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Thermal inactivation of microorganisms

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Abstract

This article serves as an overview of various aspects of thermal processing. Heat processing of foods has a long history and is still one of the most important preservation methods. To guarantee microbiological safety and stability large safety margins are often applied in traditional heat processes. Because of the need for more fresh like foods there is a need for milder preservation methods without compromising on safety and stability. The review deals with heat resistance data and mathematical models that describe heat inactivation. The effects of food composition are not yet fully clear and more knowledge of the cell physiology of the target microorganism could be of help in predicting the effects of food constituents. Finally, special

attention has been paid to biological time temperature indicators to enable proper process calculations.

Key words bacterial spores, *D* and *z* value, time temperature integrator, heat stress, sub lethal, non linear.

1. Introduction

Heat processing of foods has a long history and it is still one of the most important preservation methods. Stabilization by heat processing was already patented by Appert (1810) in the early nineteenth century without any knowledge of microbiology. In the late nineteenth century pasteurization was introduced, named after Louis Pasteur, to eliminate vegetative pathogens or spoilage organisms. In the early twentieth century well established, science based heat processes were formulated (Esty and Meyer, 1922; Bigelow et al., 1920; Bigelow, 1921; Richardson and Ball, 1920). In traditional heat processes microbiological stability margins are often unnecessarily large, because of suboptimal control of the heating process or of lack of precise knowledge of inactivation kinetics of the relevant micro-organisms.

Traditional canning methods aim at destruction of all spores (sterilization) or of all spores that can grow in the container below 40 °C (commercial sterilization or appreciation). Because of new consumer demands for fresh-like products there is a need for milder processes e.g. in combination with cold storage and a limited shelf life. As a consequence, more recently, special attention has been paid to the heat resistance of spores from sensitive cold growing spore formers as well as their vegetative cells. In this review an inventory will

be given of heat resistance data of food poisoning and food spoilage organisms. Putative mechanisms involved in thermal inactivation and stress response will be addressed.

Although mathematical descriptions of heat inactivation date already from the early twenties, development of new software during the last decades enables a better description of inactivation kinetics under different conditions. Combined with a better control of temperature in each container a milder heat process can be designed without compromising microbial safety and stability. The principles of modeling death kinetics will be addressed within the framework of quantitative microbiological risk assessment (QMRA). The effect of environmental conditions and its extrapolation to real food situations will also be discussed.

2. Classes of heat resistance,

Microorganisms are far more resistant to dry heat than to wet heat. Whereas dry heat resistance is relevant in particular for disinfection of materials such as devices for surgery, in food microbiology most attention has been paid to wet heat resistance. In dry heat treatment microorganisms seem to be inactivated by oxidation whereas protein denaturation (Setlow and Setlow, 1998) and membrane damage seems to play an important role in wet heat inactivation (Coleman et al., 2007; Zhang et al., 2010). Bacterial spores are generally the most heat-resistant forms, although the heat resistance of bacterial spores varies considerably. To compare heat resistance data of various organisms the data are mostly given in *D* values (see also section 7.1). Expressed in *D* values the heat resistance of spores varies

from < 1 min at 90 °C for *Clostridium botulinum* Type E to 3 – 4 min at 130 °C for the thermophilic *Geobacillus (Bacillus) stearothermophilus.* Most spores formed by moulds and yeasts are much more sensitive to heat than bacterial spores. Asexual spores known as chlamydospores which are formed by thickening and swelling of the hyphal cells are considerably more resistant to heat than other asexual spores of moulds. Many yeasts and moulds can form sexual forms of spores, ascospores. They are often quite resistant to heat, especially those of *Byssochlamys* species (Engel and Teuber, 1991). Ascospores survive exposure to temperatures well above 80 °C. Non-spore forming bacteria are generally not resistant to temperatures above 60 to 70 °C. For instance *D* values of some seconds at 72 °C have been reported for *Mycobacterium* in milk (Foddai et al., 2010). There is some controversy whether *Mycobacterium* would be able to form spores (Ghosh et al., 2009b, Singh al., 2010, Traag et al., 2010). *Listeria* is relatively resistant to heat, but a pasteurization treatment of 2 min at 72 ° C or an equivalent is generally regarded as safe. In Tables 2a, 2b and 2c an overview of heat resistance of non-spore forming bacteria, yeasts and moulds as well as bacterial spores is given. In general an inverse correlation exists between minimum growth temperature and heat resistance of spores (Silva and Gibbs, 2010).

3. The effect of environmental conditions on heat resistance.

3.1. *Cultivation conditions.*

It is well known that exponentially growing cells are more sensitive to heat (Black et al., 2009; Chabra et al., 2002). When exponentially growing cells are subjected to stressful

conditions such as sub-lethal heat, high salt concentration, or starvation, the cells have their appropriate (general) stress response mechanisms (Breand et al., 1998). Indeed, stationary phase cells are more resistant presumably since they are faced with stressful environmental conditions (see also paragraph 4 and 5). It is well known that incubation at higher temperature coincides with a higher rigidity of the cell membrane, which is a factor contributing to enhanced heat resistance (Alvarez-Ordonez et al., 2009a, 2009b). Spore specific factors such as metal ions in the sporulation medium play a role in the thermal resistance. Particularly Ca^{++} ions enhance heat resistance (Cazemier et al., 2001). Bacterial spores can be made reversibly heat sensitive or heat resistant by incubating them in acid environments or in Ca^{++} rich environments respectively (Alderton and Snell, 1963, 1969).

