Recovery of heat-injured *Listeria innocua*☆

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Abstract

Listeria innocua was subjected to thermal inactivation and the extent of heat-injured cells was quantified. Cultures were heated in liquid medium for different times, using temperatures in the range of 52.5 to 65.0 °C, and plated on Tryptic Soy Agar with 0.6% yeast extract (TSAYE) used as non-selective medium and on TSAYE plus 5% NaCl (TSAYE+NaCl) and Palcam agar with selective supplement (Palcam agar) as selective media. The difference observed in counts in non-selective and in selective media gave an indication of cell injury during the heat treatment. *D*- and *z*- values were calculated for all conditions considered. For each temperature, *D*-values obtained using non-selective recovery procedures were higher than the ones obtained using the two selective media. When comparing the selective media, it can be concluded that Palcam agar allowed recovery and growth of thermally injured cells and so it was less inhibitor than TSAYE+NaCl. Another important result was the influence of temperature on the degree of cellular injury. As temperature increases, the degree of heat-injured cells also increases, and consequently concern has to be taken with the temperature and the counting medium used in food processing studies. The results of this work clearly demonstrated that selective media used for *Listeria monocytogenes* enumeration/detection might not be suitable for the recovery of heat-injured cells, which can dangerously underestimate the presence of this foodborne pathogen.

Keywords: *Listeria innocua*; Thermal inactivation; Injury; Recovery

Introduction

Listeria monocytogenes is an important foodborne pathogen that has been responsible for several outbreaks of human illness. This fact, together with its ubiquitous nature, makes *L. monocytogenes* an object of many researches. Because most listeriosis outbreaks have been associated with pasteurised products, the evaluation of heat resistance of this microorganism has been the aim of several studies. It is known that cells contain several targets for heat action, but it has been proposed (Abee and Wouters, 1999) that the basal heat resistance of microorganisms might be due to the intrinsic stability of macromolecules, i.e. ribosomes, nucleic acids, enzymes and proteins inside the cell and the membrane.

It is well documented (Hurst, 1977; Smith and Archer, 1988; Besse, 2002) that not all bacteria subjected to a heat treatment are killed, but a large number actually survive being physiologically injured. Such injured microorganisms present a potential threat, since they may, under suitable conditions, repair themselves after the heat treatment (McMahon et al., 2000).

Hurst (1977) recognized that the term "injury" implied temporary damage of a structure, expressed by the loss of a function. Nevertheless, many studies were conducted aiming at establishing the injury manifestation form. Most of those studies (Abee and Wouters, 1999; Besse, 2002) are in agreement with the notion that many, if not all forms of injury, involve damages of the cell membrane. As a consequence, there is significant loss of internal solutes and increased sensitivity to unfavourable chemicals.

Rowan and Anderson (1998) stated that, while the treatment temperature had the most significant effect on thermotolerance (i.e., higher heating temperatures resulted in greater reductions in cell numbers), another factor, which provided great level of cell protection, is the recovery of thermally injured cells on the non-selective medium.

Favourable environmental conditions may be comparable to a non-selective medium, where the injured cells usually undergo repair and become functionally normal. However, on selective agars containing bile salts, such as NaCl or antibiotics, injured cells suffer additional stresses and fail to repair the initial

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damage (Smith and Archer, 1988). The rate at which a population of injured cells undergoes repair will vary with incubation temperature, pH and salt concentration of the medium (Chawla et al., 1996). Some studies demonstrated that organisms repair sublethal damage more readily in simple minimal media than in a complex one (Sörqvist, 1993; Hansen and Knøchel, 2001). The reason behind this phenomenon is not well understood, but some authors (Hurst, 1977; Stephens, Druggan and Caron, 2000) believe that this is related to the development of DNA lesions attributed to the toxicity of ingredients in complex media.

Several studies have been done to improve techniques for recovering sublethally injured cells. A thin agar layer method was developed by Kang and Fung (2000) to recover heat-injured *Salmonella thyphimurium* cells, which consisted on a layer of selective medium and an overlaid of non-selective medium placed on top of it, to allow repair and subsequent growth of characteristic colonies. This seems to be a promising procedure, since it allows recovery of heat-injured cells and also avoids the overgrowth of other microorganisms. Stephens et al. (2000) applied a flow cytometry technique in studies of heat stressed *Salmonella*. This methodology provides information about the physiological state of the cells and consequently the type of injury, thus allowing expectation of the recovery ability.

