Comparison of recovery methods for the enumeration of injured *Listeria innocua* cells under isothermal and non-isothermal treatments

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ABSTRACT

This study compares the feature of different media with the combination of selective with non-selective media in a TAL method for recovery of *Listeria innocua* cells exposed to thermal treatments. Experiments were conducted in broth at constant temperature (52.5 and 65.0 °C) and pH (4.5 and 7.5) conditions, using NaCl or glycerol to adjust water activity to 0.95. Four different media were used in bacterial cell enumeration: (i) a non-selective medium − TSAYE, (ii) two selective media − TSAYE + 5%NaCl and Palcam Agar and (iii) TAL medium (consisting of a layer of Palcam Agar overlaid with one of TSAYE).

Two food products were used as case studies aiming at comparison of results obtained on selective and TAL media enumeration. Parsley samples were inoculated with *L. innocua* and subjected to posterior thermal treatments both under isothermal (52.5, 60.0 and 65.0 °C) and non-isothermal (heating rate of 1.8 °C/min from 20.0 to 65.0 °C) conditions. The recovery capability of TAL method was also studied when a pre-cooked frozen food (i.e. *meat pockets*) was fried (oil temperature of ∼180 °C). TAL method proved to be better than Palcam Agar in terms of capability to recover injured cells and was effective in *L. innocua* enumeration when non-sterile samples were analysed.

Keywords:
Food products
Listeria innocua
TAL method
Thermal inactivation

1. Introduction

Listeria innocua is a non-pathogenic specie of *Listeria* that is usually used as a surrogate for *Listeria monocytogenes*, the cause of listeriosis. Both species are widespread in the environment and are commonly found in the same food products. The majority of *L. innocua* strains may be used as safe marker organisms for *L. monocytogenes,* since their heat resistance is higher (Kamat and Nair, 1996; Piyasena et al., 1998; Margolles et al., 2000; Friedly et al., 2008; Lecompte et al., 2008).

Heat treatments are the most common methods for food preservation, causing a significant reduction in bacterial cell numbers. However, most of the industrial food processes include non-isothermal heating up phases that usually are not considered in bacteria heat resistance calculations. Predictions of industrial food processes aiming at microbial inactivation are often carried out using information obtained on the basis of isothermal experiments. Microbial responses under such conditions may differ from the ones observed when time-varying temperature processes are applied, thus compromising safe standards.

Time-varying temperature conditions are observed in cooking processes (frying is one example). For frozen pre-cooked meals, if the time of frying is not enough for the coldest point to reach the required temperatures, serious outbreaks may occur if the product is contaminated. *Listeria* can be one of the microorganisms involved, due to its ubiquitously and resistance to refrigeration and even frozen temperatures.

The efficiency of a thermal food processing on bacteria inactivation can also be compromised if selective media is used for enumeration, since injured cells may not be detected. It is well documented (Chawla et al., 1996; McKellar et al., 1997; McMahon et al., 2000) that *Listeria* cells can be injured when exposed to a variety of stressing conditions, such as the ones observed in thermal processes, freezing, acidification or drying. This injury can be manifested as the inability to growth on selective media. One of these selective media, PALCAM, is recommended by the International Organization for Standardisation (ISO) (Anonymous, 1996), and consequently has been widely adopted in the protocols for *Listeria* cells detection/enumeration. Although the use of this medium allows the differentiation and enumeration of *Listeria* spp., it contains selective agents which may inhibit the repair of injured cells and therefore may increase the risk of under-estimation. Attending to this fact, the majority of published studies use non-selective media for the enumeration of pathogenic bacteria.

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However, when working with food products in which the presence of background microflora is inevitable, the media selectivity is required.

The methods to recover sublethally injured *Listeria* cells have been investigated by many researchers (Busch and Donnelly, 1992; Mendonca and Knabel, 1994; Han et al., 2002; Rijpens and Herman, 2004; Yuste et al., 2004). These methods were based on traditional microbiological techniques with enrichment procedures, which are extremely time-consuming. Kang and Fung (2000) proposed the Thin Agar Layer (TAL), one-step method which was efficient in the recovery of *Salmonella typhimurium*. Other researchers (Hajmeer et al., 2001; Yuste and Fung, 2003; Yuste et al., 2004) applied the same method to other microorganisms and various types of injuries and proved the effectiveness of the recovery method. The TAL method consists of a layer of selective medium overlaid with one of a non-selective one. During the first hours of incubation, injured cells recover and start to grow on the non-selective medium top layer. After diffusion of agents from the selective medium to the top layer, typical target bacterium colonies are formed with inhibition of other microorganisms. This is a more effective procedure for enumeration of microbial food contaminants.

The objective of this work was to evaluate the TAL method to recover heat-injured *L. innocua* cells in liquid broth, after isothermal treatments. The degree of recovery was investigated for four different media (one non-selective, two selective and TAL media). Two food products were also used as case studies: (i) parsley, an aromatic herb with a great potential of contamination, which suffered both isothermal and non-isothermal heat treatments, and (ii) a pre-cooked frozen food (*meat pockets*), that was fried (non-isothermal conditions). Results obtained on selective and TAL media were therefore compared.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Cultures
L. innocua NCTC 10528 was subcultured in Tryptic Soy Broth − TSB (Lab M, Lancashire, UK) containing 0.6% yeast extract − TSBYE (Lab M). Cultures were maintained at 7 °C on Tryptic Soy Agar − TSA (Lab M) supplemented with 0.6% yeast extract − TSAYE.

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 exponential phase cells (Miller et al., 2009a).