3.2. *Conditions during heating and recovery.*

Many heat resistance studies have been conducted in phosphate buffer pH 7.0 but to our knowledge no attention has been paid to the specific effects of the phosphate ions on heat resistance. In low pH experiments phosphate buffer is mostly replaced by citrate buffer and it might well be that citrate can have non-specific effects on heat inactivation. In general, pH seems to be a crucial factor determining inactivation next to water activity (a_w) and temperature itself (Akterian et al., 1999). A low pH value of the heating menstruum enhances inactivation. Leguerinel and Mafart (2001) showed that the type of organic acid had specific effects on the heat resistance of *Bacillus subtilis* They found that the effect was most pronounced at pH 4 which was the lowest pH they tested, when the pKa value was higher. It should be noted that most complex rich growth media contain in all likelihood significant

amounts of organic acids. Apart from thermal death, microorganisms can be sub-lethally damaged by heat and thus become more sensitive to adverse recovery conditions such as organic acids, low pH or low water activity. Consequently, the effects of pH during heating and during recovery are cumulative. It is well known from growth studies that organic acids inhibit growth not only because of low pH but also due to undissociated acid, probably due to perturbation of the membrane as is the case with sorbic acid and benzoic acid or acidification of the cell as is the case with acetic acid or lactic acid (Brul and Coote, 1999; Ter Beek et al., 2008; Ter Beek, 2009; Mols et al., 2010; Stratford et al., 2009; Ter Beek and Brul, 2010). Hence the recovery of heated cells will be impaired and the apparent heat resistance will lower when organic acids are present in the recovery medium. To counteract low pH during recovery from thermal treatment most microorganisms try to avoid extreme acidification of the cell interior (Cotter and Hill, 2003). Protective measures alter the membrane composition, extrude protons, protect macromolecules, alter metabolic pathways and favor alkalinization. Particularly reliance on proton pumps notably F_1F_0 ATPase is of paramount importance. Clearly, it will have consequences for recovery when some of the defense mechanisms are perturbed by heat. It is still to be firmly established though whether heat inactivation of spores at low pH is even further enhanced by undissociated organic acids. Recent data by van Melis et al. (2011a , 2011b) do show that sorbic acid directly interferes with events in spore germination.

It is a well known observation that reduced water activity in general protects microorganisms during heating. At water activity values between 0.2 – 0.4 both spores (Murell and Scott,

1957; Kempf et al., 2008) and vegetative cells (Laroche and Gervais, 2003; Laroche et al., 2004) are most resistant to thermal stress. The protective effect during heating is consistent with observation on purified enzymes (e.g. Makki and Durance, 1996). However reduced water activity creates unfavorable conditions for recovery. The effect of reduced a_w on recovery seems to outweigh the protective effect during heating (Coroller et al., 2001). In general salt, e.g. NaCl, protects less than sugars. However, under harsh conditions sugars can caramelize or form Maillard compounds thus creating a toxic environment to micro-organisms (Smelt, 1980, Suorti and Mälkki, 1984). An extreme example of the effect of reduced water activity is the protective effect of chocolate on survival of *Salmonella* during heating (Barrile and Cone, 1970; Goepfert and Biggie, 1968). The effect of oil is equivocal. Whereas some reports indicate that oil has a protective effect on micro-organisms there are also reports on the sensitizing effect of oil (Rodrigo et al., 1999).

4. Target of heat inactivation / mechanisms to cope with heat stress in vegetative cells.

Heat injury of vegetative cells is multi-targeted. The site of damage can be some cell wall components, the cytoplasmic membrane, ribosomes and ribosomal RNA, as well citric acid cycle enzymes involved in cellular metabolism (Earnshaw et al., 1995; Wu, 2008). Results of differential scanning calorimetry (DSC) showed that the cell membrane seems to be most commonly affected at relatively low temperatures (Nguyen et al., 2006; Texeira et al., 1997). At higher temperatures death coincided with unfolding of the most thermally labile proteins including for instance α and β subunits of RNA polymerase. Injury is enhanced by loss of

cellular materials such as potassium, amino acids and proteins through a damaged plasma membrane. Repair is accomplished by de novo synthesis of rRNA, ATP and proteins. The repair period is reflected by a prolonged lag time before growth resumes. Most probably injury is unevenly distributed over all cells. As a result the variability of lag times of individual cells increases (Guiller et al., 2005; Metris et al., 2008; Smelt et al., 2002a; Standaert et al., 2007). This variation only becomes apparent when the initial cell numbers under study are small.

5. Response to unfavorable environments: stress response and sporulation.

To counteract unfavorable conditions most organisms activate stress response mechanisms under suboptimum culture conditions such as starvation, sub lethal heat, suboptimum pH, osmotic stress, or the presence of organic acids such acetic acid, lactic acid, benzoic acid or sorbic acid. As a result cross resistance is often seen, leading for instance to more heat resistance of the microbial population (McKellar et al., 1997). Consequently, slow heating can sometimes result in increased heat resistance. Stress response is characterized by the synthesis of special proteins and changes in the membrane. A transient growth arrest will occur and the intracellular pH drops (Vanaelst et al., 1991). For extensive reviews the reader is referred to Brul and Coote (1999), Abee and Wouters (1999), Periago et al. (2002), Ter Beek (2009) and Orij et al. (2011). Sub-lethal heating results in increased resistance as long as the stress is not too severe. Beyond a certain point the cell can no longer cope with stress and gets injured. When stress is even more severe it is no longer able to form a colony and

can be considered as dead. Injured cells need time to repair resulting in a longer lag time. Recovery under suboptimum conditions is more difficult and as a result many injured cells are no longer able to recover. Cell injury leads also to an increased variability of individual lag times (Augustin et al., 1999; Smelt et al., 2002a; François et al., 2005; Guiller et al., 2005; Guiller and Augustin, 2006; Metris et al., 2008). Entirely different from this kind of stress response is the induction of sporulation in bacilli and clostridia. These bacteria are able to activate a specific set of genes involved in sporulation. When availability of nutrients becomes highly restricted sporulation starts. Once the cell starts sporulation it is committed to it (Stewart et al., 1981). Sporulation takes many hours and proceeds in several stages eventually leading to a mature spore. It starts with the formation of an asymmetrical septum upon which the small compartment is engulfed by the mother cell. Subsequently the typical spore structure is gradually formed (Driks, 2002)**.** Spores consist of a spore core with a membrane that will later form the vegetative cell. The spore core contains 15 – 25% calcium dipicolinate (Ca-DPA). The role of DPA in heat resistance of spores is not yet fully understood. It is clear that DPA release occurs largely after heat induced spore death (Coleman et al., 2007). The spore core is surrounded by a cortex with a peptidoglycan structure and spore coat layers consisting of keratin at the outside. Sometimes the spore coat is surrounded by an exosporium (Charlton et al., 1999). The very low water content of the spore core plays a crucial role in the heat resistance of the spore and indeed an inverse correlation has been shown for a wide range of species between the hydration state of the spore and its heat resistance (Beaman and Gerhardt, 1986). The physical state of water in the different compartments is assumed to play an important role in thermal resistance. Whereas it

was assumed previously that the spore core was in a glassy state, recent work on the physical state of water in bacterial spores by Sunde et al. (2009) has shown that the spore core is likely not in a uniform glassy state. Instead it may be suggested that the heat resistance is linked to dehydration-induced conformational changes in metabolic key enzymes. The heat resistance of the spore remains largely unaltered when the spore coat is removed. When the spore cortex is also removed the spore looses its heat resistance and the spore starts to germinate.