Because current techniques for detection/enumeration of pathogens use selective enrichment media, it is important to identify the degree to which these may affect the ability of injured cells to repair damage and grow. There is a lack of information concerning quantification of injured cells and/or their ability to recover from stressing heat treatments. Smith and Archer (1988) quantified the ability of several media to support colony formation of heat-injured *L. monocytogenes*, using a recovery inhibition coefficient. Hansen and Knøchel (2001) expressed the degree of heat injury of this microorganism by percentage of injured cells.

In this work *Listeria innocua* was used, instead of *L. monocytogenes*, because apart from being non-pathogenic, it is physiologically very close to *L. monocytogenes* and both can be isolated in the same food products (Cornu and Flandrois, 2000).

Thus, the objectives of this study were: (i) to evaluate the influence of temperature on thermal inactivation of *L. innocua*, (ii) to determine the incidence of injured cells after thermal stress, and (iii) to compare two selective media in terms of recovery potential.

Materials and methods

Cultures

L. innocua NCTC 10528, supplied by Leatherhead Food Research Association (Leatherhead, UK) was subcultured (30 °C, 24 h) in Tryptic Soy Broth — TSB (Lab M, Lancashire, UK) containing 0.6% yeast extract — TSBYE (Lab M, Lancashire, UK). Cultures were maintained at 7 °C on Tryptic Soy Agar — TSA (Lab M, Lancashire, UK) supplemented with 0.6% yeast extract — TSAYE.

Preparation of cultures

The second subculture of *L. innocua* was incubated at 30 °C for 20 h to yield stationary phase cultures. This cell growth phase was chosen due to its higher stress resistance than lag or exponential phase cells. The culture was then centrifuged (4000 rpm for 10 min), the pellet was washed twice and re-suspended in TSBYE. Cells in each cellular suspension were enumerated by plating appropriate dilutions, in duplicate, on the three solid media studied (see Section 2.4).

Heat treatments

Heat treatments were carried out in an agitated water bath at different temperatures (52.5, 55.0, 57.5, 60.0, 62.5 and 65.0 °C). Ninety-nine milliliters of TBSYE used as heating medium were dropped in an Erlenmeyer flask, which was immersed in the water bath at the desired temperature. Once the heating medium temperature had attained stability, it was inoculated with 1 ml of cell suspension. Samples were removed at different time intervals and immediately placed in a mixture of ice-water. On average, 10 and 18 samples were collected in the experiments carried out at the highest and at the lowest temperatures, respectively.

There was a control for each experiment, which consisted of another 99 ml of TSBYE inoculated with 1 ml of the same suspension culture and incubated at 30 °C for the same time. This control was used to ensure that the observed death was only due to the temperature applied.

Two true replicates of all these experiments were performed.

Enumeration

Samples were serially diluted and plated in duplicate onto three different media: (i) TSAYE, (ii) TSAYE supplemented with 5% (w/v) sodium chloride − TSAYE + NaCl (NaCl, Merck, Darmstadt, Germany) and (iii) Palcam agar (Merck, Darmstadt, Germany) plus selective supplement (Merck, Darmstadt, Germany).

TSAYE medium is non-selective and was used to enumerate both injured and non-injured cells. TSAYE + NaCl and Palcam agar are selective for *Listeria* and were used for enumeration of non-injured cells. The minimal inhibitory concentration of NaCl (i.e. 5%) was experimentally determined (data not shown).

Plates were incubated at 30 °C and counted each 24 h during 5 days, or until the number of colony formation units (cfu) no longer increased.

Mean values of bacterial counts, from duplicate plate samples, were converted to log numbers for each heating time and temperature.

