



Guidelines for experimental design protocol and validation procedure for the measurement of heat resistance of microorganisms in milk



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ABSTRACT

Studies on the heat resistance of dairy pathogens are a vital part of assessing the safety of dairy products. However, harmonized methodology for the study of heat resistance of food pathogens is lacking, even though there is a need for such harmonized experimental design protocols and for harmonized validation procedures for heat treatment studies. Such an approach is of particular importance to allow international agreement on appropriate risk management of emerging potential hazards for human and animal health. This paper is working toward establishment of a harmonized protocol for the study of the heat resistance of pathogens, identifying critical issues for establishment of internationally agreed protocols, including a harmonized framework for reporting and interpretation of heat inactivation studies of potentially pathogenic microorganisms.

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1. Introduction

At the beginning of the 20th century, heat processing in the dairy industry was focused on the two main diseases known to be transmitted by milk: brucellosis and tuberculosis. *Mycobacterium tuberculosis* was considered to be the most heat resistant pathogen associated with milk (Hammer, 1948). Later, the time-temperature combinations for milk pasteurization were modified in order to inactivate also *Coxiella burnetii*, the agent of Q-fever, which is more heat resistant than *M. tuberculosis*. However, this bacterium should no longer be considered relevant as it is transmitted by inhalation (Cerf and Condron, 2006). More recently, concerns were raised by controversial research results showing occasional survival of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in pasteurized milk. The controversy regarding MAP survival in pasteurized milk is not now a major issue (Robertson et al., 2012), but such controversy might reappear on other occasions with other emerging potential pathogens.

The establishment of a thermal process is often a complex exercise, which must consider food composition, number and type of microorganisms present and anticipated storage conditions (Sindelar et al., 2013). There is a need for harmonized experimental design protocols

for heat resistance testing and a need for harmonized validation procedure for heat treatment studies.

Different researchers use different techniques, a situation that makes it difficult, if not impossible, to compare the results, and leads to disagreement that cannot be resolved easily. Examples of difference are: heating in open vs. closed vials, laboratory vs. industrial setup, laboratory vs. wild strains, strains isolated from the environment vs. from infected animals, accounting or not for non-linear death kinetics, etc.

The dairy sector would benefit from agreement on harmonized protocols for measurement of heat resistance of food-borne pathogens at the laboratory level and validation at the pilot-plant or industrial scale level. This will be of particular importance to allow international agreement on appropriate risk management of emerging potential hazards for human and animal health.

To provide a scientific and technical basis for future developments in the management of food safety for humans and animals in international trade, there is a need for better utilization of experimental data on heat inactivation of bacterial and viral pathogens. An aim of this paper is to facilitate enhancement of international databases of heat inactivation data, so that diverse data sources are complementary and more readily combined, accepted and applied in dairy processing. Such databases are important to assist industry, in particular small manufacturers, in the design and validation of their control measures and for authorities to assess the measures implemented and to assess their equivalence.

The purpose of this paper is to review the literature and express expert views relevant toward establishment of such a harmonized

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protocol, to identify critical issues for establishing internationally agreed protocols, and to provide a harmonized framework for reporting and interpretation of heat inactivation studies of potentially pathogenic microorganisms.

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2. Approach

Heat inactivation data relevant to microbial pathogens in milk are based on diverse strains, and methodologies which, *prima facie*, make comparison of the results of these studies difficult. By identifying and discussing the issues that affect the results of heat inactivation studies, the aim is to highlight the significance of those issues so that transparent and objective decisions can be made regarding establishment of experimental approaches while at the same time satisfying the objectives elaborated above.

Internationally, food-safety management approaches are moving toward science- and risk-based approaches. It follows, that experimental studies should be performed in a way that reflects or is demonstrably relevant to the thermal treatment processes and technologies that are used commercially in the international dairy industry. Experimental studies should be relevant to the hazards in the product, and while laboratory studies provide useful basic knowledge of microbial heat resistance, including variability, the results need to be validated under commercial conditions. This has benefited from a greater understanding of raw material and ingredient quality, growth and survival characteristics of microorganisms, dynamics of heat treatments and the subsequent implications for manufacture, distribution and consumer use of foods. The use of a wide range of modeling techniques has provided powerful tools to aid this understanding.

