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Exposure assessment model to combine thermal inactivation (log reduction) and thermal injury (heat-treated spore lag time) effects on non-proteolytic *Clostridium botulinum*

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ABSTRACT: *Clostridium botulinum* is one of the most severe hazards causing foodborne disease. There are well-established control procedures that have been identified to destroy or inhibit growth of non-proteolytic *Clostridium botulinum* spores. One of these used in the manufacture of chilled foods is application of the 'non-proteolytic *C. botulinum* cook', based on the characteristic that spores of non-proteolytic strains are considerably more heat sensitive than spores of proteolytic strains. It is generally agreed that a temperature of 90°C for 10 min will deliver a 6D inactivation of non-proteolytic *C. botulinum* and this is a commonly used performance standard for the heat processing of chilled foods. However, under stress conditions provided by intrinsic (e.g. pH) or extrinsic factors (e.g. storage at chilled temperatures), heat-treated surviving spores need a certain time (namely lag time) before recovering from injury, germinating and then starting to grow. The objective of this work was to explore the possibility of identifying milder heat-treatments than 10 minutes at 90°C, by incorporating this lag time with the 'traditional' thermal inactivation effect. To do so, an exposure assessment model, based on the Degree of Protection (DoP) concept was developed. Results are promising; for example, with a product having a pH of 6.0 and heat-treated at 85°C for 10 min, shelf-lives above 21 days are expected. Under the same heat-treatment condition, if the pH is reduced to 5.7, shelf-lives above 50 days are expected as a significant pH effect has been identified. These examples are based on calculations including realistic chilled supply-chain temperatures, i.e. including some retailer/consumer refrigerator temperatures at 10°C. This incorporation is possible with probabilistic methods since they take multiple-value inputs into account.

1 INTRODUCTION

1.1 Food safety context

One of the food product categories that is expanding is the chilled food category. The chilled foods market consists of food products that are stored at refrigeration temperatures until consumption. The European chilled food sector showed a 50% increase to €9.1 billion from 1991-1996 and in 2005 the European market for chilled prepared foods had a turnover of €10.6 billion (Del Torre et al. 2004, Leatherhead Food International 2007). To ensure a high quality in terms of taste and aroma, the safety of refrigerated, processed food of extended durability (REFPEDs) relies on a combination of mild heat treatment and refrigerated storage, sometimes in combination with other hurdles such reduced pH and water activity. Shelf-life of REPFEDs is generally in a range of two/three up to five/six weeks as function of the process and formulation conditions.

In REPFEDs, non-proteolytic *Clostridium botulinum*, is considered as a serious hazard because of its ability to grow at temperatures as low as 3°C (Carlin et al. 2004, Notermans, Dufrenne, & Lund

1990, Peck 1997). Non-proteolytic *C. botulinum* is an anaerobic Gram-positive sporeforming rod-shaped bacterium, able to produce toxins that cause foodborne botulism. Foodborne botulism is rare, but severe intoxication can be caused by consumption of foods containing the neurotoxin produced by *Clostridium botulinum*. This neurotoxin is also called botulinum toxin or BoNT and is one of the most poisonous natural toxins known. The toxic dose of foodborne botulism is very low. Consumption of as little as 30 ng of neurotoxin is sufficient to cause illness and even death (Lund & Peck 2000, Odlaug & Pflug 1978).

The aim of this study was to suggest combinations of heat-treatment and shelf-life which maximize product quality without compromising public health safety. To do so, an exposure assessment model incorporating the key elements of REPFEDs in term of process, formulation and supply-chain distributions, i.e. mild heat treatment (< 90°C for 10'), mild acidic pH values (5.5 to 6.5), high water activity values (>0.97), and chilled storage temperature (realistic values coming from supply-chain surveys), has been developed. Particular attention was paid to

the heat-treatment impact as this factor is one on which the food operator can rely on when setting the food safety control measures.

1.2 Heat-treatment effect on non proteolytic *C. botulinum*

Heat treatments affect the outgrowth of spores of non-proteolytic *C. botulinum* in two ways: inactivation and injury.