Cells in each cellular suspension were enumerated by plating appropriate dilutions, in duplicate, on the recovery media studied (see Section 2.1.4). The concentration of *L. innocua* in the suspension was approximately 10^7 cfu/ml.

2.1.2. Heat treatments under isothermal conditions
2.1.2.1. Experiments in liquid medium. TSBYE, adjusted with NaCl or glycerol to achieve a water activity of 0.95, was used as the basal medium for all experiments. Combinations of solutes (NaCl and Glycerol), temperature (52.5 and 65.0 °C) and pH (4.5 and 7.5) were studied according to a 2^3 factorial design (i.e. 8 conditions; Box et al., 1978), with three independent trials of each combination. The pH was always adjusted with lactic acid (0.5 M) and measured using a pH meter (GLP 22, Crison Instruments, Spain). Water activity determinations were performed with a dew point hygrometer (Aqualab − Series 3, Decagon Devices Inc., USA) at 25 ± 1 °C.

The pH and a_w of the media were measured before and after autoclaving. Autoclaving did not change the pH or a_w of the medium.

Heat treatments were carried out in a thermostatized water bath. Two covered Erlenmeyer flask containing 99 ml of stirred TBSYE, adjusted to the desired pH and a_w, were immersed in the water bath at the desired temperature. One of the flasks was used for the microbial inactivation experiments, while the other was used for temperature control. 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.

2.1.2.2. Experiments in parsley. 5 g of parsley (*Petroselinum crispum*) samples were inoculated by immersion in 250 ml of bacterial suspension, for approximately 10 s. Samples were then removed and transferred to sterile commercial available bags of 17 × 24 cm, where they were sealed and vacuum-packaged (Multivac A300/41/42, Wolfertschwenden, Germany). Bags were immersed in the thermostatic agitated water bath and heating treatments were done at 52.5, 60.0 and 65.0 °C for 180, 14 and 3 min, respectively. Bags were positioned such that entire parsley samples were submerged and each bag was removed at different time intervals and immediately placed in a mixture of ice-water. For each time, 27 ml of sterile peptone water was added to approximately 3 g of parsley and bags were pummeled for 4 min in a stomacher at normal speed.

The pH and water activity of raw parsley was measured at 25 ± 1 °C before treatments, being 6.2 ± 0.05 and 0.98 ± 0.003, respectively.

Three independent trials of the experiments were performed. Uncontaminated parsley and contaminated parsley (that was not submitted to heat treatment) were used as controls.

2.1.3. Heat treatments under non-isothermal conditions
2.1.3.1. Experiments in parsley. Parsley samples were prepared as mentioned in Section 2.1.2 and the time-varying temperature treatments were carried out in a thermostatic bath with stirring capacity (Julabo® FP40, Seelbach, Germany) and with a temperature programmer (Julabo® HC-E07, Seelbach, Germany). The temperature history varied from 20 °C to 65 °C as following: a linear increase in the temperature at a rate of 1.8 °C/min (for approximately 25 min until 65 °C was attained), with a subsequent period of constant temperature of 65 °C (for approximately 20 min).

Three independent trials of the experiments were performed.

2.1.3.2. Experiments in meat pockets. Frozen *meat pockets* commercially available (i.e. dough stuffed with sliced cooked pork and beef, which is a pre-cooked product that requires frying before being consumed) were artificially inoculated by injecting, into the centre, 250 μl of *Listeria* inoculum. The *meat pockets*, with approximately 37.5 g each and with dimensions of 7 × 5 cm, were maintained at −8 °C for 1 h, before being fried in 1 L of oil at approximately 180 °C, for a maximum of 8 min (using a common fryer). The temperature history was monitored during all frying process using K-type thermocouples, placed in the centre of the samples. The values were recorded by a squirrel datalogger (Grant Instruments 1023, Cambridge, England).

After different time intervals, the *meat pockets* were removed from the fryer and immediately placed in a mixture of ice-water (each sample was one *meat pocket*). The meat of each sample was isolated and mixed with sterile peptone water in a stomacher bag, where it was blended by Stomacher. The pH and water activity of the meat product was measured at 25 ± 1 °C, being 6.1 ± 0.05 and 0.98 ± 0.003, respectively.

Three independent trials of the experiments were performed.

Uncontaminated frozen *meat pocket* and contaminated frozen *meat pocket* (that was not subjected to frying process) were used as controls.

2.1.4. Enumeration

Samples from heat treatments in liquid medium were serially diluted and plated in duplicate onto four different media: (i) TSAYE, (ii) TSAYE supplemented with 5% (w/v) sodium chloride − TSAYE + NaCl, (iii) Palcam Agar plus selective supplement (Miller et al., 2006) and (iv) Palcam Agar overlaid with TSAYE − TAL. Samples from heat treatments in food products were also diluted and plated in duplicate, but only in Palcam Agar plus selective supplement and in Palcam Agar overlaid with TSAYE, due to the presence of natural food microflora.

All 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 combination.

2.2. Modelling procedures

2.2.1. The inactivation model

Depending on the experimental conditions used, the microbial inactivation kinetics may vary from a linear tendency till a notorious sigmoidal behaviour (with an initial shoulder preceding a maximum inactivation rate period, tending to a tail). A Gompertz model, reparameterized according to these features, is able to describe these different patterns (Miller et al., 2009a, 2009b).