3. Issues and recommendations

3.1. Selection and preparation of challenge organisms

Raw food products may often naturally contain a variety of pathogenic microorganisms and data collection should be focussed on pathogens relevant for milk and dairy products. The sensory quality of food is influenced by the metabolic activities of spoilage organisms, limiting the food's shelf-life. As a consequence, a thermal process may be applied for the destruction of not only microorganisms of public health concern, but also of those capable of growth and spoilage.

3.1.1. Choice of organisms

The choice of organism(s) should be based on the risk presented by specific microorganisms due to their likely presence and growth, or an association with known illness outbreaks or spoilage, to levels likely to compromise product safety and suitability. That potential is a consequence of the inherent characteristics of the organism. For example, while spore formers are much more heat resistant than vegetative cells, their potential for growth in refrigerated dairy products is usually less than vegetative psychrotrophs. Within vegetative cells, inherent characteristics such as cell wall composition and metabolic activity will contribute to differences in heat resistance.

3.1.2. Pathogens versus surrogates

Ideally, inactivation studies would employ the specific pathogen of concern. However, this is not always possible, particularly in a food production environment. In such cases, non-pathogenic strains of the same species (generic *Escherichia coli* versus Enterohaemorrhagic *E. coli*), or closely related non-pathogenic species (e.g. *Listeria monocytogenes* and *Listeria innocua*) may be used. Ideally, other studies e.g. laboratory based, would have demonstrated a high level of similarity

in heat resistance of the surrogate and specific organism of interest. Additional limited experiments should be undertaken with the pathogenic organism in order to confirm the results obtained with the non-pathogenic organism.

3.1.3. Strain variation

Variation in heat resistance among different strains of the same species is well documented. Some reasons for this are known, such as attenuation of strain vigor due to long term storage and repeated sub-culture on nutritious media. As such, use of recent isolates from factory environments, where possible, is usually advocated. This also has the advantage that, if the target organism in the factory or in the region has specific thermal adaptation, this will be incorporated into controls most appropriate to the enterprise, consistent with risk-based approaches. Such wild-type strains should be identified and fully characterized to ensure a basis for comparison between studies. Controls for comparisons are necessary within a given laboratory and more importantly in studies between laboratories. Readily available type cultures with a minimum number of subcultures provide for consistency between studies.

Other considerations are the representativeness of the strain used for the species as a whole. One approach is to attempt to isolate the most heat resistant strain as the basis for inactivation data, because it should provide the most conservative assessment, providing more confidence in the level of safety achieved by control measures. There may not be a single strain, however, that exhibits higher heat resistance under all conditions relevant to all products, or even at all temperatures because *z*-values may differ between strains. Striking differences in *z*-values between species such as for *Bacillus stearothermophilus* and *Bacillus sporothermodurans* are well known (Dogán et al., 2009; Huemer et al., 1998). In the absence of certainty that the most heat resistant strain has been isolated, a mixture or “cocktail” of strains may be employed to increase confidence that the upper limit of heat resistance is established. Yet, the strain with the highest heat resistance is sometimes unacceptably different from the “average” strain. Using its characteristics might lead in most cases to a uselessly conservative heat treatment. As an example, the rare strain *Salmonella* Senftenberg 775 W is 30 times more resistant than *Salmonella* Typhimurium (Ng et al., 1969; Silva and Gibbs, 2012).

