Influence of pH, type of acid and recovery media on the thermal inactivation of *Listeria innocua*

Fátima A. Miller, Bárbara Ramos, Maria M. Gil, Teresa R.S. Brandão, Paula Teixeira, Cristina L.M. Silva *

CBQF / Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

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A B S T R A C T

Acidification of foods with organic acids, either by fermentation or by intentional addition, is an important and common mechanism for controlling foodborne pathogens in a diversity of food products. The objective of this work was to study thermal inactivation of *Listeria innocua*, an acid tolerant microorganism, at 52.5, 60.0 and 65.0 °C, at different pH values (4.5, 6.0 and 7.5), using three types of acid (lactic, acetic and hydrochloric) and three different plating media (Tryptic Soy Agar with 0.6% yeast extract—TSAYE; TSAYE plus 5% NaCl—TSAYE + 5%NaCl; and Palcam Agar with selective supplement—Palcam Agar), according to a 3^4 factorial experimental design. Survival data experimentally obtained were fitted with a Gompertz-inspired model and kinetic parameters (shoulder, maximum inactivation rate—k_{max}, and tail) were estimated for all conditions considered. The influence of temperature, pH, type of acid and enumeration media on kinetic parameters was assessed. Results showed that, with the exception of the type of acid, all the remaining factors and their combinations significantly affected the shoulder period and k_{max}. In relation to tail, temperature and recovery media were the affectable factors. It was concluded that the survival of this bacteria is higher when combining low temperature with neutral pH, and when TSAYE is the enumeration medium. Bigelow-inspired models were successfully developed and describe accurately the temperature and pH effects on the kinetic parameters.

Introduction

Listeria genus is composed by facultative anaerobic gram-positive bacteria that are widespread in the environment. The closely related species *Listeria monocytogenes* and *Listeria innocua* are frequently found in the same food products. They can grow over a range of temperatures varying from 0 to 45 °C and pH values from 4.5 to 9.2 (Norrung, 2000). The presence of *L. monocytogenes* in ready to eat foods can cause listeriosis, a severe infectious disease characterised by meningoencephalitis, spontaneous abortion, stillbirth, septicaemia and a high fatality rate of 30% (Rodríguez-Lázaro et al., 2004). Listeriosis predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients. Despite attempts to eliminate the pathogen from the food chain, human listeriosis outbreaks frequently occur and are mainly associated with the consumption of contaminated ready to eat food products. Gandhi and Chikindas (2007) mentioned several factors that have influenced the incidence of this disease. Changing the food habits of the consumer, trending towards consumption of minimally processed and ready to eat food products, may be considered a critical factor.

L. innocua, has been used in several thermal inactivation studies instead of *L. monocytogenes*, because it is non-pathogenic and its presence in foods is not a hazard to human health (Kamat and Nair, 1996; Margolles et al., 2000; Piyasena et al., 1998).

The heat resistance of such bacteria can be influenced by important environmental factors that included temperature, pH and type of acid. Many studies have been done to evaluate the thermal resistance of *Listeria* spp. at different pH values (Chhabra et al., 2002; Hassani et al., 2005; Juneja and Eblen, 1999), but few include the effect of the type of acid used (Juneja et al., 1998).

It is well known that the heat resistance of vegetative cells and spores is reduced by low pH and, consequently, milder heat treatments may be used to achieve target safe standards (Casadei et al., 2000). Therefore, several additives are commonly used with the purpose of reducing foods' pH. According to The Miscellaneous Food Additives Regulations (Anon., 1995), which describes the legislation controlling additives in the European Union, organic acids such as acetic and lactic acid can be used as additives in most foods and mostly *quantum satis* (without specific limitations). The use of hydrochloric acid is also permitted to lower the pH of some foods (i.e. carbonated and non-carbonated drinks). In July 2006, the European Commission proposed new legislation on food additives with the objective of updating and simplifying the existing Community Legislation.

The possibility of using different types of acid to decrease the pH of food products makes this study of extreme importance. Heat

* Corresponding author. Tel.: +351 22 5580058; fax: +351 22 5090351.
E-mail address: clsilva@esb.ucp.pt (C.L.M. Silva).

treatments applied to foods with organic acids may differ from the one that has the presence of a strong acid (such as hydrochloric acid).