When conditions become favorable the spore starts to germinate and the spore core will eventually develop to a new vegetative cell. Whereas the lag time of vegetative cells is only related to adaptation to a new environment or repair from injury of the vegetative cell, the extension of the lag time of the bacterial spore can be due to perturbation of the germination mechanism or an impaired development to the vegetative state. It is not well described how the history of sporulation plays a role in the outgrowth time of the spore. Whereas the first germination step can be very fast when the conditions are favourable there are always spores that germinate after only a very long lag time. In fact the first germination step can be the limiting factor in development of the spore to an exponentially growing population (Stringer et al., 2009). In bacterial spores the germination mechanism can be damaged i.e. molecules such as a spore lytic enzyme; SleB, (Moriyama et al., 1996) located in the inner membrane and a cell wall hydrolase; CwlJ, (Ishikawa et al., 1998) located in the integuments related to triggering of germination or to lysis of the cortex resulting in phase darkening. Observation on heat inactivation of spores of *C. botulinum* or *C. butyricum* have shown that lysozyme can

enhance recovery of heated cells by at least a factor of 1000 indicating that cortex lytic enzymes are damaged (Sebald and Ionesco, 1972; Peck et al., 1993; Fernandez and Peck, 1999). As shown by microscopic observations of Stringer et al. (2009) the largest variability in recovery time is due to variation in the first stage of germination when lethal heat treatments are applied. Observations by Smelt et al. (2008) suggest that in many instances heat damage of the core as reflected by outgrowth and development to dividing cells is often more prominent than damage of the germination mechanism. In general DNA in spores remains intact after heating. When studying heat resistance it should be borne in mind that relatively mild sub-lethal temperatures may activate spores to germinate rather than destroy them. Zhang et al., (2009) showed by Raman spectroscopy that the so called heat activation indeed causes reversible denaturation of proteins and several DPA bands in *Bacillus cereus* and *Bacillus subtilis* spores. There is an inverse correlation between propensity to germinate and heat resistance (Ghosh et al., 2009a). As more heat resistant spores need a higher activation temperature to germinate, these spores can be overlooked when no or too low activation treatment is given.

6. Setting criteria for heat inactivation.

The criteria for minimum thermal treatment of a food product should be set within the frame of a microbiological risk assessment, preferably quantitative microbiological risk assessment (QMRA), as outlined in Fig. 1. One element of risk assessment is exposure assessment. Exposure assessment generally includes contamination of raw materials, contamination

during processing, growth and/or inactivation of the microorganism, storage and distribution conditions and infectivity of the microorganism. To this purpose a Food Safety Objective (FSO) is set. FSO is defined as 'the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP). FSO's are defined by Codex (CAC, 2003). The FSO equation was developed by ICMSF (the International Commission on Microbiological Specifications for Foods, 2002) as:

$$H_0 - \Sigma R + \Sigma I \leq \text{FSO} \qquad (1)$$

Where H_0 = initial level of the hazard; ΣR = the cumulative (total) decrease in level ΣI = the cumulative (total) increase in level FSO = Food safety Objective.

As shown by formula (1) and Fig 1 all elements in the whole exposure chain from 'farm to fork' will determine the required ΣR which is generally the required heat treatment. Ideally the various aspects of the hazard should be known: the variability of heat resistance among individual strains, the presumed effects of the environmental conditions under which the contaminating microorganisms had been developed, the effect of the food matrix during heating and during recovery as well as a proper description of the death kinetics. During the last 20 years much attention has been paid to a proper description of the death kinetics both under static and dynamic conditions as will be addressed below. Less attention has been paid

to the preparation of the appropriate inoculum tailor-made for different food categories (Anonymous, 2010; François et al., 2005, 2006).

Although many elements of risk assessment for safety can be used in risk assessment for spoilage the approach is not identical. When spoilage risk is estimated an objective similar to the FSO should be formulated. In general food spoilage risk may be higher than food poisoning risk. Whereas criteria for the risk of food poisoning are based on recommendations by committees of specialists (e.g. ICMSF, 2002), criteria for food spoilage are mostly set by the producer in order to guarantee a stable product on the market. It should be realized that the most resistant spoilage organisms are in general more heat resistant than the food poisoning organisms.

7. Mathematical inactivation models.

Although the mechanism of inactivation of vegetative cells is different from that of spores the structure of the models describing inactivation is essentially the same. In most cases these models are descriptive with no or hardly any mechanistic element. Although it is highly unlikely that entirely mechanistic models will ever replace descriptive models, mechanistic elements may be incorporated thanks to advances in microbial physiology (Brul et al., 2010). The advantage of mechanistic elements might be that environmental effects can be inferred, facilitating experimental design. For purely thermal processes aiming at food safety and stability, relatively simple models are generally to be preferred above complex mechanistic

models. To achieve milder thermal inactivation processes the hurdle concept is often applied (Leistner, 2000; Leistner and Gorris, 1995). Here there seems scope for more mechanistic models that describe the relevant elements of the cell machinery at the boundary of growth, survival and death. Hence, such models will facilitate the robustness of predictions at the growth/no growth boundary necessary for the application of hurdle concept based mild preservation strategies in food manufacturing (discussed amongst others by McMeekin et al., 2010).

The inactivation models presented below are deterministic. Probabilistic elements, both for processing parameters (Cronin et al., 2002) and for microbiological parameters (Membré and van Zuijlen (2011) will certainly contribute to a more realistic description of the margin of safety.

7.1. *Classical thermal death model*

Esty and Meyer (1922) observed that the heat resistance of a population of *Clostridium botulinum* was dependent on the initial number of microorganisms and from their results it could be inferred that the decrease was exponential with time. Bigelow (1920) formulated the death kinetics of bacterial spores in mathematical terms. Inactivation of microorganisms is still described in terms of decimal reduction time, i.e. the time to reduce the number of microbial cells by a factor of 10 or D value. The increase in temperature corresponding to a decrease of the D value by a factor of 10 is called the z value. Thus thermal inactivation can be described by two linear equations:

$\log (N_t) = \log (N_0) - t/D$

or:

$$\log (N_t/N_0) = - t/D \quad (2)$$

in which: N_0 = the initial number, N_t = the number at time t, D = the decimal reduction time, $-(1/D)$ = the slope of the curve.