Ideally, one may prefer to take a more “risk-based” approach, to characterize the heat resistance of a range of isolates, establishing the distribution of heat resistance e.g. characterized by its mean and standard deviation. Such information can be combined within stochastic modeling techniques to identify less conservative controls that still achieve the required food safety outcomes. When operating in a non-sterile environment, strains that are easily identifiable, e.g., by the presence of antibiotic resistance markers, or inclusion of genes for green fluorescent protein, may be useful to ensure that the organisms enumerated at the end of the treatment were those that were introduced as the challenge organism. It is important to ensure that such genetic modification does not demonstrably change the heat resistance of the strain being tested.

3.2. Preparation of challenge culture(s)

3.2.1. Growth conditions

The conditions of preparation of the inoculum for challenge are known to affect the tolerance of organisms to subsequent stress. These include the effect of temperature, pH, and availability of nutrients on the composition of the cell and its metabolic activity. When exposed to a stress, many bacteria instigate a series of responses that make them generally more resistant to a range of stresses (Ait-Ouazzou et al., 2012; Henge-Aronis, 2004). Cells in the exponential growth phase are less resistant to a range of stresses than stationary phase cells. It may be appropriate to select conditions for growth that mimic the likely physiological state of the cells in milk prior to processing.

This may be difficult to achieve as vegetative bacteria in milk will include all stages of growth depending on their origin. For example, cells exhibit higher resistance when grown at higher temperature, but that is not the situation expected in commercial operations (Juneja, 2004). Alternatively, a conservative approach may be taken with cells prepared in a way that will maximize their heat resistance. Having decided the culture preparation conditions when designing the challenge study, it is important to document those conditions when results are reported.

3.2.2. Storage

Long term storage of cultures on laboratory media, and repeated sub-culturing can select for strains that differ in vigor, including heat resistance, from wild-type isolates, reinforcing that, where possible, challenge organisms should be recently isolated. However, in some cases (e.g. MAP and other very slow growing organisms) inocula may have to be prepared and stored frozen prior to challenge studies and used directly after thawing. Frozen storage and thawing can induce injury, which is not repaired prior to the challenge. Less damage occurs at lower temperature, and $-80\text{ }^{\circ}\text{C}$ storage is widely accepted as appropriate for long-term storage of cultures. Equally, freeze-drying (“lyophilisation”) of cultures is another method for long-term storage but may introduce the need for additional steps for resuscitation and preparation of cultures prior to their use in thermal inactivation challenge studies.

3.2.3. Clumping organisms and intracellular pathogens

Some organisms normally grow in close association, resulting in aggregates of cells rather than individual cells. This may be exacerbated in broth media, particularly for organisms that form pellicles. Clumping has been suggested to provide protection from heat to cells on the inside of the clump, although several studies have demonstrated that the effect is negligible for HTST processes (Davey, 1990; Cerf et al., 2007; Hastings et al., 2001). Clumping leads to underestimation of cell numbers by plate count methods. Clumping during heating leads also to the tailing of survivor curves, leading to the wrong interpretation that there is a more heat-resistant spore fraction (Klijn et al., 2001).

While it is desirable to disrupt stable aggregates to generate valid data, and methods including sonication and homogenization have been used, the effect of any shear forces or heat generated during such treatments is uncertain (Asséré et al., 2008). Homogenization is preferable to facilitate dispersal of organisms where homogenization is used in commercial processing of the product of interest.

Studies involving organisms known to form clumps should adopt strategies to assess the contribution of clumping and to minimize physical injury to the cells. Those strategies should be documented when results are reported.

Although it has been suggested that intracellular pathogens in leukocytes in milk might demonstrate higher heat resistance, several studies have found that the effect, if it occurs, is minor and of little significance (Hope et al., 1996; Stabel, 2003).

It is well established that there is a tailing of Foot and Mouth Disease (FMD) virus inactivation after the treatment of milk from infected cows following pasteurization treatment (Callis, 1996; Donaldson, 1997; Hyde et al., 1975). The failure of pasteurization to inactivate all virus particles has been explained by virus attachment to fat micelles and cellular debris (Callis, 1996) due to reduced water activity in the fat during heating.