A significant factor that influences microbial recovery to thermal treatments is the plating media that is used. If a selective media is utilized to grow stressed bacteria colonies, injured cells present after the treatment may not recover and under-estimated values may be found (Miller et al., 2006). Consequently, all experiments must be done using a non-selective media to circumvent this problem. However, this is not possible when working with unsterile food products. Thus, the behaviour of microorganisms in different media must be known. Applying different types of media can also help to identify the level of cell damage.

The microbial kinetic behaviour (assessed on the log base10 transformed number of viable cells) often deviates from linearity, depending on the treatment conditions applied (Huang, 2009; McKellar and Lu, 2004; Xiong et al., 1999). Nevertheless, and erroneously in many situations, *D*-values (decimal reduction time, or time required to inactivate 90% of the population) and *z*-values (temperature necessary to reduce *D*-value by 10-fold) are frequently calculated, assuming first-order kinetics. Some mathematical models, able to describe non-linear kinetic behaviour, have been used by several researchers (Albert and Mafart, 2005; Geeraerd et al., 2000; Gil et al., 2006). A complete (or incomplete) sigmoidal tendency with an initial delay, followed by a maximum inactivation rate period tending to a residual population is often mentioned in literature (Janssen et al., 2007; Peleg, 2003).

If model parameters are estimated on the basis of experimental data and if the effects of environmental factors on those parameters are evaluated, the influence of those factors on the kinetic behaviour can be quantified. Consequently, one can draw conclusions concerning the most relevant factors that significantly contribute to thermal inactivation changes.

The main objective of this study was to evaluate the influence of pH, type of acid (organic and inorganic) and plating media on the thermal inactivation of *L. innocua*, using a convenient factorial experimental design.

Materials and methods

Experimental design

A 3^4 factorial design (Box et al., 1978) was applied to assess the effect of (i) temperature, (ii) pH, (iii) type of acid and (iv) enumeration media, on the inactivation behaviour evaluated by shoulder, maximum inactivation rate and tail parameters of the Gompertz-inspired model (Eq. (1); Section 2.3.1). The levels assumed for the variables were: (i) 52.5, 60.0 and 65.0 °C for temperature, (ii) 4.5, 6.0 and 7.5 for pH, (iii) lactic, acetic and hydrochloric acid, and (iv) TSAYE, TSAYE + NaCl and Palcam Agar (resulting in 81 combinations).

Experimental procedures

Cultures

L. innocua NCTC 10528 was subcultured (30 °C, 24 h) 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.

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

Cells in each cellular suspension were enumerated by plating appropriate dilutions, in duplicate, on the three solid media being studied (Section 2.2.4).

Inactivation experiments

The media : TSBYE was used as the basal medium for all experiments. Combinations of temperature (52.5, 60.0 and 65.0 °C), pH (4.5, 6.0 and 7.5) and type of acid (lactic 0.5 M, acetic 3 M and hydrochloric 0.5 M—Merck) were studied according to the experimental design. The pH of the media was adjusted with the desired type of acid and measured before and after autoclaving using a pH meter (GLP 22, Crison Instruments, Spain). The added volume of acid solutions did not affect significantly the volume of the media. Autoclaving did not change the pH of the medium.

Unacidified TSBYE (pH 7.5) served as a control broth for all combinations.

The treatment : Heat treatments were carried out in a thermostatized water bath. An Erlenmeyer flask containing 99 mL of stirred TSBYE, adjusted to the specified pH, was immersed in the water bath. Once the heating medium temperature had stabilized, it was inoculated with 1 mL of cell suspension. Samples were removed at different time intervals and placed in a mixture of ice–water.

Three replicates of all experiments were performed.

The initial concentration of *L. innocua* was determined to be approximately 10^7 CFU/mL for all conditions tested.

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 + 5%NaCl and (iii) Palcam Agar plus selective supplement (Miller et al., 2006). 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.

Modeling procedures

The inactivation model

When microbial inactivation follows a sigmoidal behaviour, experimental data can be mathematically described by a Gompertz-inspired model (Bhaduri et al., 1991; Char et al., 2009; Gil et al., 2006; Linton et al., 1995):

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

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.