Data analysis

Calculation of D-andz-values

D-values (decimal reduction time, or time required to inactivate 90% of the population) were calculated as the negative

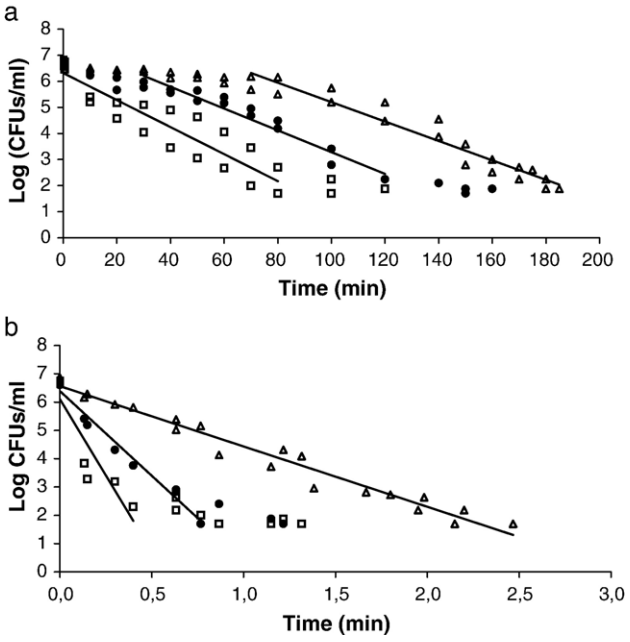

Fig. 1. Typical thermal inactivation behaviour of *L. innocua* 10528 at the temperature extremes of 52.5 °C (a) and 65 °C (b), using TSAYE (Δ), TSAYE+NaCl (□) and Palcam agar (●) as recovery media.

inverse slope of the linear portion of survivor curves (obtained by plotting decimal logarithms of survival counts versus their corresponding heating times). Linear regression lines were fitted to the linear portion of two sets of independent data.

z-values (temperature necessary to reduce *D*-value by 10-fold) were calculated as the negative reciprocal of the slope of the regression line between decimal logarithm of *D*-values and the treatment temperatures.

Determination of degree of injury

The difference from selective (TSAYE+NaCl and Palcam agar) to non-selective (TSAYE) media gives an indication of cell injury during the heat treatment.

Percent injury during the process can thus be calculated by using the following equation (Hansen and Knøchel, 2001):

$$\%\ \text{injured cells} \equiv 100 \times \frac{\text{cfu}_{\text{TSAYE}} - \text{cfu}_{\text{selective}}}{\text{cfu}_{\text{TSAYE}}} \quad (1)$$

With the purpose of quantifying an average value of injured cells for a specific heat treatment, a *Time-averaged Injured Cells Coefficient* (TICC) was defined. For each temperature, this coefficient was calculated by:

$$\text{TICC} \equiv \frac{\int_{t_{\text{initial}}}^{t_{\text{final}}} (\%\ \text{injured cells}\ (t))\,\mathrm{d}t}{t_{\text{final}} - t_{\text{initial}}} \quad (2)$$

where t is the process time. The first and last experimental sampling times are denoted by t_{inital} and t_{final}, respectively.

Smith and Archer (1988) defined a *Recovery Inhibition Coefficient* (RIC) of a medium, as the difference between the areas below experimental inactivation curves, obtained respectively in selective medium and in the non-selective one, used as control:

$$\text{RIC} \equiv \int_0^{\text{total process time}} \log(\text{cfu}(t))_{\text{selective medium}}\,\mathrm{d}t - \int_0^{\text{total process time}} \log(\text{cfu}(t))_{\text{TSAYE}}\,\mathrm{d}t \quad (3)$$

This coefficient varies between negative values and zero. The higher the values of RIC, the better the medium in terms of capability of recovering injured cells.

Statistical analysis

To study the effects of the temperature and selective media on the degree of injured cells and capacity of recovering injured cells, expressed by TICC and RIC respectively, a two-way analysis of variance (ANOVA) with two replicates was performed (Walpole and Myers, 1993).

Microsoft® Excel 2000 (Microsoft Corporation, 1999) was used for all calculations and statistical analysis procedures.