3.3. Experimental design

3.3.1. Inoculum density

Ideally, the density of the inoculum used in the challenge study should reflect levels that would be expected to occur naturally. This is because there are situations where microbial density affects the behaviour of the population as a whole, e.g. through quorum sensing. Also, as

discussed below (see “Heating menstruum”), the relative level of challenge organisms and other microbiota may affect the inactivation kinetics of the challenge organism. Very high levels of competing flora appear to be necessary to affect the kinetics. The concentration of contaminating Gram negative bacteria needs to be $>10^8$ CFU/mL to exert a protective effect on the thermal destruction of *Salmonellae* (Duffy et al., 1995). Pragmatic considerations regarding enumeration, however, and in particular for validating a process lethality step such as heat processing, mean that high inoculum levels are usually required, for example 10^6 to 10^7 CFU/g of products, to be able to measure the extent of reduction in challenge organisms. If the interest is only in establishing time to non-detection of ‘realistic’ natural levels of contamination, lower inoculum levels can be employed together with enrichment methods for demonstrating absence of the challenge organism in the treated sample. Alternatively, lower cell densities may be used and enumerated if large sample volumes can be processed, e.g. using most probable number (MPN) methods (Duquet et al., 1987) or cell concentration methods such as filtration.

Inocula should also be suspended in the smallest practical volume of medium that allows even distribution of the organism in the heating menstruum. This is to minimize the effect of the suspending medium on the properties of the heating menstruum and also to minimize time for heat transfer (see “Thermal treatment apparatus” below).

3.3.2. Treatment conditions and variables

In the design of inactivation studies, consideration should be given to the variability in processes and product formulations that could affect process lethality. The challenge trial should be designed to encompass the full range of conditions, including the least lethal combination of processing and product formulation.

3.3.3. Heating menstruum

In the context of the current paper, the preferred heating medium will be milk, but questions involve the composition of the milk used and whether the milk should be sterile, at one extreme, or at the other extreme unpasteurized (raw) and containing “natural” levels of other microbial contaminants, and possibly active natural antimicrobial systems.

Ideally, milk with natural contamination levels should be used as the presence of other microbiota could provide protection, although this effect may not be significant. This can create difficulties in enumeration of the test organism because, if other organisms are present in the milk, selective media may be required to enumerate the challenge organisms. Selective media often contain antibiotics or other selective agents that can prevent the growth of stressed and/or injured surviving cells, leading to overestimation of the lethality of the treatment. For this reason, some investigators have preferred to use heat treated milk (e.g. UHT) that has very low levels of microbial contaminants. The heating process affects the properties of milk, however, and may reduce the lethality of subsequent treatments. Pearce et al. (2001) centrifuged milk samples and disinfected the pellet with cetylpyridinium chloride, before re-suspension, to inactivate “extraneous” contaminants. Some authors have suggested the use of irradiated milk, which while having low microbial levels, has properties closer to raw milk.

The properties of milk can vary according to region and season etc. Such differences include fat levels and the influence of fat in food in increasing microbial heat resistance is well documented (van Asselt and Zwietering, 2006). Conversely, the presence of free fatty acids may increase the inactivation effect of heat (Lekogo et al., 2010). To minimize the effects of these differences, rehydrated standard spray-dried milk powders have been suggested as a preferred heating menstruum (Cordier et al., 2004), but it has disadvantages such as absence of any natural flora or denaturation of proteins or use of unsterile milk powders.

Ultimately, it may be possible to demonstrate that systematic differences in heat resistance of pathogens in milk can be related to fat levels,

or calcium levels, homogenized or not etc. Thus, whatever heating menstruum is chosen, it is recommended that properties such as these are measured and reported together with the determinations of microbial inactivation.