The Gompertz-inspired model presented in Eq. (1) is versatile in fitting linear data and those that contain shoulder and/or tailing effects (Zwietering et al., 1990).

Temperature and pH effects

The parameters k_{max} and L are temperature and pH dependent.

The maximum inactivation rate is the reciprocal of the *D*-value (i.e. the time required for 1-log reduction in microbial load, at a given temperature), being this parameter often preferred by microbiologists. The Bigelow model can be used to express the dependence of k_{max} (or *D*-value) on temperature and pH (Gaillard et al., 1998; Mafart and Leguerinel, 1998):

$$\log\left(\frac{1}{k_{max}}\right) = \log(D) = \log(D_{ref}) - \left(\frac{T-T_{ref}}{z_T}\right) - \left(\frac{pH-pH_{ref}}{z_{pH}}\right)^2 \quad (2)$$

Herein D_{ref} is the D-value at a reference temperature (T_{ref}) and at a reference pH (pH_{ref}), and z_T and z_{pH} are, respectively, the temperature and pH required for a 10-fold reduction of D-value.

The shoulder parameter can also be related to temperature using a Bigelow-type relation:

$$\log(L) = \log(L_{ref}) - \left(\frac{T - T_{ref}}{z_T^*}\right) - \left(\frac{pH - pH_{ref}}{z_{pH}^*}\right)^2 \quad (3)$$

where L_{ref} is the shoulder at a reference temperature (T_{ref}) and at a reference pH (pH_{ref}), and z_T^* and z_{pH}^* are, respectively, the temperature and pH required for a 10-fold reduction of *L. innocua*.

2.3.3. Data analysis

The parameters of the Gompertz-inspired inactivation model, i.e. L, k_{max} and $\log(N_{res}/N_0)$, were estimated by non-linear regression analysis, fitting Eq. (1) to experimental inactivation data at the temperatures studied.

The parameters of the temperature and pH effect models (i.e. log (D_{ref}), z_T and z_{pH} for log ($1/k_{max}$); L_{ref}, z_T^* and z_{pH}^* for shoulder) were estimated by fitting Eqs. (2) and (3), respectively, to $\log(1/k_{max})$ and log (L) values previously estimated at each temperature, by multiple regression analysis.

The temperature and pH values of reference were assumed to be 60.0 °C and 7.5, respectively, in all cases.

The quality of the regressions was evaluated by the coefficient of determination (R^2), randomness and normality of the residuals.

Parameters' precision was evaluated by the coefficient of determination (R^2), randomness and normality of the residuals.

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

Results from 3^4 factorial experimental design were analysed by ANOVA procedures.

Statistica© 6.0 (StatSoft, USA) and Microsoft® Excel 2000 (Microsoft Corporation, USA) were used for all calculations, regression procedures and statistical analysis.

Results and discussion

In the majority of processes used in food industry, temperature has been considered one of the most relevant factors on microbial survival. However, a number of studies clarified the influence of pH on thermal inactivation. The works of Chiruta et al. (1997), Hassani et al. (2005) and Juneja and Eblen (1999) are examples dealing with *Listeria* spp. behaviour in unfavourable temperature and pH conditions. However, these studies are confined to the influence of such conditions on overall microbial thermal resistance, neglecting the direct impact on particular features of a complete sigmoidal tendency (situations included in this work). The influence of temperature, pH, type of acid and plating media on those features (assessed by shoulder, maximum inactivation rate and tail parameters) was studied using the conditions defined according to the 3^4 experimental design. The Gompertz-inspired model (Eq. (1)) was fitted to experimental inactivation data, and kinetic parameters were estimated for all situations considered. These results are included in Tables 1–3, respectively for pH 4.5, 6.0 and 7.5. Typical inactivation behaviour can be visualized in Fig. 1 (for some of the conditions studied). Kinetic model parameters (i.e. shoulder, maximum inactivation rate and tail) were estimated by non-linear regression analysis. The quality of all model fits was assessed by residual analysis (with validated residuals randomness and normality in all cases) and by the coefficient of determination (R^2 was above 0.92 in all cases). The precision of parameter estimates was also assessed (based on the standardized half width at 95%, aiming at a better perception of the parameter variance, avoiding the magnitude of the estimates themselves). Analysing all studied cases (Tables 1–3), one can conclude that satisfactory precision was attained. Shoulder time presented higher $SHW_{95\%}$ when compared to inactivation rate or tail. In general, the worse cases were observed for TSAYE + 5%NaCl medium and for all parameters. This can be explained by the unfavourable recovery conditions of this medium that implied less experimental data points (a design drawback that can be visualized in some situations included in Fig. 1; the case of $T = 60.0$ °C and pH = 6.0 is one example) and, consequently, precision of the estimates is sacrificed.