It is generally agreed that a temperature of 90°C for 10 min will deliver a 6D inactivation of non-proteolytic *C. botulinum* and this is a commonly used performance standard for the heat processing of chilled foods (Gould 1999). The z values used to calculate process equivalence differ in different guidelines and codes of practice, but for example the US-FDA refer to a z value of 7°C for temperatures below 90°C (U.S. Food & Drug Administration 2001), which are the temperatures of concern in this present study.

In addition to this killing or 'cidal' effect and depending on the heat treatment applied, some spores remaining after the heat treatment may be injured. Due to this injury, the time for the spores to recover is prolonged and the length of the lag time is increased (Peck et al. 1995, Peck 1997). This injury heat-treatment effect depends also upon the recovery conditions such as temperature (chilled conditions), pH and water activity (a_w). If the shelf-life of the REPFED is short enough, it is biologically possible that the injured spores of *C. botulinum* have insufficient time to recover, germinate, multiply and potentially produce toxins.

When performing a risk assessment of REPFEDs, both heat-treatment effects, i.e. inactivation and injury, should be taken into account in the exposure assessment model as both contribute to significantly reduce the exposure level. In the following section, the methodology to combine the two heat-treatment effects is presented.

2 METHODOLOGY

2.1 Combining thermal inactivation and thermal injury in an overall Degree of Protection

The principle of the approach used to calculate the Degree of Protection (DoP) combining thermal inactivation effect and thermal injury effect is based on similar work previously reported in the literature (Hauschild 1982, Lund 1993, Schillinger, Geisen, & Holzapfel 1996). Details are provided in Equations 1 to 4.

$$\text{DoP} = \Delta R + \text{ThI} \quad (1)$$

where, ΔR is the log reduction due to thermal inactivation, and ThI the degree of protection due to thermal injury. Both terms can be expressed as the

decimal logarithm of the reciprocal of a probability (Equations 2 & 3).

$$\Delta R = \log (1/\text{Pr}) \quad (2)$$

$$\text{ThI} = \log (1/\text{Pi}) \quad (3)$$

where Pr is the probability that one spore survives the heat treatment, and Pi is the probability that lag time (recovery time) is shorter than the shelf-life (SL) as indicated in Equation 4.

$$\text{Pi} = \text{Probability} \{ \text{Lag} \leq \text{SL} \} \quad (4)$$

When the exposure assessment model is built, the output can be presented in two ways.

The first is to present the Degree of Protection, including thermal inactivation and injury, for different conditions of process, formulation (pH and a_w) and chilled storage conditions at various shelf-lives.

The second is to choose a targeted Degree of Protection, and present the model output as a set of combinations of process, formulation, storage conditions and shelf-life which deliver this targeted DoP. This second output visualization could be compared to an iso-probability method, as DoP are derived from probability calculations, and these iso-probabilities could be a basis for future operational Guidelines or user-friendly tools.

It is the second approach which has been selected in this study. An overall degree of protection of 6 has been chosen as it is a reference value currently applied in food safety management, in the form of inactivation (Gould 1999).

To calculate ΔR , the widely-used Bigelow model has been applied (Bigelow 1921). To calculate ThI, a lag model has been developed, details are given in the following sections.

2.2 Lag model development: data used

322 data points from 15 different studies (Table 1) were incorporated in the model input data set. Time-to-toxin data were taken into account in the model as left-censored data, since these data are less conservative than time-to-growth determined by plate count method. Time-to-growth (turbidity) and time to gas production have been considered as close enough to the actual lag time to be kept in the model input list without being censored. This is based mainly on the fact that often, more than one tube was studied and the response kept in our analysis is based on the shortest time. However, this introduces uncertainty in the model output which should be considered before any interpretation (see Discussion section).

Also, right-censored observed data were taken into account, which extended the data collection to experiments which were stopped before any growth or toxin production were observed. From the 322 data points, 156 were non-censored and 166 censored. Both heat-treated and non-heat-treated data were taken into account to assess the impact of for-

mulation and chilled storage conditions. From the 322 data points, 97 were heat-treated and 225 were not heat-treated.