3.3.4. Enumeration

The sensitivity and specificity of microbial enumeration methods vary. Factors causing differences in quantification include media composition (e.g. selective versus non-selective media, selective/differential medium), use of solid or liquid media, and incubation times and temperatures. While similar methods usually provide consistent data, the use of different methods may result in poor repeatability of inactivation determinations. Issues concerning the use of selective media were mentioned above (see “Heating menstruum”). Thus, enumeration methods should be detailed when results are reported and, where possible, the sensitivity, specificity and reproducibility of the methods employed should be detailed, or related to known and widely accepted “standard” methods. Sensitivity refers to the ability of the media to enumerate all cells, or colony forming units, of interest. Specificity refers to the ability of the method to correctly discriminate between the organism of interest and other, similar, organisms that may be present in the heating menstruum. The latter issue should not be relevant if a sterile heating menstruum and axenic culture is used.

3.3.5. Sampling frequency

As intimated above, for validation studies in particular it may only be necessary to demonstrate absence of the challenge organism after treatment (in a stated number of samples) or preferably a particular level of reduction. Determination of kinetics of inactivation (e.g. the rate of inactivation) as a function of temperature and other environmental conditions will provide more fundamental information that can be used to evaluate the effects of different time-temperature combinations and processes. From this information, equivalent processes can be designed. The latter approach will require a series of determinations of survival after different treatment times. Importantly, monitoring survival over time will reveal whether inactivation follows simple log-linear kinetics or whether more complex inactivation kinetics occur. This is discussed further, below (see “Analysis/interpretation of results”).

3.3.6. Replication

Variation between trials can arise from slight differences in sampling and sample processing, from differences in the batches of the heating menstruum and milk, from differences in the preparation of the challenge strain, etc. as well as the variables that are deliberately changed to determine their effect on inactivation. Experimental designs should include sufficient independent replication to characterize the extent of these sources of variability, both:

- to be able to determine whether differences in results between trials and between laboratories represent systematic (and important) differences between experimental systems and treatments or are simply normal variation, and
- to characterize the variability of treatments so that processes can be designed that satisfy inactivation criteria with known levels of confidence by application of statistical analysis of the variability (e.g. to establish statistical confidence limits).

Independent duplicate determinations are commonly employed and are the minimum required. It is important that such replicate determinations are completely independent, and not repeats of the same experiment. Greater replication (independent triplicate or quadruplicate, for example) enables a characterization of variability and, thus, greater confidence in decisions based upon the results of those trials (van Asselt and Zwietering, 2006).

3.4. Thermal treatment apparatus

The choice of thermal treatment apparatus will depend on the type of determination being undertaken, viz. whether to establish basic pathogen thermal inactivation data or to validate a commercial scale process. In the case of verification of commercial pasteurization processes, the evaluation of pasteurization performance would ideally be undertaken by direct challenge of the equipment with naturally contaminated milk. This is almost always impractical. First of all, naturally contaminated product would not be expected to have consistent levels of pathogens. The process will have to include a margin of safety for exceptional cases of high levels of contamination and thus, as discussed above (“Inoculum density”), it is usually more practical to test systems against high levels of challenge organisms. Moreover, testing commercial systems directly involves greater expense due to the high volume of samples that are required, the need to decontaminate the plant between challenge trials and the problems of deliberately introducing high levels of challenge organisms into factory environments.

Consequently, pasteurization efficiency of commercial systems is usually inferred by extrapolation of the results of studies conducted in simpler, or smaller, experimental systems. Examples of systems used for basic data collection include test-tubes, vials, capillary tubes, and submerged coils. Process verification can be undertaken with laboratory scale pasteurizers and pilot scale pasteurizers. Systems that are not totally immersed in the heating medium can cause artefacts, described by Cerf et al. (2007).

The extrapolation of results from simpler, or smaller, systems to full scale commercial pasteurizers is not straightforward, however, because of potential physiological changes (i.e. induction of stress responses during warming) in cells experiencing dynamic temperature processes compared to exposure to static temperature and, more particularly, because of the differences in the time-temperature profile in simple systems compared with fluid milk passing through modern commercial pasteurizers. In addition, turbulent flow, associated with increased Reynold's numbers (R_e), provides for more efficient residence times and heat transfer.