The equation can be written in line with enzyme kinetics in the form:

$$\ln(Nt/N_0) = - kt \quad (3)$$

In which

$$k = \ln(10)/D \quad (4)$$

The relation between D and temperature (T) can be described as

$$\log (D/D_{ref}) = -(1/z)(T-T_{ref}) \quad (5)$$

where; D = the decimal reduction time; D_{ref} = reference D value corresponding to the reference temperature = T_{ref} z is the temperature increase that corresponds to a 10 fold reduction of the D value. z is the reciprocal of the slope in equation (5); D can be expressed in seconds or minutes; z is expressed in °C or in older literature in °F; T = temperature,

mostly in °C or sometimes in K. The first aim of these models was to ensure virtual absence of *Clostridium botulinum* (often a 12 *D* reduction, which is arbitrary) and the excellent safety record of canned foods with respect to *C. botulinum* has proven the value of these two models. So far botulism due to under processing has not occurred unless the processing rules formulated in the twenties and the thirties were not followed. In fact these equations form the basis of heat processing. *D* and *z* values are empirical terms. Yet these simple equations are still satisfactory for designing most current heat processes. It should be borne in mind, however, that there are many spoilage organisms that are more heat resistant than *C. botulinum.* Therefore processing to prevent spoilage is an extra safeguard to avoid botulism. Besides, quite a few deviations from log linearity exist and alternatives will de dealt with in the next paragraph. Unfortunately in many articles no inactivation curves are given, but only *D* values**.** Consequently deviations from log linearity are not always visible. Hence, descriptions in terms of *D* and *z* values should often be used with care especially when significant initial microbial loads are present. Compared to other sources of variation, however, *D* values give useful, global information. When the shape of the inactivation curves is different for different organisms (and also for the same organism under different conditions) comparison of heat resistance can be a problem. A practical solution for this problem has been given by van Asselt and Zwietering (2006)**.** Taking into account that large differences exist for various strains and various products they introduced the concept of $D_{average}$:

$$D_{average} = (5\,D + \lambda)/5. \qquad (6)$$

$D_{average}$ is the amount of heating time needed to obtain one log reduction (min) and λ is the length of the shoulder. The latter is defined as the time before micro-organisms start to be inactivated.

In most cases more than 5 D is the target. Then the approach leads to a conservative estimate of heat resistance if shoulders are present, but if tails are present the equation cannot be used for a final description. As there are large differences in heat resistance, even among different strains of one species, the approach is valuable to identify the most relevant microorganism(s) in a certain process. When the most relevant organism(s) is (are) determined, a refined approach can be followed taking into account possible non linear inactivation.

7. 2. *Non linear models.*

In fact the classical model is identical to the model describing radioactive decay: every cell has at any moment the same probability of survival. It implies a first order reaction. In biological terms it means that inactivation of a micro-organisms is caused by a single hit. Observations such as more fastidious requirements for recovery of heat injured cells seem to conflict with the single hit model. Moreover, genetic or phenotypical heterogeneity (Hornstra et al., 2009; Stringer et al., 2009) of the cells in a population may result in non linearity. Cerf (1977) discussed many possible causes of tailing. As stated before there are many examples of shoulders and also sigmoid curves. In view of these considerations a number of non linear models will be discussed in this section. Li et al. (2007) has made an inventory of a number

of non linear inactivation models. They are mentioned in Table 3a. Geeraerd et al. (2000) have analyzed 8 inactivation models each with their own limitations.

7.2.1. Weibull like models.

A simple way to describe non linear curves is the Weibull equation (Heinz and Knorr, 1996; Peleg and Cole, 1998; Mafart et al., 2002; van Boekel, 2002; Fernandez et al., 2002; Collado et al., 2006). This equation can properly describe inactivation curves that are either upward or downward concave. Weibull and Weibull like equations have been applied for the description of inactivation of bacterial spores as well as inactivation of vegetative cells. They could well be described by a Weibull equation as was proposed by Mafart et al. (2002):

$$\log(N_t/N_0) = -(t/\delta)^p \qquad (7)$$

in which Nt = the number of microorganisms at time t, N_0 = the initial number of microorganisms, t = heating time, δ is the scale parameter and p the shape parameter. van Boekel (2002) investigated 55 inactivation data sets of various non spore forming bacteria. All sets contained heating curves within a range of 3 – 9 temperatures and only two curves could be considered as linear. Based on his observations he presented almost simultaneously with Mafart et al. (2002) an equation that was essentially the same: $\log (N_t/N_0) = -(1/2.303)(t/\alpha)^{\beta}$ in which α and β are the scale parameter and shape parameter respectively. To reduce the number of parameters, N_t and N_0 are not always estimated separately but only combined as N_t/N_0. (Mafart et al., 2002; van Boekel, 2002). The scale parameter (δ) has some

resemblance to the inactivation constant and becomes identical to the *D* value when the shape parameter (*p*) becomes 1. The same holds also for the first decimal reduction even when $p \neq 1$. Sometimes a Weibull like equation is written as $\ln(N_t/N_0) = - kt^p$ (Peleg and Cole, 1998), but it is expected that the estimation of both parameters *k* and *p* is more difficult because inactivation rate (*k*) is probably more correlated with *p* than δ and *p*. A high correlation might have serious consequences for further development of secondary models including the effect of temperature or pH or a_w. Several studies have shown that the shape parameter (*p*) of the Weibull equation in most cases can be considered as independent of temperature and pH. That has been observed both for spores (Peleg and Cole, 1998; Fernandez et al., 2002; Mafart et al., 2002) and for vegetative cells (van Boekel, 2002; Albert and Mafart, 2005; Leguerinel et al., 2007), whereas the relationship between δ and temperature or pH can be described as a linear relation. Thus the development of secondary models is relatively simple.