2.2.1.1. Isothermal conditions. Under constant temperature conditions, the Gompertz-inspired model becomes (Gil et al.,

$$
\log(N) = \log(N_0) + (\log(N_{res}) - \log(N_0))\exp\left(-\exp\left(\frac{-k_{max}e}{(\log(N_{res}) - \log(N_0))}(L - t) + 1\right)\right) \tag{1}
$$

2006):where N is the microbial cell density at a particular process time, t. The indexes 0 and res indicate initial and residual (or tail) microbial cell density, respectively, L is the initial shoulder and k_{max} the maximum inactivation rate constant.

2.2.1.2. Non-isothermal conditions. When temperature varies with time, the number of microorganisms at a particular time, can be calculated by encompassing the time-temperature [i.e. $\log(N)_{non-isothermal} = \int_0^t \frac{d(\log(N)_{isothermal})}{dt}dt'$]. The expression that relates $\log(N)$ with time is the following:

$$
\log(N)_{non-isothermal} =
$$
$$
\int_0^t \left[-kmax(t')e\exp\left(-\frac{kmax(t')e}{(\log(N_{res}) - \log(N_0))}(L(t') - t') + 1\right)\exp\left(-\exp\left(-\frac{kmax(t')e}{(\log(N_{res}) - \log(N_0))}(L(t') - t') + 1\right)\right)\right]dt' \tag{2}
$$

Since kinetic parameters (k_{max} and L) are time−temperature dependent, the Gompertz-inspired model under time-varying temperature conditions ($T(t)$) becomes more complex. It was assumed that shoulder and k_{max} temperature dependency were respectively:

$$
L(t) = c(T(t) - d)^2 \tag{3}
$$

and

$$
k_{max}(t) = k_{ref}\exp\left(-\frac{Ea}{R}\left(\frac{1}{T(t)} - \frac{1}{T_{ref}}\right)\right) \tag{4}
$$

The model presented in eq. (3) was first suggested by Ratkowsky et al. (1982) and has two parameters (c and d). The model presented in eq. (4) is the well-recognized Arrhenius equation, being k_{ref} the inactivation rate at a finite reference temperature, Ea the activation energy and R the ideal gas constant.

If the temperature history $T(t)$ is known, the model valid for non-isothermal conditions can be obtained by merging equations (2)−(4) (Gil et al., 2006).

2.2.2. Determination of degree of injury

An indication of cell injury during thermal treatment can be obtained by the differences between the following selective/non-selective media: (i) TSAYE + NaCl/TSAYE; (ii) Palcam Agar/TSAYE; (iii) TAL/TSAYE.

$$
\%\text{injured cells} = \frac{N_{TSAYE} - N_{selective}}{N_{TSAYE}} \times 100 \tag{5}
$$

Miller et al. (2006) defined a *Time-averaged Injured Cells Coefficient* (TICC) with the purpose of quantifying an average value of injured cells. For each temperature, this coefficient can be calculated by:

$$
TICC = \frac{\int_{t_{initial}}^{t_{final}} (\%\text{injured cells}(t))dt}{t_{final} - t_{initial}} \tag{6}
$$

where t_{inital} and t_{final} are the first and last experimental sampling times, 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:

$$
RIC = \int_0^{totalprocesstime} \log(N(t))_{selective\ medium}dt
$$
$$
- \int_0^{totalprocesstime} \log(N(t))_{TSAYE}dt \tag{7}
$$

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

2.2.3. Data analysis

The parameters of the Gompertz-inspired inactivation model assumed for isothermal conditions (i.e. L, k_{max} and $\log(N_{res}/N_0)$) were estimated by non-linear regression analysis using Statistica© 6.0 (StatSoft, USA).

For non-isothermal conditions, the model parameters (i.e. k_{ref}, Ea, c, d and $\log(N_{res}/N_0)$) were also estimated by non-linear regression analyses by programming least squares method using FORTRAN 77 (Fortran 5.1, Microsoft Corporation®, 1990).

Parameters' precision was evaluated by the standardised half width (SHW) at 95%, i.e. halved confidence interval divided by the estimate $\equiv \frac{\text{confidence\_interval}_{95\%}}{2} \times \frac{1}{\text{estimate}} \times 100$.