Table 1. List of publications reviewed before including (at least partially) the data as model inputs

Experimental medium	References
Beef stew	(Schmidt, Lechowich, & Folinazzo 1961)
Different medium	(Carlin & Peck 1996)
Eel and mackerel	(Smelt & Rijke 1994)
Meat	(Eklund, Wieler, & Poysky 1967)
Meat	(Peck & Fernandez 1995)
Meat slurry	(Peck 1997)
Potato, mushroom, cauliflower	(Elliott & Schaffner 2001)
PYGS	(Graham, Mason, & Peck 1996)
PYGS	(Stringer, Haque, & Peck 1999)
PYGS, potato, broccoli	(Carlin et al. 2000)
Soya peptone broth medium	(Lawson, Adair, & Cole 1992)
TPSY	(Emodi & Lechowich 1969)
Turkey	(Lawson, Adair, & Cole 1992)
Vegetable	(Austin et al. 1998)
Vegetable	(Carlin & Peck 1995)
PYGS ¹	(Stringer & Peck 1997)
Meat ¹	(Notermans, Dufrenne, & Lund 1990)
Crabmeat and Broth ¹	(Solomon et al. 1977)

1. Observed data are uncertain (several days or even weeks before two observations), consequently these data have been used as additional information/validation and not strictly as model inputs.

2.3 Lag model development: model structure

From the literature review on lag time and also on the more general predictive microbiology knowledge, several assumptions have been made to define the model structure:

- ✓ the factors that influence the lag time include temperature (chilled storage temperature), pH, a_w , heat-treatment time and temperature;
- ✓ the Gamma concept (Wijtzes et al. 1998, Zwietering 2002), widely employed to combine the effect of several factors on the growth rate, has been applied to the lag, considering an inversely proportional relationship between lag and growth rate, the Gamma concept is still relevant to combine the effect of several factors on lag;
- ✓ the square root transformation has been chosen to stabilize the variance. The logarithm transformation is also a relevant and parsimonious (no need to add an extra parameter) method to stabilize the variance, but when dealing with numerical values such as 0 in the estimation

process, the square root transformation is easier to implement.

The lag time model equations are detailed in Equation 5 to 12.

$$\sqrt{\text{Lag}} \sim N(\sqrt{\text{Lag_mean}}, \sigma) \quad (5)$$

$$\sqrt{\text{Lag_mean}} = a_1 \times f_{11}(T) \times f_{12}(\text{pH}) \times f_{13}(a_w) \times f_{14}(\text{HT}) \quad (6)$$

$$\sqrt{\text{Lag_mean}} = a_2 \times f_{21}(T) \times f_{22}(\text{pH}) \times f_{23}(a_w) \quad (7)$$

Equation 6 is applied in presence of heat-treatment, i.e. when the temperature is equal to or greater than 70°C (Elliott & Schaffner 2001), Equation 7 is applied otherwise.

In Equations 6 and 7, functions f are defined as $f_{ij}(x) \geq 1$ in the domain of application/use of the model, to consider that the Lag_mean would be at its minimum if the optimal conditions were applied (ambient storage temperature, neutral pH, maximum a_w , heat-treatment at 70°C). The model has been developed using data generated under different conditions, collected by different research teams, and unfortunately the 322 data set analyzed does not cover the whole range of factors in a systematic manner, including all relevant combinations. Because of the data gaps, and also due to uncertainties (e.g. on pH and a_w values), the model outputs have been analyzed from a statistical perspective and then, from a practical application and microbiological perspective.

From a statistical perspective, terms f_{11} and f_{21} are similar (relative effect of chilled storage temperature is the same in presence or absence of heat treatment). The terms f_{12} and f_{22} on one hand, and terms f_{13} and f_{23} on the other hand, are significantly different. This might reflect that the effect of pH (and a_w) in the presence or absence of heat treatment, is different, but at this stage, this is only an assumption.

Effects attributed to the food matrix have already been reported in the literature but they have not been comprehensively analyzed and are not well-understood. As the general model structure chosen here is a Gamma concept structure, the matrix effect is only associated to two parameters, parameters a_1 (Equation 6) and a_2 (Equation 7). From a statistical perspective, the parameter a_2 was significantly different when applied to data collected using broth, vegetable or meat; while the parameter a_1 was not significantly different. To facilitate the practical application, it has been decided to merge the effect of broth, vegetable and meat on the lag time, in the case of heat treatment (unique term a_1 in Equation 6).