7.2.2. Biphasic inactivation.

One of the causes of tailing that were suggested by Cerf (1977) was the existence of two phenotypically different subpopulations. The majority is relatively heat sensitive whereas a minority is relatively heat resistant.

$$\ln(N_t/N_0) = \ln((1-f)\exp(-k_1 t)+f\exp(-k_2 t)) \quad (8)$$

N_t = number of microorganisms at time t; N_0 = initial number of microorganisms; $1-f$ = majority fraction of heat sensitive cells; k_1 = inactivation rate constant of sensitive (majority),

f = minority fraction of resistant cells; k_2 = inactivation rate constant of resistant (minority) fraction.

A convincing example of biphasic inactivation of non sporing microorganisms was presented by Humpheson et al. (1998). They showed that a very small fraction proved to be more resistant than the majority of the population. The proportion between the number of heat sensitive and heat resistant cells was independent of the inoculum size. It should be noted that the biphasic model contains 3 or 4 parameters, whereas the Weibull model contains 2 or 3 parameters – depending on the choice of N_t/N_0 as the dependent variable or of N_t and N_0 as an dependent variable and a separate parameter respectively. Humpheson's results could be fitted more satisfactorily with the biphasic model than with the Weibull model even taken into account the extra parameter of the biphasic model. Moreover the biphasic model has some biological meaning. It has been often observed that the observed heterogeneity in heat resistance is not caused by genotypic differences (Attfield et al., 2001). The observation has consequences when small probabilities of survival are considered and as a consequence that risk of food poisoning can be underestimated when the tail is ignored.

7.2.3. Normal distribution model.

The classical model can also be considered as an exponential distribution where cumulative frequency of dead cells can be plotted against time. Similarly, distribution of the number of dead cells against time can be cumulative log normal. Augustin et al. (1998) proposed a model based on lognormal distribution of probability of death with time. From that

assumption he could develop inactivation curves that were mainly characterized by tailing.

The formula is given in Table 3a.

7.2.4. S shaped curves.

In most cases Weibull like equations solve problems regarding description of inactivation. However, when inactivation takes the form of an S shape, the Weibull model is no longer appropriate. There are a few solutions to describe these curves. A few models have been described that fit an S shape curve such as the logistic or Gompertz equations as described by Linton et al. (1995). These models are adapted from growth models with negative growth rate. Whereas an upper asymptote in growth can be attributed to depletion of nutrients or formation of toxic substances there is no reason to assume a lower asymptote in inactivation. However as long as no extrapolation is done the model is still suitable as a descriptive model. A solution that eliminates the asymptote has been proposed by Coroller et al. (2006) by combining one convex and one concave Weibull equation.

7.2.5. Sapru model (Sapru et al., 1992).

This model has derived specifically to describe the activation of bacterial spores during the sterilization process, which implies an initial increase of the activated spore population. Two types of spores are distinguished: (i) a dormant, viable population n_d (cfu/ml) potentially able of producing colonies on an appropriate growth medium after heat activation and (ii) an active population n_a (cfu/ml) able of forming colonies without activation on an appropriate growth medium. The model consists of two equations mentioned in Table 3a. Geeraerd et al.

(2000) extended the model by assuming n_d as the resistant population and n_a as the sensitive population. Thus they were also able to describe tailing. As pointed out by Geeraerd et al. (2000) the model has two limitations: it structurally impossible to describe a more or less flat shoulder and the length of the shoulder is completely determined by the slopes before and alter the 'peak' of the curve.

7.2.6. Critical sites model

Inactivation of vegetative cells is often characterized by a shoulder or a slow decrease (more or less a shoulder) followed by an apparent log linear decrease. In this case the use of a binomial distribution might be justified as was originally suggested by Moats (1971) and adapted by Smelt et al. (2002b). In that model the microorganism contains a number of critical sites (n). Such critical sites might be crucial enzymes or (parts of) ribosomes (Anderson et al., 1991). Damage of the membrane is certainly also involved in heat inactivation (Earnshaw et al., 1995; Setlow and Setlow, 1998; Zhang et al., 2010). The cell can only survive if it contains a minimum (m) of intact critical sites. In the model described here, a log linear inactivation of the *critical sites* is assumed, but such assumption is not a necessary prerequisite though. The survival of critical sites can be described analogous to log linear inactivation of whole cells. $N_{crit}/N_{crit0} = \exp(-k_{crit}t)$ or in terms of probability of survival of critical sites:

$$P_{crit} = \exp(-k_{crit}t) \quad (9)$$

In which N_{crit} is the number of critical sites per cell after any heating time, N_{crit0} the number of critical sites per cell at time 0,

k_{crit} the inactivation rate constant of a critical site; P_{crit} the probability of survival of a critical site.

The probability of inactivation of a critical site:

$$1 - P_{crit} = 1 - \exp(-k_{crit}t) \quad (10)$$

The probability that less or equal to n – m sites are inactivated equals the probability of the whole cell to survive (P_{cell}) can be given as a binomial relation:

$$P_{cell} = \text{Bin}[(1 - P_{crit}); (n - m); m)] \quad (11)$$

$1 - P_{crit}$ = probability of inactivation (equation (10), n = total number of 'critical'sites ('number of trials'), m = maximum number of critical sites that can be inactivated before the cell is no longer able to develop in the most suitable conditions.

When the recovery conditions are suboptimal the minimum number of critical sites necessary for recovery will be higher. As long as the kinetics of inactivation of the critical sites themselves is still unknown, the inactivation of the sites assumed to be exponential (log linear). k_{crit} will be dependent on temperature, and possibly on the composition of the heating medium although the heating medium is now the inside of the cell and hence constant as long

as the cell is viable. So far the model is still only descriptive. An example is given in Fig 2. This approach can be developed to a mechanistic model if sufficient data on *in situ* thermostability of cellular contents becomes available. *E. coli* and *Saccharomyces cerevisiae* are probably the first candidates to test the model (McMeekin et al., 2010; Medini et al., 2010).

A summary of a set of inactivation models is given in Tables 3a and 3b.

7.3. *Secondary models*

The oldest secondary model is the z concept as described above in the paragraph on classical inactivation models. Contrary to the log linear equation between D and T the relation between k and $1/T$ is expressed by the Arrhenius equation:

$$k = k_\infty \exp(-E_a/RT) \quad (12)$$

or

$$\ln(k) = \ln(k_\infty) - E_a/RT \quad (13)$$

E_a is the activation energy ($kJmol^{-1}$); R is the gas constant ($8.314446 x Jmol^{-1}K^{-1}$); T is the absolute temperature (K); k_∞ is the rate constant at infinite temperature.