All required numerical calculations were done in Microsoft® Excel 2000 (Microsoft Corporation, USA).
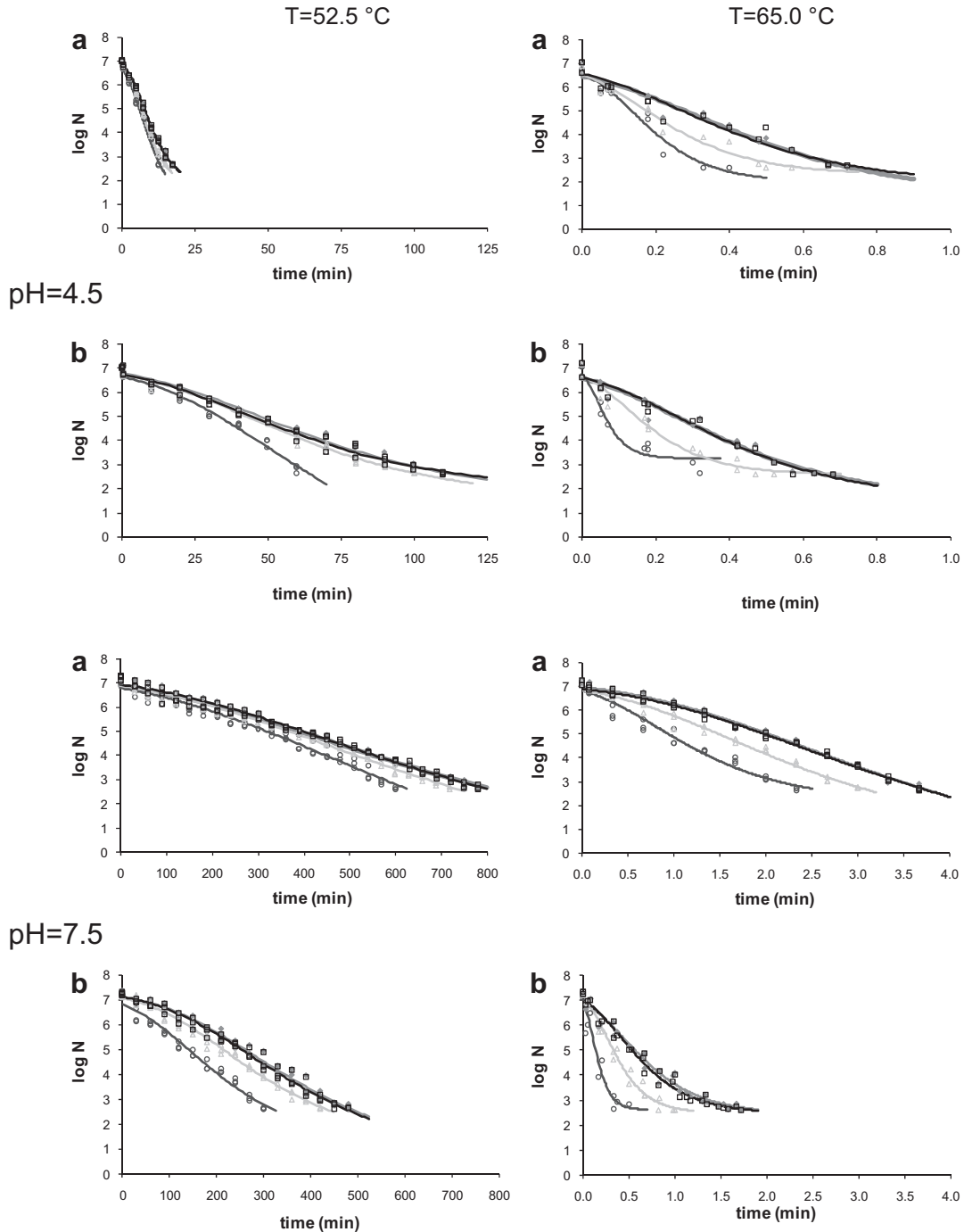
Fig. 1. Thermal inactivation data of *L. innocua* in TSBYE adjusted to a_w of 0.95 with NaCl (a) and glycerol (b), using the four recovery media: (◆)TSAYE; (○) TSAYE + NaCl; (△) Palcam Agar; (□)TAL method. The lines represent model fits (eq. (1)).

Table 1
Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 in liquid medium and parsley, under isothermal conditions.

Solute	pH	T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
				L (min)	$SHW_{95\%}$	k_{max} (min^{-1})	$SHW_{95\%}$	$Log(N_{res})$	$SHW_{95\%}$
NaCl	7.5	52.5	TSAYE	32.29	53.7	0.01	3.8	0.38	249.6
			TSAYE + NaCl	41.22	102.9	0.01	9.5	−1.86	171.8
			Palcam	33.04	59.4	0.01	4.0	−0.41	327.7
			TAL method	39.59	41.7	0.01	3.4	0.09	*
		65.0	TSAYE	0.48	25.4	1.43	5.0	−0.50	310.5
			TSAYE + NaCl	0.00	*	2.31	27.4	2.16	9.6
			Palcam	0.20	53.3	1.65	6.8	0.83	148.2
			TAL method	0.50	21.6	1.41	4.6	−0.20	682.1
	4.5	52.5	TSAYE	1.61	23.9	0.32	5.8	1.73	22.2
			TSAYE + NaCl	0.94	63.4	0.37	8.6	0.90	167.7
			Palcam	1.34	24.1	0.36	5.5	1.62	25.4
			TAL method	1.60	23.2	0.33	5.7	1.73	21.1
		65.0	TSAYE	0.00	*	6.51	53.9	1.41	17.4
			TSAYE + NaCl	0.03	178.5	15.33	36.2	1.97	72.5
			Palcam	0.00	*	10.23	29.2	2.31	39.0
			TAL method	0.01	372.7	7.13	49.3	1.95	18.7
Glycerol	7.5	52.5	TSAYE	68.16	18.5	0.01	5.2	0.48	230.8
			TSAYE + NaCl	5.42	242.3	0.02	9.0	1.20	102.8
			Palcam	45.78	24.3	0.01	6.1	1.16	68.1
			TAL method	62.08	20.5	0.01	5.3	0.45	251.3
		65.0	TSAYE	0.00	*	3.98	20.0	2.45	6.4
			TSAYE + NaCl	0.00	*	16.48	48.5	2.61	39.0
			Palcam	0.03	158.2	7.48	18.8	2.50	18.6
			TAL method	0.00	*	4.39	13.9	2.49	13.4
	4.5	52.5	TSAYE	3.69	92.6	0.05	7.6	1.59	39.0
			TSAYE + NaCl	8.82	98.5	0.08	20.5	−2.13	271.2
			Palcam	4.10	74.9	0.05	7.4	1.50	42.2
			TAL method	2.81	132.2	0.05	8.8	1.93	27.1
		65.0	TSAYE	0.00	*	7.61	57.0	1.54	17.7
			TSAYE + NaCl	0.01	563.0	33.29	64.2	3.23	14.7
			Palcam	0.00	*	13.37	35.4	2.57	13.2
			TAL method	0.00	*	7.80	55.4	1.47	16.2
Parsley	−	52.5	Palcam	13.31	88.3	0.04	21.6	1.50	65.3
			TAL method	0.34	*	0.04	15.2	2.05	60.5
		60.0	Palcam	0.35	179.3	0.55	14.5	1.50	44.5
			TAL method	0.10	614.2	0.60	12.7	2.20	33.9
		65.0	Palcam	0.37	30.8	1.93	9.8	0.65	171.5
			TAL method	0.24	66.8	1.92	13.8	2.08	52.3

* Considerable high meaningless value.