Results and discussion

Typical experimental inactivation data obtained at the extreme limits of the temperature range studied (i.e. 52.5 and 65.0 °C) are presented in Fig. 1. Results showed a gradual loss of resistance of *L. innocua* with the increase of the heating medium temperature, for all recovery media used. This was confirmed by calculation of decimal reduction times. *D*-values were determined from counts recovered on TSAYE, TSAYE+NaCl and Palcam agar (Table 1). It can be observed that, for a given temperature, *D*-values calculated from data of different media are of the same magnitude. Those values were in agreement with the ones obtained by Murphy, Marks, Johnson and Johnson (2000) for *L. innocua* M1, using TSBYE supplemented with rifampicin and streptomycin as selective agents. Nevertheless, as temperature increases, a tendency can be distinguished: *D*-values calculated from non-selective medium are higher than the ones calculated from the selective media. The differences become higher as the temperature increases, being greater from TSAYE+NaCl than from Palcam agar. At the highest temperature of 65 °C, *D*-values calculated

Table 1
Estimated *D*- and *z*-values of *L. innocua* 10528, using different growth media

Temperature (°C)	Non-selective		Selective			
	TSAYE		TSAYE+NaCl		Palcam agar	
	D (min)	R^2 [a]	D (min)	R^2 [a]	D (min)	R^2 [a]
52.5	26.88	0.93	19.31	0.85	23.98	0.95
55.0	16.84	0.89	10.66	0.97	14.37	0.96
57.5	7.65	0.97	3.53	0.95	5.60	0.97
60.0	2.73	0.97	1.08	0.96	1.72	0.94
62.5	0.94	0.98	0.23	0.76	0.46	0.97
65.0	0.47	0.97	0.09	0.82	0.17	0.98
z (°C)	6.73		5.10		5.56	

[a] Coefficient of determination of the linear regression analysis on survival data.

for non-selective media were three times greater than *D*-values obtained from Palcam agar, and five times greater than those calculated from TSAYE+NaCl. These occurrences call for caution in results obtained on the basis of selective media. An apparent lower thermal resistance of the microorganism may be estimated and consequently the safety of food products may be assessed erroneously.

Concerning temperature sensitivity, evaluated by *z*-value, results were of the same magnitude.

The yeast extract (YE) was incorporated into TSA medium, because it facilitates cell repair. Busch and Donnelly (1992) suggested that YE could act as an important source of B-complex vitamins, thus allowing maximum recovery of heat-injured *Listeria*. The addition of NaCl to TSAYE, to form a selective medium, was due to its importance as inhibitor, which has been reported as preventing recovery of stressed *Listeria*. The use of Palcam agar, as another selective media, is justified by its application in current microbiological analysis techniques for *Listeria* detection.

The analysis of the results in Fig. 1 showed better cell recovery from the heating process using TSAYE than both selective broths, as expected (i.e. higher number of colony forming units in TSAYE medium). This was observed for all temperatures tested. Palcam agar was less able to support colony formation of injured cells than TSAYE, but was not as inhibitory as TSAYE+NaCl (i.e. it allowed the repair and growth of some, but not all, of the thermally injured cells). This can be explained by different sensitivities of *Listeria* to the selective agents presented in TSAYE+NaCl and Palcam agar, which are NaCl and LiCl, respectively. These salts have different antimicrobial mechanisms of action. Sodium chloride may cause disturbance in cells permeability, by increasing osmotic stress and promoting cellular plasmolysis (Lin and Chou, 2004). Other proposed mechanisms include dehydration effects, oxygen removal and toxicity of the chloride or sodium ions when salt ionisation occurs. However, there is a minimal inhibitory concentration of NaCl, that allows growth of bacteria (this value was experimentally determined for the case study of *L. innocua* and it is referred in Section 2.4. Enumeration). The presence of LiCl, even in low concentrations, inhibits bacterial growth by competing with essential divalent cations, such as calcium and magnesium. It is possible that critical metalloenzymes are inactivated when the monovalent cation Li^+ replaces divalent cations on these enzymes (Mendonca and Knabel, 1994).

One feature of heat injury and recovery of bacteria is the observation of longer lag phases required for repair, while no cell growth occurs. This fact was experimentally observed in this work as counts on TSAYE+NaCl did not increase significantly during the first 72 h, while counts on TSAYE and Palcam agar did increase significantly after 24 h, indicating that severely injured cells were recovering. It is also important to point out that for both TSAYE and Palcam agar higher differences in counts were observed between 48 and 72 h (data not shown).