It is essential that data derived from one (experimental) pasteurization system can be compared to data from others. While the “residence time” in the holding tube at the nominal pasteurization temperature is the main process step governing the extent of inactivation, other stages before and after this can also contribute to pathogen inactivation. This requires, for example, that inactivation during “come-up” and “cool-down” is determined or can be calculated by full characterization of the time-temperature profiles experienced by cells in the experimental system.

A liquid flowing in a closed system has a distribution of residence times (“residence time distribution”, RTD) for microorganisms suspended in the milk. Pasteurization processes are designed to ensure that the “fastest moving particle” (FMP), i.e. that particle which has the minimum residence time, still is exposed to the required time-temperature conditions to achieve the required thermal inactivation. Laminar flow results in shorter minimum residence time than does turbulent flow. As flow becomes more turbulent the residence time of the FMP more closely approaches the average of the RTD and the movement of milk through the pipe may ultimately approximate “plug” flow, i.e. when all particles effectively take the same time to pass through the tube. The flow characteristics can be deduced from calculation of the R_e value which is a function of the average flow speed through the tube, tube diameter, fluid viscosity and fluid density. A higher R_e indicates more turbulent flow. Thus, each of these characteristics should be reported when inactivation data derived from flow-through systems are presented. Methods for characterization of flow characteristics in HTST pasteurizers and calculation of their effects are available (Asteriadou et al., 2010; Kiesner, 2004; Tomasula and Kozempel, 2004). For complex systems, with different geometries, temperature ranges and times, the combined effect can be estimated as the sum of

the effects of the component steps. This approach considers the warming, heating and cooling processes as several individual simpler processes with different residence times (Kiesner, 2004).

When validating a process, steps should also be taken to verify that the process is operating as planned, e.g. to ensure the integrity of the system so that cross-contamination of raw to pasteurized product does not confound the results.

Traditional D and z value determinations on a laboratory scale are derived from plots of survival versus time over a range of temperatures. If these determinations were to be scaled up to pilot-scale turbulent flow pasteurization, each time point would require changing the holding-tube with associated sterilization before and after. As this approach is impractical, an alternative strategy was pioneered by Pearce et al. (2001). They developed kinetic data for a pilot-scale pasteurizer operating under commercial conditions of turbulent flow and used survival data at different temperatures to derive the required parameters from the appropriate mathematical model. The general applicability of this approach will need to be confirmed by other research groups.

3.5. Analysis/interpretation of results

The preceding discussion has set out principles for the reproducible determination of thermal inactivation. This section sets out approaches for interpretation and application of the data generated.

While commercial processes can be verified by testing for the absence of the challenge strain after the process, or by demonstration of the achievement of the required reduction, the knowledge gained is limited in its utility and cannot be readily extrapolated to other conditions or processes. This type of experiment shows the absence of survivor in the treated volume. It gives no indication of the probability of survivor presence in larger volumes as those of continuous flow plants. Therefore, unless there is some replication of the process verification experiments, there is little certainty that the process is reliable under all realistic pathogen loads and operating conditions. The effect of minor process deviations will also be unpredictable. It cannot be used, for example, to optimize the process or to develop alternative processes. This requires a more fundamental and detailed knowledge of the effects of time and temperature on the survival of the challenge organism. Knowledge of the kinetics of inactivation, i.e. the change in microbial numbers as a function of heating time, is also required. This knowledge can be integrated with detailed knowledge of the times and temperatures experienced (see “Thermal treatment apparatus”) by those organisms during commercial processes to assess processes or to design novel processes that, while assuring product safety, optimize other product quality attributes.