3. Results and discussion

Experimental inactivation data of *L. innocua* obtained at isothermal conditions (of 52.5 and 65.0 °C), for different pH's (4.5 and 7.5) and water activity of 0.95 (adjusted with NaCl and glycerol) are presented in Fig. 1. The overall tendencies depended on the experimental conditions used, varying from a linear kinetics ($T = 52.5$ °C, pH = 4.5, $a_w = 0.95$ adjusted with NaCl and using TSAYE + NaCl medium) till a pronounced complete sigmoidal behaviour ($T = 65.0$ °C, pH = 4.5, $a_w = 0.95$ adjusted with NaCl and using TSAYE + NaCl medium). A Gompertz-inspired model was adequate in data fitting, and the parameters estimates are included in Table 1. In all cases, the adequacy of the model was assessed by residual analysis (randomness and normality was verified) and by the coefficient of determination, R^2. The values of R^2 varied from 0.94 to 1.0, thus revealing good model fits. Precision of the parameters estimates was evaluated by standardising confidence intervals at 95% in relation to the parameters themselves, avoiding the magnitude of the estimates and seeking easier comparison. Results revealed that k_{max} was estimated with satisfactory precision. However, the highest $SHW_{95\%}$ were obtained for experiments at $T = 65.0$ °C and pH = 4.5 (maximum of 64.2% when glycerol and TSAYE + NaCl medium were used). This may be explained by the low number of experimental points (please check Fig. 1b) in the maximum rate period. For the remaining experiments, $SHW_{95\%}$ for k_{max} varied between 3.4 and 48.5%, averaging 10.9%.

Estimates of shoulder parameter lacked precision for the same conditions at which k_{max} estimates were also less precise. Curiously, satisfactory estimates of tail were obtained. This is related to the kinetic pattern and the experimental design, since a number of experimental points was gathered at the final residual tendency (with improvements in tail precision).

Comparing the recovery media used, results showed that major differences were observed when the most severe conditions were used (i.e. for a temperature of 65 °C and pH 4.5). The medium TSAYE + NaCl presented the worse scenario concerning bacterial survival. For this medium, the inactivation rates were higher than the ones obtained for the remaining cases. Thus, to quantify the degree of injured cells and the capability of the media used in recovering injured cells subjected to different treatments, the coefficients TICC and RIC were calculated. Values of TICC and RIC from the three media used are represented in Figs. 2 and 3, respectively, for all the conditions studied.

Results showed that as temperature increased, TICC also increased for TSAYE + NaCl and Palcam Agar, for both pH's used. This means that as temperature increases, the number of injured cells also increases. For a given pH, TICC values varied with the solute used to change a_w, being this effect more evident for the lowest temperature studied. TICC values from TSAYE + NaCl
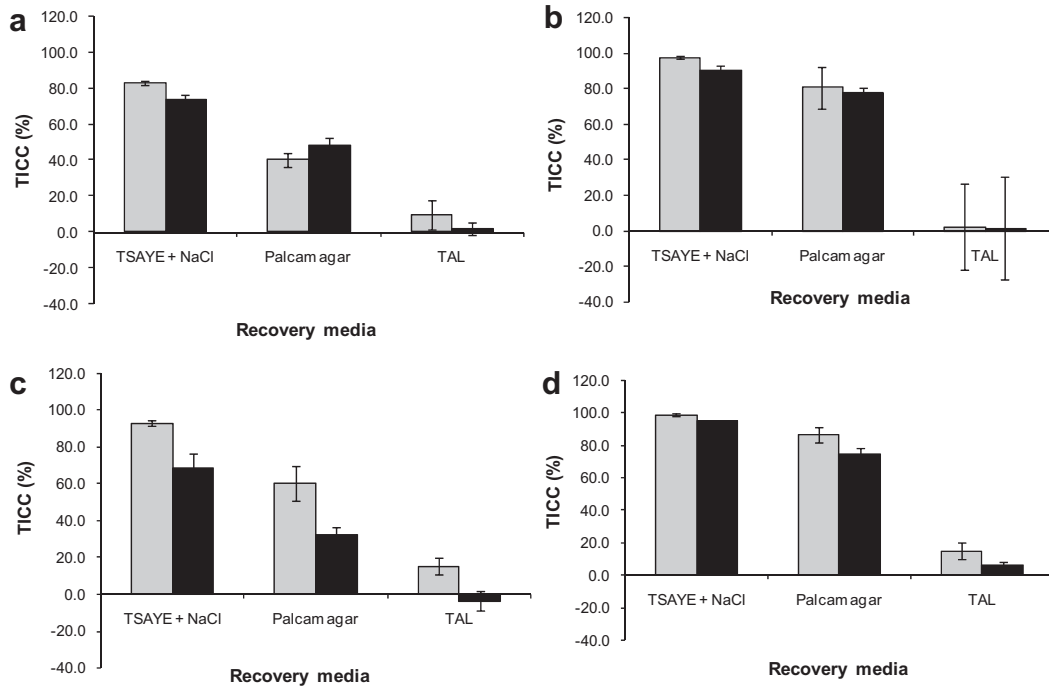
Fig. 2. TICC values for the recovery media used: (a) pH = 4.5 and T = 52.5 °C, (b) pH = 4.5 and T = 65.0 °C, (c) pH = 7.5 and T = 52.5 °C, and (d) pH = 7.5 and T = 65.0 °C, using glycerol (▢) or NaCl (■) to adjust a_w. The bars represent the standard deviations.