Equation (5) can also be written as:

$$z = (\mathrm{T}\text{-}T_{ref})/\log(D_{ref}/D) \quad (14)$$

Explanation of the symbols is given under equation (5)

The relation between and the Arrhenius equation can be written as:

$$z = (\ln(10))RTT_r/E_a.$$

In heat processing the difference between T_r and T is relatively small and hence z may be considered as

$$z = (\ln(10))RT^2/E_a. \quad (15)$$

The classical D and z models have no biological meaning and are purely empirical. However, it is questionable whether the parameters in the Arrhenius equation have a real biological meaning when applied to thermal death of bacteria. Hence D and z models are generally to be preferred because of their simplicity.

Secondary models are necessary to develop models that describe inactivation under dynamic conditions i.e. the effect of heating up and cooling during processing (Corradini et al., 2007; Peleg et al., 2008a, 2008b). Valdramides et al., (2007) developed a model for *E. coli* K12 for

static and dynamic conditions that incorporated the effect of heating up on microbial thermoresistance.

As was mentioned previously the shape parameter can be considered as independent of environmental factors such as temperature and pH. In most cases the relation between the scale parameter (α), pH or a_w can be described by a simple linear equation comparable to the *D* value. More extensive models for combined effects of temperature, pH and water activity during heating have been developed by Leguerinel et al. (2005, 2007), Gaillard et al. (2005), Mafart et al. (2002) and Valdramides et al. (2006).

Leguerinel et al (2005) developed a model that described the effect of three environmental parameters: water activity (a_w), pH and temperature. They distinguished between the effect of pH or a_w during heating and during recovery. The inactivation equations proposed by Mafart's group are in line with the gamma concept as suggested by Zwietering et al. (1996), Wijtzes et al. (2001) and confirmed by Lambert and Bidlas (2007a, 2007b) for growth and can also by applied for inactivation.

8. *Models describing sub-lethal inactivation*

Apart from complete inactivation, temperature treatment above the maximum growth temperature results in impairment of the physiological state of the cell varying from mild injury to complete inability to multiply under the most suitable recovery conditions. The models mentioned above as described by Mafart's group (i.e. Couvert et al., 2005) show injury as reflected by an impaired recovery under suboptimal conditions. Apart from the

inability of heated cells to grow under suboptimal conditions, sub lethal injury is also characterized by a prolonged lag time even under the most suitable conditions as mentioned in section 5. Lag times can be even more prolonged when the cells are recovered under suboptimal conditions. Many results of experiments have shown that heterogeneity is mainly phenotypic (Blackburn et al., 1997). The group of Mafart (Coroller et al., 2005; Gaillard et al., 1998a, 1998b, 2005; Leguerinel et al., 2005, 2007) has actually studied the separate and the combined effects of heating conditions and of recovery conditions and thus they established in fact the effect of sub-lethal heating on growth/no growth boundaries. So far they have not yet taken into account the *variability* at the growth /no growth boundaries which is a necessary prerequisite for a sound risk assessment. This variability can be caused by small variations in the environment, homogeneity of foods, small differences in strains of a species or phenotypic variation within one clonal population (McMeekin et al., 2010). Growth/no growth models have been developed by Gysemans et al. (2007) and Vermeulen et al. (2009). Their data show that and the variation in response can be quite considerable at the growth boundaries, but their models do not include the effect of previous heating.

9. Real food situations.

Although many data on heat resistance in buffer systems are available, data in real foods are comparatively scarce. The big challenge of predictive microbiology is to design experiments in model systems that give reliable information for real food situations. An overview has

been given by Silva and Gibbs (2010). *D* values can vary by a factor 10 in different food matrices, but is not known whether that is due to interlaboratory variation or due to differences in food matrices. Although the results of experiments in buffer are not always the same as in real food, the data are comparable taking into account pH and water activity. Besides, Silva and Gibbs (2004) presented an overview of the heat resistance of bacteria in acid fruit products. They suggested *Alicyclobacillus acidocaldarius* as the reference micro-organism to design heat processes for food products. For most vegetative cells the reported *z* values are generally around 5 to 6 stretching the range from 4 to 9. For *Listeria monocytogenes* D_{55} values of 3.2 min are reported for vacuum-packed minced meat to 47 min in ground pork with z values between 4 and 5. For *E. coli* O157:H7 and *Salmonella* similar values have been reported ranging about 6 min in minced meat and in ground beef or ground pork. *Yersinia enterocolitica* in ground beef and milk or in liquid egg (Toora et al., 1992; Bolton et al., 2000; Huang and Juneja, 2001; Favier et al., 2008) and *Aeromonas hydrophila* in liquid egg (Schuman et al., 1997) are even more sensitive to heat with D_{60} values of 0.55 min and 0.03 min respectively. The values are lower than those given in the compilation of table 2a given by Sørquist (2003).

10. Processing technology and inactivation of microorganisms.

A proper heat process should be based on a thorough knowledge of the Behaviour of the death kinetics of the target microorganism(s) and on the time temperature profiles in each spot of the container to be processed. A theoretical approach to combine food engineering

and predictive microbiology has been presented by Mafart (2005). Thermocouples and mathematical description of heat penetration profiles can offer much knowledge of such profiles. For many cases, e.g. rotational sterilization and sterilization of inhomogeneous contents these tools are currently not yet sufficient. If heat penetration cannot be described properly the use of time temperature integrators (TTIs) may offer a sound alternative to the above mentioned methods. The use of TTIs involves measurement of the concentration or some other attribute before and after processing of a heat sensitive substance or device present in the product or introduced into the food sample chosen to mimic the thermal degradation of the substance of interest. The requirement is to deploy easily measurable, irreversible, time-temperature dependent changes that can be attributed to a biological, chemical or physical phenomenon (Taoukis and Labuza., 1989). The basic assumption in using TTIs for thermal process evaluation is that the activation energies or z values of the TTI and the substance of interest are the same (Hendrickx et al., 1995). Another requirement for TTIs is the stability before and after heat processing. Particularly intrinsic TTIs which are homogeneously distributed are to be preferred allowing the evaluation of the volume average process impact (Claeys, 2002). Whereas there are several TTI candidates to estimate the effect of pasteurization, TTIs for sterilization are more difficult to find. Guiavarc'h et al. (2004), however, developed a TTI by coating glass beads with *Bacillus licheniformis* α amylase in a dehydrated form. Even when temperature profiles are well-known, calculations of thermal death of microorganisms become more complicated when inactivation is not log-linear. Modern software enables also accurate description of inactivation when heating is not isothermal (Aragao et al., 2007, Corradini et al., 2005; Corradini et al., 2006; Corradini et al.,