One more factor, that supported the lower survival of *Listeria* on selective broth, is the overall shape of the survivor curves, which depended on the temperature and on the recovery medium used (Fig. 1). At lower temperatures, the survivor curves exhibited shoulder or lag phases before attainment of a maximum inactivation rate, expressed by the log-linear period. At higher temperatures, the shoulder was not observed. In the temperature range studied, the shoulder behaviour occurred more frequently from TSAYE medium, followed by Palcam agar counts. For TSAYE+NaCl broth, log-linear survivor curves were obtained, which confirms the non-ability of recovery of heat-injured cells.

Geeraerd et al. (2000) gathered literature knowledge about the shoulder and tailing phenomena. The existence of a slower initial death rate may reflect the presence of cell clumps, which require to be destroyed before inactivation of cells, individually. Another explanation, for the occurrence of a shoulder phase, is the decrease of the efficiency of enzymatic repair mechanisms. This happens if cell damages are extensive either in number or in destruction level, which restricts cell repair and microbial viability. In the shoulder period the rate of destruction exceeds the rate of synthesis and repair. A further point, that must be remarked, is the fact that not all the cells are in the stationary phase and, as a consequence, loss of cell viability might not be in comparable stages.

The observed tailing of the curves, may be due to a subpopulation of cells that are more heat resistant than the majority of the population. It is also possible that, during lethal heating, heat shock proteins production occurs in a small proportion of cells and results in tailing of survival curves. These considerations show the importance of including the shoulder and tailing phenomena when evaluating the efficiency of a thermal food processing.

With the purpose of quantifying the degree of injured cells and capability of the media in recovering heat-injured cells, the coefficients TICC (Eq. (2)) and RIC (Eq. (3)) were calculated. Values are included in Table 2 for the six temperatures studied.

Results showed that as temperature increases, TICC slightly increases for both selective broths. However, for a given temperature, TICC values from TSAYE+NaCl were higher than those calculated to Palcam agar. These results confirmed that Palcam agar is not as inhibitory as TSAYE+NaCl, for the concentration of NaCl applied.

It is interesting to verify that RIC values varied greatly with temperature (e.g. for TSAYE+NaCl broth, RIC varies from −531.12 at 52.5 °C to −6.14 at 65.0 °C). Examination of the RIC values allowed also identifying general trends concerning

Table 2
Evaluation of the ability of the media to recover injured cells (RIC) and degree of injured cells (TICC) of heat-injured *L. innocua* 10528

Temperature (°C)	RIC		TICC (%)	
	TSAYE+NaCl	Palcam agar	TSAYE+NaCl	Palcam agar
52.5	−531.12	−279.33	96.47	83.83
55.0	−327.71	−197.70	96.95	82.09
57.5	−120.22	−63.94	97.73	84.39
60.0	−56.11	−39.95	99.19	93.49
62.5	−14.17	−8.89	99.91	94.58
65.0	−6.14	−5.46	99.82	98.96

compounds that prevent repair and subsequent colony formation by heat-injured *L. innocua*. Results also reveal that Palcam agar has a major capability of recovering injured cells, when compared to the other selective medium, as higher RIC values were obtained. So, although the non-selective media are better to recover heat-injured *L. innocua*, Palcam agar known to inhibit background microflora still detects injured cells.

To clarify the effect of the factors temperature and media on TICC and RIC values, an ANOVA was performed. It was concluded that both factors significantly affected the TICC (at a significance level of 5%), being the major effect due to the selective media. Besides identical results were obtained for RIC, temperature had the major effect in the capability of the medium to recover injured cells.

This study has shown that injured *Listeria* cells might be present, but escape detection, since they do not grow in selective media, which means that the potential for hazard is still present due to its capacity to repair.

Conclusions

To achieve accurate results concerning thermal inactivation of microorganisms, it is crucial to recognize the presence of potentially injured cells, since sublethal damaged bacteria may recover in food and regain their pathogenicity. This study confirms that when selective media are used to estimate *Listeria* thermal inactivation, the food microbiologist may either fail to detect it or will underreport its numbers. Comparing both selective media used in this work, it can be concluded that Palcam agar is more appropriate for the recovery of injured cells than TSAYE+NaCl medium. It was also shown that temperature has a major effect on the recuperation of thermally injured microorganisms.

It is mainly unacceptable that microorganisms that indicate the hygienic quality/safety of a food product may escape detection. Thus, every attempt should be made to improve the isolation and enumeration procedures to detect all survivors of a thermal treatment.

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