One commonly mentioned drawback of the Gompertz model is its inadequacy in predicting $\log(N/N_0)$ when time reaches zero (i.e. for $t = 0$, $\log(N/N_0)$ only approaches zero). However, the over- or sub-estimation of this value is negligible when compared to the experimental variations obtained for initial population size between two duplicates.

Results of the experimental design analyses showed that, with the exception of the type of acid, all factors significantly affected the maximum inactivation rate and the shoulder period (at a significance level of 2.5%). Combined effects of temperature/pH, temperature/media and pH/media were also important. In relation to the tail

Table 1
Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 at pH 4.5 for all type of acids and temperatures tested.

Acid	T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
			L (min)	$SHW_{95\%}$	k_{max} (min^{-1})	$SHW_{95\%}$	Log (N_{res}/N_0)	$SHW_{95\%}$
Lactic	52.5	TSAYE	4.23	20.9	0.22	7.2	−6.2	21.2
		TSAYE + 5% NaCl	1.57	38.1	0.37	10.9	−5.1	12.9
		Palcam Agar	3.16	29.6	0.25	10.2	−5.8	22.9
	60.0	TSAYE	0.30	20.8	3.06	6.4	−6.7	22.8
		TSAYE + 5% NaCl	0.07	114.3	3.58	11.2	−6.1	37.0
		Palcam Agar	0.17	37.7	3.28	8.8	−6.0	21.5
	65.0	TSAYE	1.26	9877.6	21.19	29.4	−4.3	13.5
		TSAYE + 5% NaCl	0.00	4490.3	500.93	4424.4	−4.5	9.3
		Palcam Agar	0.00	2087.9	50.25	45.3	−4.2	12.5
Acetic	52.5	TSAYE	2.23	0.8	0.22	9.2	−5.8	18.1
		TSAYE + 5% NaCl	1.97	54.3	0.43	21.7	−5.1	18.9
		Palcam Agar	1.77	41.3	0.31	11.4	−4.8	9.1
	60.0	TSAYE	0.37	29.2	3.36	10.9	−8.5	37.8
		TSAYE + 5% NaCl	0.15	75.4	4.40	14.9	−7.4	57.2
		Palcam Agar	0.22	31.0	3.73	8.4	−6.8	31.4
	65.0	TSAYE	0.00	[a]	21.54	38.4	−4.0	15.4
		TSAYE + 5% NaCl	0.00	5407.7	500.70	5372.7	−4.4	10.0
		Palcam Agar	0.00	8228.2	52.54	57.6	−4.3	15.2
HCl	52.5	TSAYE	6.19	17.1	0.17	5.1	−8.1	19.6
		TSAYE + 5% NaCl	2.79	26.3	0.22	8.1	−5.2	10.8
		Palcam Agar	3.38	20.7	0.17	4.5	−6.3	13.3
	60.0	TSAYE	0.72	24.0	2.63	13.2	−9.5	40.6
		TSAYE + 5% NaCl	0.24	27.7	3.40	8.3	−6.4	24.7
		Palcam Agar	0.44	13.2	3.10	5.4	−7.6	21.0
	65.0	TSAYE	0.00	1189.0	9.30	23.4	−4.9	17.9
		TSAYE + 5% NaCl	0.01	166.2	116.01	39.6	−4.3	3.1
		Palcam Agar	0.00	3653.7	18.60	23.2	−4.5	14.9

[a] Considerable high meaningless value.

Table 2
Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 at pH 6.0 for all type of acids and temperatures tested.