medium were always the highest, followed by the ones from Palcam Agar. These results confirmed that TSAYE + NaCl was more inhibitory than Palcam Agar, as it was less able to support colony formation of injured cells. This can be explained by different sensitivities of *Listeria* to the selective agents presented in the two media. Sodium chloride may cause disturbance in cells

permeability and, as a result, cells may be inhibited by the occurrence of osmotic stress and cellular plasmolysis (Lin and Chou, 2004). The sensitivity observed on Palcam Agar is probably related with the concentration of LiCl (15 g/l) and the presence of acriflavine. Even in low concentrations, LiCl inhibits bacterial growth by competing with essential divalent cations, such as calcium and
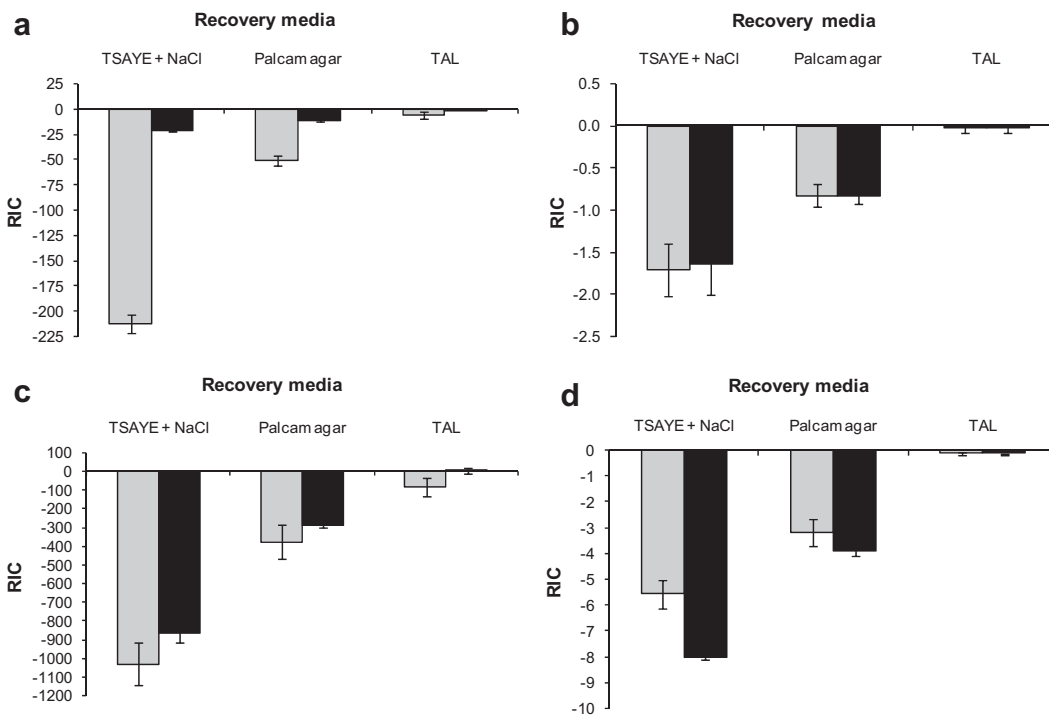
Fig. 3. RIC values for the recovery media used: (a) pH = 4.5 and T = 52.5 °C, (b) pH = 4.5 and T = 65.0 °C, (c) pH = 7.5 and T = 52.5 °C, and (d) pH = 7.5 and T = 65.0 °C, using glycerol (▢) or NaCl (■) to adjust a_w. The bars represent the standard deviations.
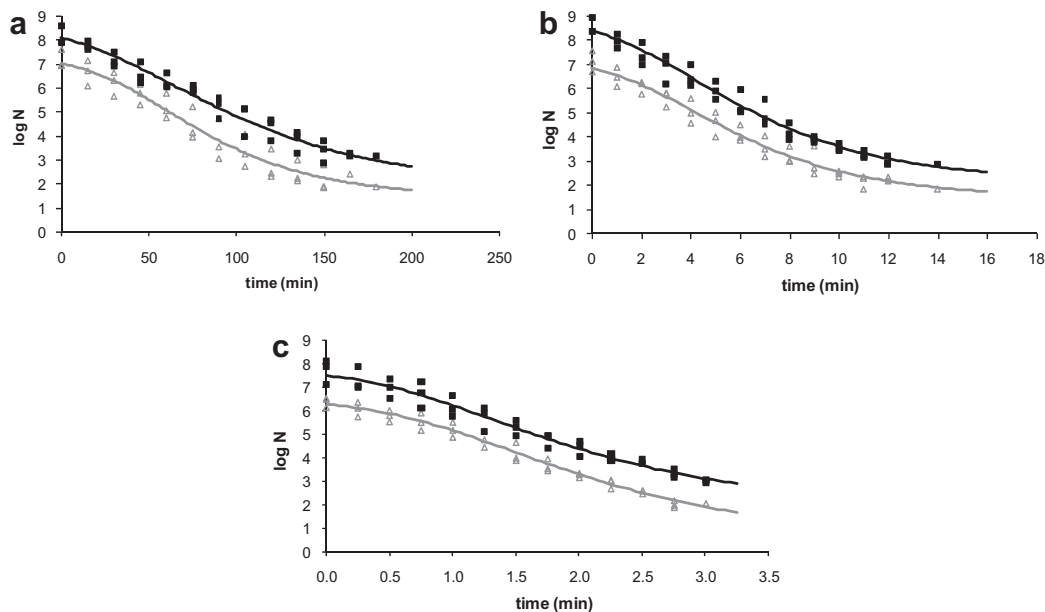
Fig. 4. Thermal inactivation data of *L. innocua* in parsley under isothermal conditions at 52.5 °C (a), 60.0 °C (b) and 65.0 °C (c), using Palcam Agar ($\triangle$) and TAL method ($\blacksquare$) as the recovery media. The lines represent model fits (eq. (1)).