3.6. Thermal inactivation models

Thermal inactivation of pathogens is accepted by many as being an exponential process, i.e. in a given time interval at a given temperature a constant proportion of the surviving cells will be killed. If the logarithm of survivors is plotted against time, a straight line is expected. From the slope of that line can be derived the D -value or “Decimal Reduction Time” (DRT) which is the time required to decrease the population ten-fold, i.e. by 90%. The slope of the line is a function of temperature, and the dependence of the D -value on temperature is expressed in the z -value, which is the increase in temperature that leads to a ten-fold decrease in D -value. These values are readily derived from appropriate experimental data and are convenient because they can be easily combined, mathematically, with time-temperature data describing pasteurization processes to estimate expected pathogen reduction in commercial pasteurizers. The effect of combined environmental factors such as temperature, pH and a_w on the heat resistance can be modeled through generalized equations such as those of Cerf et al. (1996) or Gaillard et al. (1998). It is well known, however, that

data describing thermal inactivation of populations of microorganisms often do not form a straight line when plotted as described. Delays before the start of the exponential decline, described as “shoulders” are observed as is a reduction in the rate of inactivation as treatment time increases, described as “tailing”. Other deviations from exponential decline have also been described. Theoretical reasons for these variations are well described in the literature (Stumbo, 1973) and a short critique of the promulgation of the exponential inactivation hypothesis is presented in McClure et al. (2004), with more detailed discussions in Cerf (1977). Importantly, if shoulders or tailing are reproducible properties they need to be described so that patterns of inactivation kinetics can be discerned and, thus, predicted. To this end, a range of mathematical models that describe more complex inactivation kinetics and enable their summary to be evaluated is presented e.g. in Valdramidis et al. (2004); Geeraerd et al. (2005); Coroller et al. (2006). If properly formulated and validated such models, in conjunction with secondary models, facilitate prediction of the effects of a new treatment regime or regimes other than those specifically tested. McMeekin et al. (1993) provide a useful introduction to the development and use of predictive models in food microbiology. McKellar and Lu (2004), Mafart et al. (2010) or EFSA (2012) provide comprehensive reviews of the state of the science.

Most mathematical models require assumptions about the nature of the process that they describe and, when fitted to the data, will provide an equation that represents the best interpretation of the data given the assumptions or hypotheses inherent in the model. As such, reporting of raw results, as opposed to derived results such as D - and z -values or other fitted model parameters, is important because it can reveal reproducible behaviour that the fitted model “ignores”. Apart from the benefits of data summary and revealing patterns of behaviour, fitting data to a model can also quantify variability in the data, which can be used to determine confidence intervals for the predictions of the model or the inherent variability in the process and thereby to specify operating limits that consistently provide the required level of safety. As noted above, reliable specification of confidence limits is enhanced by greater replication or more data on the time course of inactivation under a given set of conditions.

While available models can account for complex inactivation kinetics, greater complexity could be caused by use of a mixture of strains, as advocated above, and experimenters need to be aware that the interpretation of theoretical models for thermal inactivation may not be valid, or may need to be modified, when multiple strains are used in experiments.

Thus, design of experiments for process verification can be based on reasonable expectations of performance based on inactivation data and models that are integrated with mathematical descriptions of time-temperature conditions in the pasteurizer. More importantly, good inactivation data and models combined with detailed process models provide a rigorous framework for identifying reasons for unexpected process failures and a rational basis for modification of the process to overcome them.

From the foregoing discussion, it is apparent, as identified by Hastings et al. (2001) that pasteurization process design and verification will require the combined expertise and perspectives of engineers and microbiologists.

4. Conclusions

Selection of microorganisms to use in challenge testing for determination of thermal inactivation depends on the knowledge gained through commercial experience and/or epidemiological data. In addition, the technological parameters like pH, water activity, temperature, fat content, etc. should be considered. All experimental data need to come from a carefully designed protocol, where one specific microbial species is added to a chosen matrix under conditions relevant to commercial operations. All experimental variables including strains

used, inoculum preparation and addition, heating apparatus, composition of heating menstruum, etc. must be documented.

The implementation of the recommendations above would contribute to an improved comparability of published results and to a better reliability of the mathematical quantitative risk assessment models that will be developed in the future, although additional work is required to develop a harmonized protocol for the study of the heat resistance of pathogens.

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