied in the recent years (Elfwing et al., 2004; Francois et al., 2005, 2003; Guillier and Augustin, 2006; Koutsoumanis, 2008; Koutsoumanis and Lianou, 2013; Métris et al., 2008, 2005; Pin and Baranyi, 2006; Smelt et al., 2008). Pin and Baranyi studied the deviation time of *Escherichia coli* K-12 individual cells and proposed a stochastic birth model to describe the heterogenicity of individual cells in a microbial population (Pin and Baranyi, 2006). Koutsoumanis and Lianou also reported high heterogenicity in growth behaviour of *Salmonella enterica* serotype Typhimurium (Koutsoumanis and Lianou, 2013). Through their stochastic model, which provides probabilistic growth curves, they manage to demonstrate that the growth of individual cells or small population is ‘a pool of events, each one of which has its own probability to occur’. Moreover, authors reported that variability in growth depends on the initial population size and it is inversely proportional; with variability of initial populations bigger than 100 cells being almost eliminated and the system behaves almost deterministically. Furthermore, it was shown that cells of the pathogens under favourable conditions have a higher probability to grow and form colonies, in contrast to stressed cells or those which grown under unfavourable conditions.

The above findings are in line with the findings of Baranyi and Pin, who underlined the importance of cell heterogenicity especially in small population exposed to stress (Baranyi and Pin, 2004). Koutsoumanis also reported a significant heterogenicity in ability of *Salmonella enterica* serotype Enteritidis cells to initiate growth at different concentration of NaCl (Koutsoumanis, 2008). The previous mentioned findings have been confirmed for *L.*

monocytogenes individual cells varied from 0.940 to 0.997 and 0.951 to 0.997 for unheated and heat stressed cells, respectively (Aguirre and Koutsoumanis, 2016).

Kakagianni and colleagues developed a stochastic model to describe the impact of storage temperature of lag time of *Geobacillus stearothermophilus* individual spores. The developed model demonstrates the importance of individual spore heterogenicity in growth prediction, by introducing a distribution of individual spore's lag time values. In this study, both variability and duration of lag time of individual spores decreased as the number of spores increased up to 100. Nevertheless, the number of surviving spores in the food product is usually low and thus, individual spore behaviour is of a great importance (Kakagianni et al., 2017).

In case of inactivation, individual cell heterogenicity is reflected in the inactivation curve of the microbial population. The gradual decrease in the concentration level reflects the variability in individual cell inactivation times (ti), with the rate of decrease indicating the spread of ti distribution (i.e. the higher the rate, the narrower the ti distribution). Based on the above, the inactivation curve of the microbial population is defined by the shape and position of the probability distribution of individual cell inactivation times ti. Aspridou and Koutsoumanis developed a stochastic model for *Salmonella enterica* serotype Agona based on ti distribution.

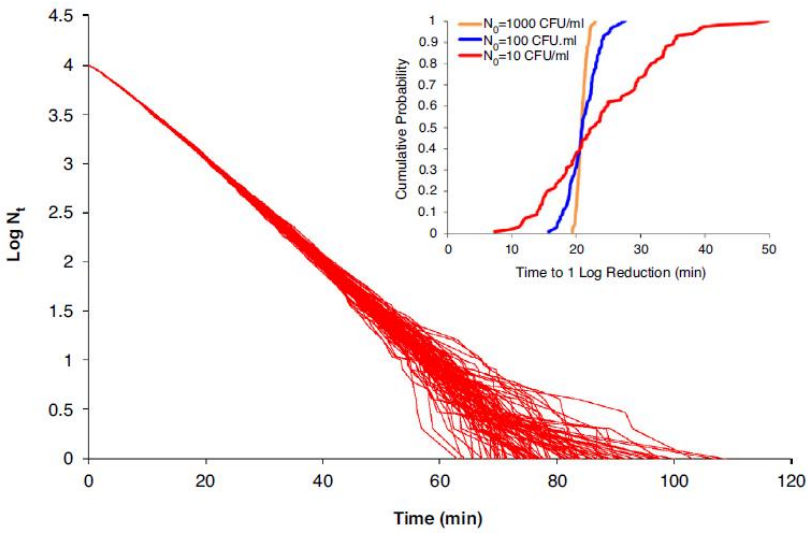

Figure 5: Inactivation of Salmonella enterica *ser. Agona as predicted by the stochastic model of Aspridou and Koutsoumanis, 2015. Inner graph shows the cumulative distributions of the time required for 1 - log cycle reduction (D-value) of the pathogen's populations with different initial concentrations (N_0). Adapted from Aspridou and Koutsoumanis, 2015.*

The developed model demonstrated that variability in the inactivation behaviour is significant for initial population size bellow 100 cells and therefore the D-value of small size population can be better characterised by a probabilistic distribution (Aspridou and Koutsoumanis, 2015). Abe and colleagues reported individual spore heterogenicity regarding heat resistance. In the latest study variability of the reduction time of bacterial sporular populations of *Bacillus simplex* during heat process was demonstrated. Moreover, heterogenicity in survival behaviour was also reported (Abe et al., 2018).