The set of equations to describe storage temperature, pH, a_w and heat treatment effect are provided in Equations 8-11

$$f_{11}(T) \text{ and } f_{21}(T) = \left(\frac{(T_{\text{opt}} - T_{\text{min}})}{T - T_{\text{min}}} \right)^{p_1} \quad (8)$$

$$f_{12}(\text{pH}) = \left(\frac{\text{pH}_{\text{opt}} - \text{pH}_{\text{min}}}{\text{pH} - \text{pH}_{\text{min}}} \right)^{p12} \quad f_{22}(\text{pH}) = \left(\frac{\text{pH}_{\text{opt}} - \text{pH}_{\text{min}}}{\text{pH} - \text{pH}_{\text{min}}} \right)^{p22} \quad (9)$$

$$f_{13}(a_w) = \left(\frac{a_{w\text{opt}} - a_{w\text{min}}}{a_w - a_{w\text{min}}} \right)^{p13} \quad f_{23}(a_w) = \left(\frac{a_{w\text{opt}} - a_{w\text{min}}}{a_w - a_{w\text{min}}} \right)^{p23} \quad (10)$$

$$f_{14}(\text{HT}) = (\text{HTT} - \text{HTT}_{\text{min}})^{p41} \times \text{HT}_{\text{t}}^{p42} \quad (12)$$

Finally, in order to assess realistic exposure level in the market place, when calculating $f_{11}(\text{T})$ and feeding it in P_i (Equation 4), realistic supply-chain chilled conditions were applied. In the illustration presented in the Results part (section 3.2), the US survey on domestic refrigerator temperatures (Food and Drug Administration 1999) was used.

2.4 Statistical methods and software

322 data were used to estimate the lag model, some of them are right-censored (the data recording was stopped before the lag finished) some of them are left-censored (experimental data corresponding to toxin production, meaning that the observed response is longer than the lag time). Despite a relatively large amount of data to solve the model, some additional expert opinion information was valuable. Also, even if the pH factor was often reported in the literature, sometimes it was not. In this latter case, the minimum-to-maximum range known for similar foods (Jay 1992) was used. Then, a Bayesian approach has been chosen for this flexibility when dealing with such a data set.

The Bayesian Markov Chain Monte Carlo procedure was run with the Winbugs package (version 1.4, Medical Research Council, UK). To check the convergence of the iteration process, visual analyses (history function and Gelman and Rubin diagnostic) of three independent chains were performed. 20,000 iterations were run, the first 10,000 iterations were eliminated (burning period). No convergence problems were detected.

To explore the consequence of heat-treatment (inactivation and injury) on the Degree of Protection and to suggest some process and formulation conditions which might deliver a 6-log Degree of Protection, a Monte Carlo simulation process was carried using Excel and @Risk (Palisade Corporation, Newfield, USA). For each scenario 100,000 values were sampled by using the Latin Hypercube method.

2.5 Model verification: graphical methods

The model verification has been carried out using graphical methods. The correlation between parameters has been checked by analyzing scatter plots for all the combinations of parameters. Strictly speak-

ing, a model can be valid even if there are high correlations between some parameters, but high correlated parameters lead to difficulties in interpreting the model outputs. No “high correlation” problems have been detected for the subset of equations applied to heat treatment effect, while for the subset of equations without heat treatment, this situation was more borderline. This might be a direct consequence of the data set used to run the model (different studies reported in literature, with some data gaps).

The second graphical method used to verify the model relevance, was to analyze the predicted versus observed values, and check if there was any bias or underlying structure. This was done also by analyzing the residual errors (predicted – observed values). No systematic bias or apparent structure has been detected.

3 RESULTS

3.1 Lag model outputs: predicted versus observed values

Observed values are plotted against predicted values, with the 90% credibility interval. Data have been collected at 75, 80, 85 and 90°C. At 90°C, whatever the conditions of pH, a_w or storage temperature, the lag times reported were longer than 90 days. Results obtained at 75°C and 85°C are presented in Figures 1 to 4.