magnesium. It is possible that critical metalloenzymes are inactivated when the monovalent cation Li$^+$ replaces divalent cations on these enzymes (Mendonca and Knabel, 1994). Although Jacobsen (1999) reported that the strongest inhibition of *L. monocytogenes* was due to the presence of LiCl, this researcher also observed that acriflavine was slightly inhibitory to the bacterium.

Concerning the TAL method, microbial counts were nearly equivalent to the ones obtained in non-selective medium; this was proven by the small TICC values, which are indicative of a scarce number of injured cells.

From results included in Fig. 3, it is possible to verify that RIC values varied greatly with temperature and pH. Results also showed that although Palcam Agar had a major capability of recovering injured cells (when compared with TSAYE + NaCl), if it is used to enumerate *Listeria* cells, some sublethally injured microorganisms would escape to detection. TAL medium was very effective in recovering injured *Listeria* cells, since RIC values were close to zero. Thus, TAL medium provided a good method to enumerate *L. innocua*, since it allowed recovery of injured cells and suppressed the growth of other microorganisms due to the presence of the selective agents. This is in agreement with the findings of Kang and Fung (2000), Yuste and Fung (2003) and Yuste et al. (2004), who also recovered high numbers of injured bacterial cells using the TAL method.

To clarify the effects of temperature, pH, type of solute used to change a_w and the difference between the utilization of Palcam Agar or TAL medium on TICC and RIC values, results were analysed according to a factorial design at two levels (i.e. 2^4 factorial design; (Box et al., 1978)). It was concluded that, with the exception of pH, all factors significantly affected TICC (at a significance level of 5%). Media had the most significant effect (i.e. TAL media differed significantly from Palcam agar). This main effect was followed by temperature, combination of media/temperature and solute (the media effect was three times higher than the temperature effect). Using NaCl to change a_w implied lower TICC values.

In relation to RIC, only the type of solute did not significantly affect the parameter. Although the main effect was due to temperature (higher temperature implied higher recovery capability), pH and recovery media were also significant (significance level of 5%). For higher pH values, lower RIC values were observed (i.e. lower recovery capability). TAL medium allowed the highest cell recovery.

Because a non-selective medium cannot be used for bacteria enumeration in food products due to the presence of endogenous microflora, the TAL method can be a wise choice; results observed in liquid broth were suggestive of the advantage of using this medium over the selective one. This was assessed by studying *L. innocua*
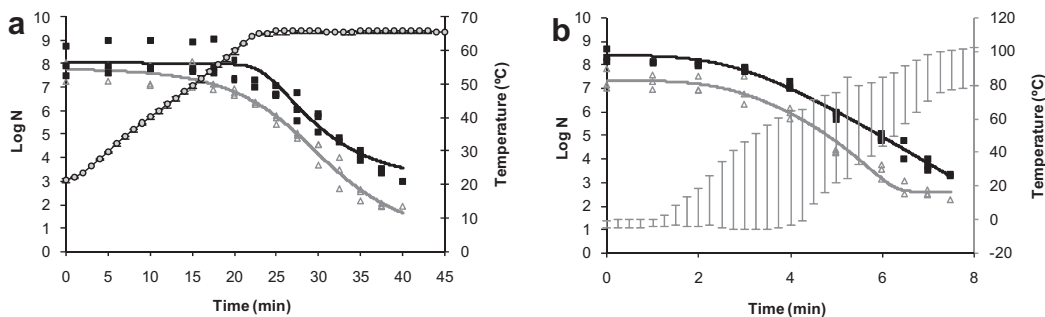
Fig. 5. Thermal inactivation data of *L. innocua* in parsley (a) and in frozen *meat pockets* (b) under non-isothermal conditions, using Palcam Agar ($\triangle$) and TAL method ($\blacksquare$) as the recovery media. The lines represent model fits (eq. (2)). The bars represent the standard deviation of the temperature histories ($\bullet$).

Table 2
Estimates of k_{ref}, Ea, c, d and tail parameters of *L. innocua* 10528, under non-isothermal treatments; Precision assessed by standard half width (SHW) at 95%.

Food Product	Media	k_{ref} (min^{-1})	SHW$_{95\%}$	Ea (J mol^{-1})	SHW$_{95\%}$	c (min K^{-2})	SHW$_{95\%}$	d (K)	SHW$_{95\%}$	Log (N_{res})	SHW$_{95\%}$
Parsley	Palcam	−0.23	3.80×10^1	5.51×10^4	8.35×10^1	6.66×10^{-3}	5.67×10^2	280.3	5.57×10^1	1.70	3.56×10^2
	TAL method	−0.25	1.13×10^6	6.65×10^4	3.09×10^6	6.20×10^{-3}	9.08×10^6	394.4	6.92×10^5	1.66	4.31×10^6
Meat	Palcam	−2.50	4.35×10^7	1.11×10^4	4.59×10^7	2.63×10^{-4}	7.60×10^7	169.8	2.24×10^7	2.05	1.08×10^2
Pocket	TAL method	−1.13	1.87×10^7	1.44×10^3	1.53×10^8	5.59×10^{-5}	7.58×10^7	53.4	1.42×10^8	1.87	1.32×10^2