4 Predictive modelling software tools

The last three decades an increased number of predictive models have been developed. This significant increase in available predictive models led to the development of several user-friendly software tools. These predictive modelling software tools allowed knowledge to be transfer from academics and researchers to stakeholders of food industry (Tenenhaus-Aziza and Ellouze, 2015). The development of these software tools gave the opportunity to stakeholders to benefit from predictive microbiology without requiring prior extended knowledge both on advanced mathematics and modelling. However, the most important benefit for the users is that predictive modelling software tools allow almost real time decision making. Table 1 provides an overview of the available predictive modelling software tools. Predictive software tools based on both deterministic and stochastic approach. The available software tools vary based on their utilities for the prediction of the majority of foodborne pathogens responses. Based on the need, available software tools may be used for prediction of growth, growth/no growth, inactivation as well as growth and inactivation fitting tools, as well as risk assessment or sensitivity modules. The availability and the source of these predictive modelling software tools are presented in Table 1.

Table 1. Overview of the available predictive modelling software tools. Adapt from (Tenenhaus-Aziza and Ellouze, 2015).

Software	Modelling approach	Description of utility						Accessibility	Source-link
		Prediction of growth	Prediction of growth/no growth	Growth fitting tool	Prediction of inactivation	Inactivation fitting tool	Risk assessment module		
Baseline	Deterministic	✓			✓			Free internet access	www.baselineapp.com
ComBase	Deterministic		✓	✓	✓	✓		Free internet access	http://www.combase.cc
Dairy Products Safety Predictor	Stochastic						✓	Commercial, internet access	www.aqr.maisondulait.fr
FDA-iRISK	Stochastic						✓	Free internet access	https://irisk.foodrisk.org
FILTREX	Stochastic			✓		✓		Free, downloadable	http://w3.jouy.inra.fr/unites/miaj/public/logiciels/filtrex/
FISHMAN	Deterministic	✓		✓				Free, downloadable	http://www.azti.es/downloads/downloads/fishmap/#tab-description
Food Spoilage and Safety Predictor (FSSP)	Deterministic	✓	✓					Free, downloadable	http://fssp.food.dtu.dk
GInaFiT	Deterministic					✓		Free, downloadable	http://cit.kuleuven.be/biotec/downloads.php
GroPIN	Deterministic and Stochastic	✓	✓		✓			Free, downloadable	www.aua.gr/psomas/gropin
Listeria Meat Model	Deterministic	✓						Commercial, downloadable	www.cpmf2.be
MicroHibro	Stochastic	✓	✓		✓		✓	Free internet access	www.microhibro.com
Microbial Responses Viewer (MRV)	Deterministic	Growth rate estimation based on ComBase						Free internet access	http://mrviewer.info/
NIZO Premia	Deterministic	✓		✓	✓	✓		Commercial, no internet access	No internet access
PMM-Lab	Deterministic	✓	✓	✓	✓	✓		Free internet access	https://sourceforge.net/projects/pmmlab/
Prediction of Microbial Safety in Meat Products	Deterministic	✓	✓		✓			Free internet access	http://dmripredict.dk
Pathogen Modelling Program (PMP)	Deterministic	✓			✓			Free internet access	http://pmp.errc.ars.usda.gov/PMPOnline.aspx
Sym'Previus	Deterministic and Stochastic	✓	✓	✓	✓	✓		Commercial, internet access	www.symprevius.org

Conclusion

In a nutshell, microbial responses to environmental conditions can be monitored by implementing predictive microbiology. Environmental distributions such as temperature increase, increased frequency and intensity of extreme weather events, precipitation patterns, ocean warming and acidification, are expected due to climate change and can be monitored through predictive microbiology. Due to the complexity of both microbial dynamics and climate change, a multidisciplinary research approach is the most suitable. To assess the impact of climate change on food safety and spoilage, uncertainty driven by the lack of scientific data (Miraglia et al., 2009) and unknowns related to this complex issue, should be taken into account (Tirado et al., 2010). Hence, the use of stochastic modelling approach may prove to be an asset.

Furthermore, microbial dynamics observed in heterogeneous environments and/or under stress conditions due to heterogeneous populations and stress adaptation phenomena cannot be explained using the macroscopic approach which is generally applied in predictive microbiology. Thus, predictive microbiology must take the modelling one step further by including more micro- and/or mesoscopic information to understand these cell dynamics. The applicability and reliability of existing models under more realistic conditions will definitively be improved by looking inside the black box and unravelling the underlying mechanisms. Incorporating intracellular information in predictive models, following a top–down systems biology approach, will result in more widely applicable mechanistic models. Once this systems biology approach is successfully validated, it will be extended to develop next generation predictive models for more complex systems in the context of climate change.

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