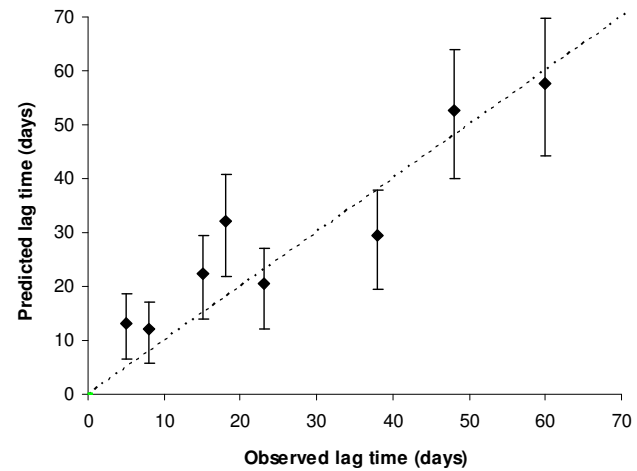


Figure 1: Lag time data points at 75°C. The data at 60 days are censored (lag is longer).

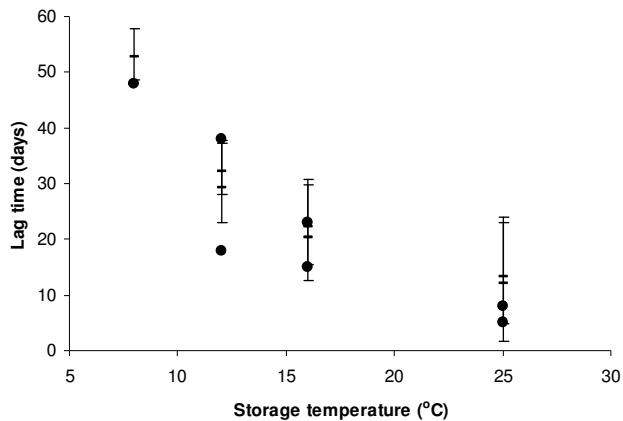


Figure 2: Observed (solid dots) and predicted (dashed dots with credible intervals) values of lag time versus storage temperature. The censored data at 60 days are not plotted.

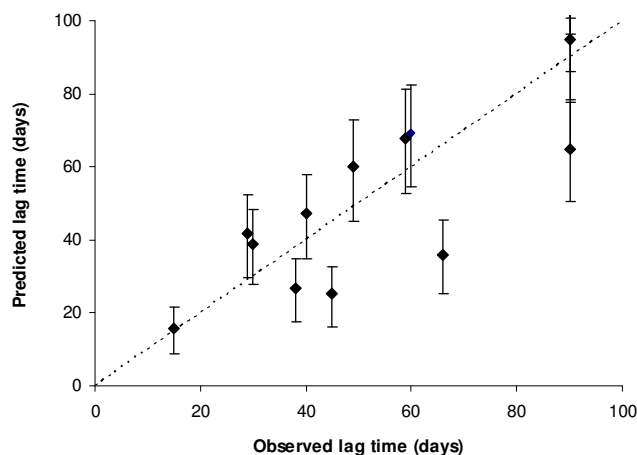


Figure 3: Lag time data points at 85°C. The data at 60 and 90 days are censored (lag is longer).

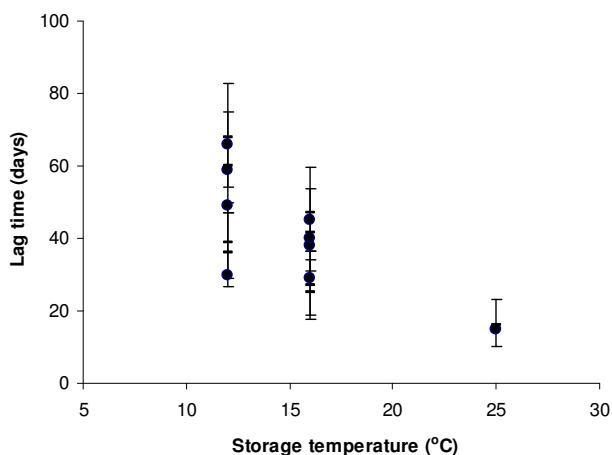


Figure 4: Observed (solid dots) and predicted (dashed dots with credible intervals) values of lag time versus storage temperature. The censored data at 60 and 90 days are not plotted.