Acid	T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
			L (min)	$SHW_{95\%}$	k_{max} (min^{-1})	$SHW_{95\%}$	Log (N_{res}/N_0)	$SHW_{95\%}$
Lactic	52.5	TSAYE	25.57	45.4	0.04	14.1	−8.2	52.4
		TSAYE + 5% NaCl	13.41	32.5	0.07	12.9	−6.0	27.7
		Palcam Agar	16.73	45.8	0.05	14.4	−6.1	42.5
	60.0	TSAYE	0.31	60.7	1.01	8.1	−5.9	18.6
		TSAYE + 5% NaCl	0.09	54.1	4.98	13.4	−4.6	5.6
		Palcam Agar	0.14	104.4	1.46	9.4	−5.7	16.8
	65.0	TSAYE	0.04	164.3	5.35	16.1	−5.1	15.0
		TSAYE + 5% NaCl	0.00	1464.5	22.62	38.2	−3.9	13.4
		Palcam Agar	0.05	136.5	8.50	28.9	−4.6	17.9
Acetic	52.5	TSAYE	21.06	29.1	0.04	7.7	−7.2	30.6
		TSAYE + 5% NaCl	12.66	18.7	0.07	6.4	−6.2	16.2
		Palcam Agar	13.98	21.8	0.05	6.1	−5.9	14.6
	60.0	TSAYE	0.61	20.9	1.04	5.2	−6.1	14.3
		TSAYE + 5% NaCl	0.11	40.5	6.27	16.0	−4.1	4.2
		Palcam Agar	0.51	21.9	1.60	7.9	−5.6	14.0
	65.0	TSAYE	0.04	109.8	5.51	12.4	−4.8	9.9
		TSAYE + 5% NaCl	0.01	270.3	24.62	31.9	−4.2	7.2
		Palcam Agar	0.03	128.4	10.59	24.8	−4.3	11.0
HCl	52.5	TSAYE	18.29	19.7	0.04	5.4	−6.0	13.1
		TSAYE + 5% NaCl	12.52	19.3	0.06	6.5	−5.2	9.7
		Palcam Agar	13.54	16.8	0.05	4.8	−5.4	8.4
	60.0	TSAYE	0.53	26.4	0.92	5.1	−6.1	13.8
		TSAYE + 5% NaCl	0.00	4568.9	3.14	17.5	−4.5	9.5
		Palcam Agar	0.35	41.6	1.26	7.6	−6.0	18.7
	65.0	TSAYE	0.12	50.4	3.78	8.8	−6.1	24.5
		TSAYE + 5% NaCl	0.00	9081.3	17.93	28.4	−3.7	9.1
		Palcam Agar	0.05	74.2	5.90	12.2	−4.6	9.4

parameter, the temperature and the enumeration media were the most significant factors.

Temperature had a positive effect on k_{max} and tail parameter, this means that when temperature rises, higher inactivation rates are observed and the tail tendency becomes evident. Concerning the shoulder, the temperature effect is negative. The shoulder period tends to disappear for higher temperatures.

Lowering pH values implies higher inactivation rates and narrow shoulders. The plating media effect is significant on shoulder, k_{max} and tail parameters.

The narrow shoulder periods and higher inactivation rates were observed for TSAYE + 5%NaCl. In such medium the tail was pronounced. The higher shoulder periods and lower inactivation rates were observed for TSAYE medium. In this medium, the tail was not so evident.

The type of acid used, i.e. lactic and acetic (organic) and hydrochloric (inorganic) acids did not significantly affect the inactivation parameters considered (when compared to the influence of the remaining factors studied). Therefore, lowering the pH of the heating menstruum is more important than the type of acid selected.

Chiruta et al. (1997) noted that the effect of pH on the rate of thermal inactivation was significant for *L. monocytogenes*, *E. coli* and for *Pseudomonas fluorescens*, but this was more evident at lower treatment temperatures. This is in agreement with the results presented here, with the exception of experiments using the TSAYE + 5% NaCl medium.

A number of researchers have reported the inhibitory effects of low pH and organic acids on *L. monocytogenes* (Conner et al., 1990; Ita and Hutkins, 1991; Phan-Thanh et al., 2000; Vasseur et al., 1999), without considering unfavourable temperature conditions. They concluded that the microorganism was most inhibited by acetic acid, followed by lactic and hydrochloric acids. A similar occurrence was observed in this work, for the lowest pH considered (pH = 4.5).