thermal inactivation in parsley, using TAL method and Palcam Agar for cell enumeration. Results, for the three temperatures tested, are presented in Fig. 4. Higher microbial counts were observed when the TAL method was used. For all temperatures and during the all inactivation processes, at least one log difference was observed between enumeration in Palcam Agar and TAL method. If a non-isothermal process is chosen, differences between those media were also observed. As case studies, parsley thermally processed and *meat pockets* fried were analysed (Fig. 5 includes *L. innocua* inactivation data). For quantification of the inactivation kinetics of *Listeria* under dynamic conditions, the Gompertz-inspired model (eq. (2)) was successfully fitted to experimental data and kinetic parameters were estimated (inactivation rate: k_{ref} and Ea; shoulder: c and d; tail: log (N_{res})). These values are presented in Table 2 as well as the parameters precision, evaluated by SHW of the estimates at 95%. As expected, and due to the observed similarity of survival curves shape, the kinetic parameters from the two media were not statistically different.

For parsley (Fig. 5a), and using the TAL method, *Listeria* cells started inactivation around 65 °C. Throughout the heating up phase, a shoulder period of approximately 20 min was observed. However, if Palcam Agar was used, inactivation started after a shoulder period of only 15 min, when 50 °C was attained. This means that some cells were injured during the raise temperature period, particularly at higher temperatures.

The thermal inactivation kinetics of *L. innocua* was also analysed when frozen *meat pockets* were subjected to a frying process. The temperature history (monitored at the coldest point, i.e. centre of the product) and the bacteria survival data are presented in Fig. 5b. A great dispersion of temperatures was observed. However, a sigmoidal tendency of *Listeria* inactivation was observed throughout the frying process and the Gompertz-inspired model was adequate in data fitting.

For *meat pockets*, and as observed for parsley, when TAL method was used higher microbial counts were attained (at least one log difference when compared to Palcam Agar enumerations). A shoulder of approximately 3 min was observed. At this time the temperature in the *meat pocket* centre varied between −2 °C and 51 °C. It is important to refer that besides these values were monitored at the product central point, in most external meat layers the temperature may have reached higher values, which allowed bacterium inactivation.

To establish the degree of *Listeria* cells injury when a thermal treatment is applied to a food product, TICC and RIC values were also calculated and are included in Table 3. For the determination of

these values, TAL method was assumed as non-selective medium (in eqs. (5) and (7)) since its similarity to TSAYE was proven in broth experiments.

Percentages of injured *L. innocua* cells (TICC) from isothermal and non-isothermal treatments were not statistically different. As happened in liquid medium, RIC values also increased with the increase of temperature in parsley experiments.

The heating up phase of non-isothermal experiments may influence injured cells recovery, which can be assessed by the RIC values calculated. For instance, when a constant temperature of 65 °C was assumed for parsley thermal processing (RIC = −3.76 ± 0.3), the capability to recover was higher than the one observed when a non-isothermal process until the same final temperature was applied (RIC = −39.62 ± 8.1).

Another interesting fact that deserves to be mentioned is related with the plating technique used in both methods for the enumeration of *L. innocua* in food products. When Palcam Agar was used, a pour-plating technique was employed contrasting to the surface-plating utilized in TAL method. Although in both techniques typical *Listeria* colonies were observed, in TAL plates the colonies were bigger, allowing an easier bacteria enumeration. The TAL method efficacy is due to the recovery and growth of injured cells on TSAYE during the first hours of incubation, with posterior diffusion of the selective agents of Palcam Agar to TSAYE. As a result, typical reactions can be observed as well as the inhibition of endogenous microflora.

4. Conclusions

To attain safe standards of thermal processed foods, particular attention should be given to the potential presence of injured microorganisms. Palcam Agar is one of the selective media used in current detection/enumeration of *Listeria*. However, results showed that this medium may not be effective in the resuscitation of the totality of *L. innocua* injured cells. On the other hand, and for microbial detection in food products, media without selective or differential ingredients that can support the growth of debilitated cells cannot be used alone.

The TAL method tested in this work allowed enumeration of almost all thermal injured *L. innocua* cells (when experiments were carried out in broth), since small values of TICC were obtained. The high RIC values achieved in those conditions were close to non-selective medium (TSAYE) results, which confirmed the TAL medium recovery capability.

The use of Palcam Agar as enumeration medium of *L. innocua* cells in food products thermally processed (both isothermally and non-isothermally) resulted in microbial counts lower than the ones obtained when TAL medium was chosen. Therefore, it was proven that the combination of selective/non-selective media in TAL method is efficient in detecting injured *L. innocua* cells in food products, without compromising the selectivity of the medium.

Table 3
Evaluation of the ability of the media to recover injured cells (RIC) and degree of injured cells (TICC) of heat-injured *L. innocua* 10528 in food products.

Treatment conditions	Food product	T (°C)	RIC ± standard deviation	TICC ± standard deviation
Isothermal	parsley	52.5	289.75 ± 210.6	92.66 ± 6.6
		60.0	−19.31 ± 4.5	94.32 ± 3.8
		65.0	−3.76 ± 0.3	91.96 ± 2.0
Non-isothermal	parsley	Fig. 5a	−39.62 ± 8.1	87.22 ± 5.5
	meat pockets	Fig. 5b	−8.55 ± 1.3	89.88 ± 7.0

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