At 85°C, all the data showing relatively short lag times are actually collected at relatively high storage temperatures (Figure 4). A detailed analysis of censored data shows: i) with heat-treatment times of either 18min or 23min, pH 6.5, a_w 0.999, lag times are longer than 60 days at storage temperature of 5 and 8°C; also ii) in the same conditions but pH 6, lag time was reported longer than 90 days; and finally iii) at pH 5.6 lag time was longer than 90 days even at storage temperature of 12 or 16°C (Peck et al. 2006).

In conclusion, at 85°C, conditions with pH between 5.5 and 6.0 and chilled temperatures ($\leq 10^\circ\text{C}$) seem key conditions to significantly extend the lag times. This set of conditions has been investigated further in the next section.

3.2 Application of the Degree of Protection concept

The Degree of Protection (DoP) concept can be compared to an iso-probability method, as DoP is derived from probability calculations. For example, there is the same probability ($1 \cdot 10^{-6}$) of having non-proteolytic *C. botulinum* surviving a heat treatment of 88°C for 10 min, at pH 6.3 and recovering within 30 days, as having non-proteolytic *C. botulinum* surviving a heat treatment of 84°C for 10 min, at pH 5.7 and recovering within 50 days (Figure 5). The chilled temperature conditions used to illustrate this approach are taken from the US survey (Food and Drug Administration 1999).

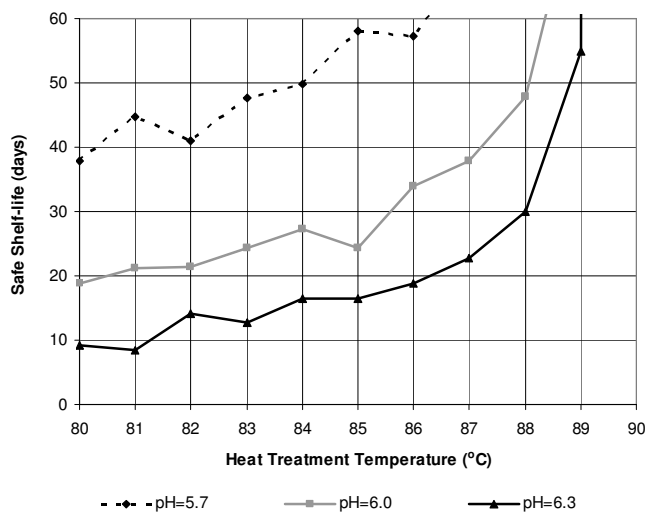


Figure 5: Combinations of heat-treatment temperatures (applied for 10 min), pH and shelf-life which maximize product quality without compromising public health safety. Illustration of the concept with a Degree of Protection chosen as 6.

These results, although very encouraging from a risk management perspective in a food safety context, have to be interpreted with care, and validated before being applied at the operational level, for instance by challenge-testing.

4 DISCUSSION

In this paper we have described a model that predicts the effects of sub-‘90 for 10’ heating on lag time of spores of non-proteolytic *C. botulinum*. The data used to generate the lag model, and predictions from the model show that significant lag times can be expected after heating at temperatures $> 75^{\circ}\text{C}$ and that additional inhibitory effects are afforded by intrinsic and extrinsic factors such as reduced pH, a_w and storage at chill temperatures. The mechanism of sub-lethal injury and inhibition to psychrotrophic spore-forming bacteria such as non-proteolytic *C. botulinum* is not well-understood. For bacterial spore-formers in general, heat injury results in specific requirements for germination stimulants, optimum growth temperatures, nutrients and a higher sensitivity to growth inhibitors (Johnson & Busta 1984). From studies showing improved recovery of heat-treated non-proteolytic *C. botulinum* spores using lysozyme in recovery media (Schaeffer & Ionesco 1972) it is thought that damage to spore-related lytic enzymes plays some role in some injury effects. Such effects are not as significant with spores of mesophilic (proteolytic) *C. botulinum*. It is likely that adverse effects other than damage to lytic enzymes in the spore may contribute to the increase in lag time observed in the studies involving heating non-proteolytic *C. botulinum* spores in sub-optimal growth conditions.