2008; Hassani et al., 2007; Peleg et al., 2008). However, extrapolation beyond the observed values remains almost impossible. It should be borne in mind that heat resistance studies are always conducted with relatively high numbers of organisms. The tacit assumption is that high number per ml represent the same numbers in low concentrations in many containers. If the inactivation is dependent on the initial concentration this extrapolation is not allowed. If indeed the initial concentration has an effect tailing is not the same in practice. Only if the cause of non linearity is known some extrapolation seems to be justified. There are many examples of the effect of heating rate on microbial survival. In slow heating vegetative cells can adapt to stress conditions resulting in a lower inactivation rate. Also changes in composition due to heating can cause changes in the thermal resistance of microorganisms.

11. Concluding remarks

Although heat inactivation processes are well established for more than 90 years, better control of existing processes will enable smaller safety margins and hence milder processes. As a consequence a better description of the heating process is required as well as a more thorough understanding of the molecular processes involved in the heat inactivation of microorganisms. The availability of better tools to take into account processing conditions, more advanced software and better mathematical models is an aid in modeling inactivation kinetics of micro-organisms. Much knowledge is still lacking with respect to phenotypic heterogeneity in genetically homogeneous strains as well as variation among strains. Besides, more knowledge is required of the mechanisms responsible for the emergence of new highly heat resistant microbes.

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Table 1. Steps in quantitative risk assessment

1. *Hazard identification*

   Which microorganisms are relevant for that type of food?

2. *Exposure assessment*

   What are the steps in the process where microorganisms enter,

   Multiply or decrease?

3 *Dose response assessment* (*Hazard characterization*)

   What are the health problems at different level of exposure?

4. *Risk characterization*

   Estimation of the probability of occurrence and severity of

   known or potential adverse health effects in a population

Table 2a

Heat resistance of some non sporing food pathogens and enterococci.

Bacterium	T/°C	D value/s (mean)
Enterococcus faecium	55 60 65 72	3813 1150 347 65
Enterococcus faecalis	55 60 65 72	1393 415 123 23
Listeria innocua	55 60 65 72	1635 162 16 0.6
Listeria monocytogenes	55 60 65 72	643 87 12 0.7
Escherichia coli	55 60 65 72	266 39 5.6 0.4
Yersinia enterocolitica	55 60 65 72	168 30 5.4 0.5
Salmonella ssp.	55 60 65 72	222 24 2.6 0.1
Campylobacter jejuni/coli	55 60	50 8.2

	65 72	1.3 0.1

z values of many vegetative cells are ranging between.,9 to 6.7. z values of enterococci are higher (9.6 – 13.0)

Table 2b

Approximate heat resistance (*D* values, min) of vegetative cells of moulds and yeasts. (Ingram, 1971, Tomlins and Ordal, 1976, Put et al., 1976, Beuchat and Rice,1979, Bayne and Michener, 1979; Rayashekara et al., 2000, Panagou et al., 2002).

	Temperature (°C)				
	55	60	65	70	80
moulds and yeasts					
Torulopsis mogii			*4*		
Penicillium verniculatum			*4*		
Saccharomycopsis lipolytica			*4*		

Ascospores of moulds and yeasts		
Hansenula anomala	4	
Saccharomyces cerevisiae	>4	
Saccharomyces chevalier	>4	
Byssochlamys nivea		5
Byssochlamys fulva		5
Neosartoria fisheri		2
Monascus ruber		1 -2

Table 2c.

Approximate heat resistance (*D* values, min) of bacterial spores in low acid substrates a_w >0,95 (based on data of Ingram (1971), Michels and Visser; Brown, 2000, Scheldeman et al. 2006).

	Temperature (°C)				
Species	80	90	100	110	120
Cold growing aerobes					

Bacillus megaterium	17.5				
Cold growing anaerobes					
Clostridium botulinum Type E and B		0.3 – 3			
Mesophilic aerobes					
Bacillus polymyxa	4.5	0.1–0.5			
Bacillus megaterium		1.0			
Bacillus cereus			5.5		
Bacillus subtilis				11.0	6.9
Bacillus sporothermoacidurans			11–30		
Alicyclobacillus terrestris	15				
Bacillus coagulans		2			
Bacillus sporothermodurans				3	
Mesophilic anaerobes					
Closridium butyricum					
Closridium tyrobutyricum	13				
Clostridium perfringens		0.2-2			
Clostridiuum A, B and F					0.1-0.2
Clostridium sporogenes					0.1-1.5
Thermophilic aerobes					

Geobacillus strearothermophilus	4-5
Thermophilic anaerobes	
*Clostridium thermosaccharolyticum**	5
*Desulfotomaculum nigrificans**	13 - 55

*Much higher heat resistance data have been reported

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Table3a.

Overview of some primary inactivation models. (adapted from van Gerwen, 1998 and Li et al., 2007)

Exponential (Bigelow, 1921) $\ln(N_t) = \ln(N_0)-kt$

Weibull model (Peleg and Cole,1998, Mafart et al, 2002, van Boekel,2002.)	$\log(N_t/N_0)=-(t/\delta)^p$
Gompertz equation (empirical) (Linton et al, 1995)	$\log(N_t) = \log(N_0) + a\exp[-\exp(b+ct)]-a\exp[-\exp(b)]$
Critical sites model Moats, 1971, Smelt et al, 2002)	see text (paragraph 8.2.5)
Logistic model	$\log(N_t) = A- D/[1-\exp(-B(t-M))]$
Biphasic model (Cerf, 1977)	$\ln(N_t/N_0) =\ln((1-f)\exp(-k_1t)+f\exp(-k_2t))$
Augustin et al., 1998	$N_t = N_0/(1+ \exp (\log (t)- m_a)/s_a^2))$
Activation/inactivation model (Sapru et al, 1992)	$dN_d/dt = -(k_{d1}+k_a)N_d,\ N_d(t=0) = N_d(0)$ $dN_a/dt = k_aN_d – k_dN_a,, N_a(t=0) = N_a(0)$