In general, all the authors that studied the effect of pH and type of acid on the inactivation of bacteria proposed two inhibitory mechanisms: *(i)* an intracellular acidification (lost of homeostasis) and *(ii)* a specific effect of the acid (non-dissociated form) on metabolic activities. Ita and Hutkins (1991) observed that low intracellular pH was not the major factor in the inhibition of *L. monocytogenes*. In fact, cells treated with organic acids or HCl at pH values as low as 3.5 were able to maintain their cytoplasmic pH near 5.0. Budde and Jakobsen (2000), Shabala et al. (2002) and Siegumfeldt et al. (1999) also concluded that *L. monocytogenes* maintains its intracellular pH within a narrow range of 7.6–8.0 at extracellular pH values of 4.0 to 8.0. Failure to maintain intracellular homeostasis leads to loss of cell viability (Chitarra et al., 2000).

The inhibitory activity of organic acids against *L. monocytogenes* is mainly related with their dissociation constants (pKa value) and with the greater permeability of the cell membrane to weak acids in their undissociated form (Vasseur et al., 1999). Looking to the acids that we have tested, HCl is totally dissociated in aqueous environments, lactic acid has a pKa of 3.86 and acetic acid a pKa of 4.76. Because acetic acid has the highest pKa, this acid would be present in higher amounts of undissociated molecules, which accumulation in the cells leads to a higher antimicrobial activity. Indeed, the results of the present work indicate that weak organic acids are more efficient against *L. innocua* than a stronger acid, such as HCl, at the same pH.

These findings corroborate the knowledge about the way different kinds of acids pass through the cell membrane, which was clarified by Phan-Thanh et al. (2000). Strong mineral acids completely dissociate into H^+ and anions. Cell membranes have a very low permeability to H^+. Protons enter and exit the cell by interacting with the cell systems that control H^+ transport, such as $F_0F_1^-$ APTase, Na^+/H^+ antiporters, electron transport systems (respiratory chains). Weak organic acids permeate the cell membrane as undissociated molecules through other mechanisms, such as permeases or porins. The highest inhibitory effect of acetic acid can be explained by its ability to diffuse through the cell membrane, while lactic acid may be less inhibitory as it cannot passively penetrate the cell membrane (Vasseur et al., 1999). Once dissociated inside the cell, they cannot diffuse out, leading to an accumulation of the acid within the cell cytoplasm, lowering the intracellular pH to dramatic values and deregulating the metabolic activity of the cell. Phan-Thanh and Montagne (1998) showed by the measurement of intracellular pH that organic acids caused a more important decrease in intracellular pH than HCl did, at the same external pH.

Confronted with acidic conditions, bacterial cells attempted to resist by maintaining its intracellular homeostasis. This can be done in different ways depending on the kind of bacteria (Booth, 1985). In aerobic bacteria, the active transport of H^+ is coupled to the process of electron transport in respiratory chains with three major constituents, dehydrogenases, quinines and oxydoreductases. In anaerobic bacteria, H^+ transport is carried out through a specific H^+ channel in the $F_0F_1^-$ APTase molecule (proton pump) using energy from ATP hydrolysis. *Listeria*, a facultative bacterium, may use both processes to maintain its intracellular pH homeostasis (Phan-Thanh et al., 2000).

Table 3
Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 at pH 7.5, used as control, for all temperatures tested.

T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
		L (min)	$SHW_{95\%}$	k_{max} (min^{-1})	$SHW_{95\%}$	Log (N_{res}/N_0)	$SHW_{95\%}$
52.5	TSAYE	40.00	32.9	0.03	16.4	−5.2	48.3
	TSAYE + 5% NaCl	0.00	[a]	0.09	39.9	−3.4	21.9
	Palcam Agar	20.00	111.1	0.04	35.5	−8.2	79.9
60.0	TSAYE	3.93	48.4	0.36	23.5	−8.3	67.9
	TSAYE + 5% NaCl	0.10	245.5	1.24	19.0	−4.5	21.9
	Palcam Agar	0.96	55.3	0.67	12.3	−6.4	46.7
65.0	TSAYE	0.06	338.8	2.68	26.6	−4.8	30.1
	TSAYE + 5% NaCl	0.02	100.0	29.64	34.2	−3.9	15.8
	Palcam Agar	0.01	868.2	8.88	38.7	−4.0	18.8

[a] Considerable high meaningless value.

