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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.

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 subculture 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 (Dogan 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 form 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.