By combining this injury effect with more conventional, processing options, such as heating to deliver a log reduction in numbers, the Degree of Protection approach detailed in this paper, provides the ability to make much better use of combination preservation conditions that are less damaging for product quality but should still deliver the required level of safety. The importance of such interactions has been recognized for controlling mesophilic spore-formers in ambient-stable products. Braithwaite and Perigo (Braithwaite & Perigo 1971) produced a set of contour plots where limiting F_0 values were plotted against a_w and pH, clearly showing combinations of conditions that may only require relatively mild heat treatments (e.g. F_0 0.3) to deliver ambient stability. The concept of taking account of destruction and injury was first described by Pivnick and Petrasovits (Pivnick & Petrasovits 1973) in relation to safety of shelf-stable canned cured meats and developed further by Hauschild and Simonsen (Hauschild & Simonsen 1985) to estimate the ‘protection’ of a food based on probability of spores surviving the

heat process, overcoming inhibition, growing out and producing toxin. Hauschild and Simonsen (1985) expressed this ‘protection’ by deriving a safety unit (SU), a quotient analogous to the log of reciprocal of probability of toxin production multiplied by incidence of spores in raw product. This quotient was based on industrial experience with commercial products that had a safe history of use because experimental work was not sufficiently reliable to assess safety.

In our study, to recalculate the combination of heat-treatment temperatures, pH and shelf-life which maximize product quality without compromising public health safety (Figure 5), the US Audit data (Food and Drug Administration 1999) have been used. Consequently, some retailer/consumer refrigerator temperatures at 10°C , have been incorporated in the analysis. This incorporation is possible with probabilistic methods since they take multiple-value inputs into account. Probabilistic modeling techniques have been already applied to spore-forming bacteria (Barker et al. 2005, Membré et al. 2006) and their values for microbiological risk assessment applied to food safety have been already emphasized (Albert, Pouillot, & Denis 2005, Gonzalez-Martinez, Corradini, & Peleg 2003, Vose 1998). For chilled products, they are particularly valuable as they allow the integration of realistic, complex, supply-chain inputs (Nauta 2001, Nauta et al. 2003, Rosset et al. 2004).

Definitively, the Degree of Protection approach would allow shelf-lives and/or heat-treatment to be set that give new food management design windows. For example, with a product having a pH of 5.7 and heat-treated at 85°C for 10 min, shelf-lives (targeting non-proteolytic *Clostridium botulinum*) of 55 days are expected. This is significantly longer than the common shelf-lives of REPFEDs. However, there are a number of data gaps in the data set used to produce the model. The data used were taken from a limited number of published literature studies. Also, uncertainties have been generated when estimating lag time from first observed time to growth (turbidity or gas production). In some cases, we have assumed values for factors (e.g. pH and a_w) that may influence lag time where these were not reported in the studies used. We intend to fill some of these gaps with further work. Even though these additional data should improve the current model and improve model predictions, it is important that full validation of any predicted effects is made in real foods before they are launched in the market. Faille et al. (Faille et al. 1997) emphasized the importance of testing relevant strains in the food of concern because of the different responses that can be seen in other systems, such as buffer.

The Degree of Protection model proposed in this paper falls within the framework of risk assessment

(exposure assessment step) and more generally within the new food risk management approaches (CAC (Codex Alimentarius Commission) 2004, CCFH (Codex Committee on Food Hygiene) 2005, CCGP (Codex Committee on General Principles) 2005). The Food Safety Objective (FSO) signifies a maximum hazard level at consumption that still constitutes a product that is safe for consumption and a Performance Objective (PO) is similar to the FSO but is applied earlier in the farm-to-fork food supply chain.

The Performance Criterion (PC) refers to the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a PO or an FSO. The 6-log Degree of Protection defined here can be considered equivalent to a 6D inactivation, a standard PC used to assure safety of chilled foods; and consequently, the approach described in this paper is a means of articulating the FSO, PO and PC with practical and easy-to-be-implemented food safety control measures. A similar attempt has been demonstrated recently with thermal inactivation of *Salmonella* in poultry meat (Membré, Bassett, & Gorris 2007).

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