Another factor that was studied in the present work was the influence of the enumeration media on the survival of *L. innocua*. From observation of Fig. 1, it can be observed that higher cell recovery is achieved when TSAYE is used, followed by Palcam Agar and TSAYE + 5%NaCl. It is known that stressed bacteria become sensitive to many selective compounds, due to damage in their membrane and modification of their permeability, and they lose their ability to grow on selective media (Abee and Wouters, 1999; Besse, 2002). However, survival in rich media may confer protection to the microorganisms by providing energy and metabolic precursors (Shabala et al., 2002).

Based on the results, it can be concluded that shoulder times are higher for higher pH values, lower temperatures and for rich media. Generally, the shoulders of the survival curves are considered as a manifestation of damage accumulation that becomes irreparable, only beyond a critical level (Smelt et al., 2002). Thus, as the three factors went to unfavourable values for the survival of *L. innocua*, the shoulder increases.

Maximum inactivation rates can be converted into the decimal reduction time (D-value), by using the relation presented in Eq. (2). This parameter is often preferred by microbiologists, since it provides a perception of the required time to achieve a 1-log reduction of the microbial load. In Table 4, the calculated D-values obtained for all conditions studied are presented. The temperature and pH effects on the inactivation rate were then analysed through the logarithmic of D-values, using a Bigelow-inspired function (Eq. (2)).

The effects of temperature and pH on shoulder parameter were also modeled, using a similar Bigelow function (log L as function of pH and T, presented in Eq. (3)). The overall predictive ability of both models can be evaluated by the results presented in Fig. 2. It can be concluded that Eq. (2) predicts accurately log D values, for all media used. For shoulder parameter, results obtained from TSAYE + 5%NaCl medium were not included in these studies, since a great uncertainty of estimates were observed (results already discussed; Tables 1–3). However, the adequacy of using Eq. (3) in log L prediction for the remaining media is also satisfactory.

The models are presented in Table 5. Good fits were attained for log D predictions (evaluated through residual analyses and R^2 values). Besides a greater variability was observed for log L results (lower R^2 values averaging 0.86, inherent to the higher uncertainty obtained for shoulder parameter previously discussed), the adequacy of this model was proven for TSAYE and Palcam media. One typical example of the dependence (and prediction) of log D and log