Nomenclature

A = higher asymptotic log count value (log cfu ml^{-1})

B = maximum death rate or growth rate at time M (s^{-1})

D = difference in value of the upper and lower asymptotic log count (log cfu ml^{-1})

M = time at which death rate is at maximum (s, min or h)

a, b, c = parameters of Gompertz equation

f = proportion of the minor subpopulation in the initial population

k = death rate constant (s^{-1})

k_a = first-order activation constant of the dormant spores in the Sapru model, (min^{-1})

k_{d1} = first-order inactivation constant of dormant population in the Sapru model, (min^{-1})

k_{d2} = first-order inactivation constant of N_a in the Sapru model, (min^{-1})

k_s = maximum death rate constant of the major subpopulation of microbes (s^{-1})

k_r = maximum death rate of the minor subpopulation of microbes (s^{-1})

m_a = time where inactivation rate is maximum) in model of Augustin

s_a = parameter proportional to the standard deviation of heat resistance in the Augustin (1998) model, ($min^{1/2}$)

N_t = concentration of microbes at time t (cfu ml^{-1})

N_0 = initial concentration of microbes at time t = 0 (cfu ml^{-1})

N_a = active population in the Sapru model, (log (cfu ml^{-1}))

N_d = viable but dormant spore population for Sapru model, (log (cfu ml^{-1}))

t = time (s or min or h)

p = shape parameter in Weibull equation

δ = scale parameter in Weibull equation

Table 3b.

Secondary inactivation models adapted from van Gerwen (1998) and Davey (1993)

z concept	$\log(D_{ref}/D) = (T - T_{ref})/z$
	$z = 2.303RTT_r/E_a$
Arrhenius	$k = A\exp(-Ea/RT)$
Arrhenius Davey	$\ln(k) = a + b/T + c/pH + d/pH^2$

Captions to the Figures

Fig. 1. Example of effect of heat inactivation in the context of exposure assessment (Membre and Lambert, 2008)

Fig. 2. Heat inactivation of *Listeria monocytogenes* at 62 °C. In this example there is a minimum number of critical sites left after treatment below which no cell growth is possible. As a consequence the inactivation rate of whole cells (observed values (diamonds) and predicted values (drawn line) is faster than predicted inactivation of critical sites themselves (broken line).

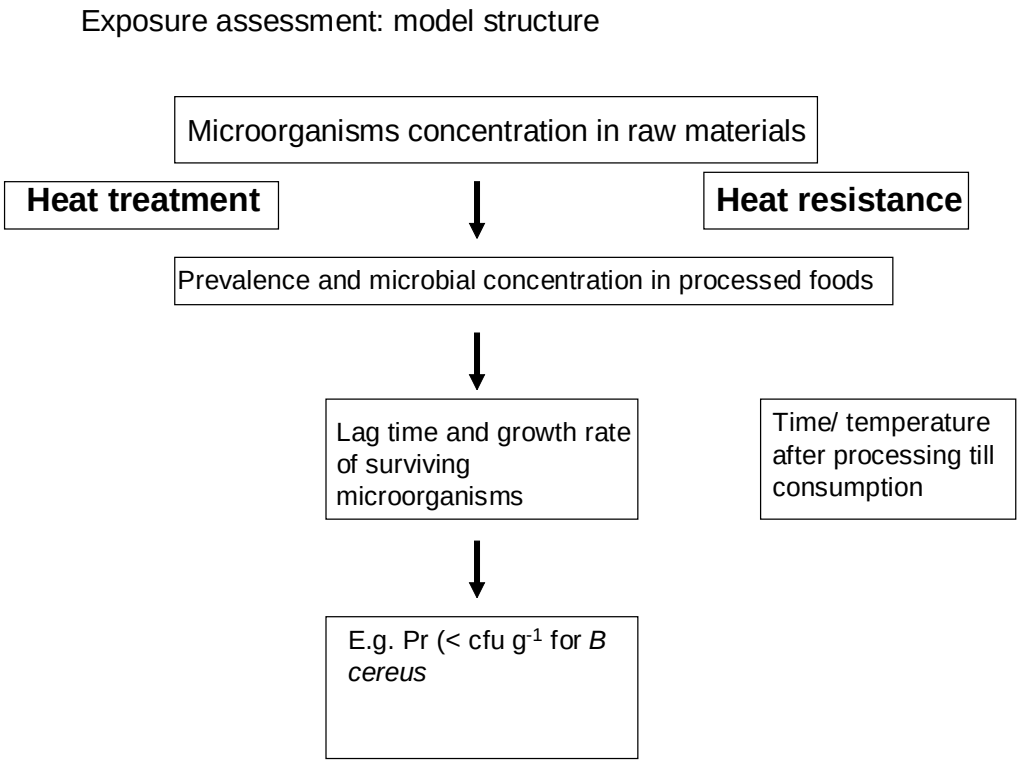

Fig. 1

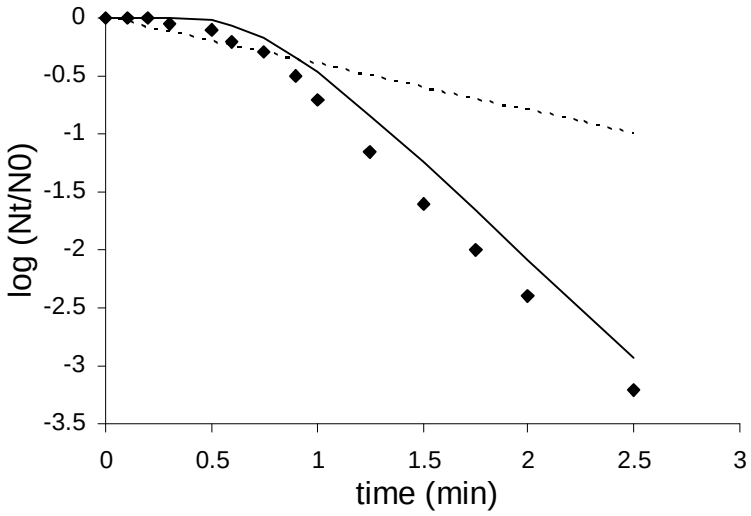

Fig. 2