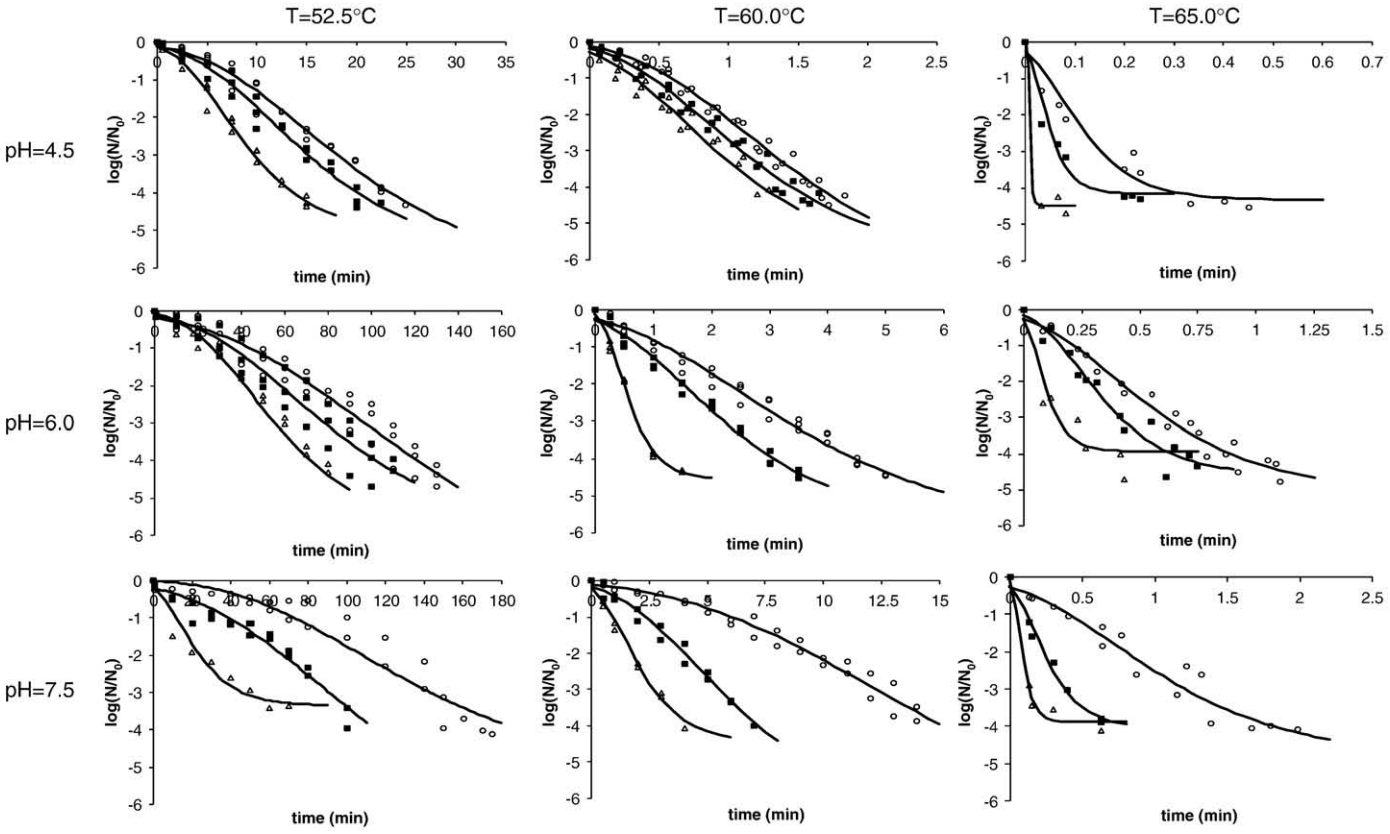

Fig. 1. Thermal inactivation data of *L. innocua* at all conditions of temperature and pH adjusted with lactic acid, and using TSAYE (○), TSAYE + 5%NaCl (■) and Palcam Agar (Δ) as recovery media. The lines represent model fits (Eq. (1)).

Table 4
Calculated *D*-values (expressed in minutes) for *L. innocua* at different temperature, pH, type of acid and enumeration media.

Media	T (°C)	pH = 4.5			pH = 6.0			pH = 7.5
		Lactic	Acetic	HCl	Lactic	Acetic	HCl	No acid
TSAYE	52.5	4.611	4.569	5.802	23.97	24.57	27.31	33.89
	60.0	0.327	0.297	0.381	0.990	0.959	1.081	2.782
	65.0	0.047	0.046	0.108	0.187	0.181	0.264	0.373
TSAYE + 5%NaCl	52.5	2.690	2.308	4.467	14.00	14.41	17.68	11.63
	60.0	0.280	0.227	0.294	0.201	0.159	0.319	0.808
	65.0	0.002	0.002	0.009	0.044	0.041	0.056	0.034
Palcam Agar	52.5	3.978	3.205	5.744	20.54	20.67	21.97	23.58
	60.0	0.305	0.268	0.323	0.686	0.623	0.791	1.501
	65.0	0.020	0.019	0.054	0.118	0.094	0.170	0.113

Table 5
Models that include pH and temperature effects on log *D* and log *L* prediction, for the enumeration media studied.

Kinetic parameter	Media	Bigelow-inspired model
Log *D*	TSAYE	$\log(D) = 0.354 - \left(\frac{T-60}{6.29}\right) - \left(\frac{pH-7.5}{3.30}\right)^2$ $R^2 = 0.99$
	TSAYE + NaCl	$\log(D) = -0.311 - \left(\frac{T-60}{4.73}\right) - \left(\frac{pH-7.5}{3.54}\right)^2$ $R^2 = 0.94$
	Palcam Agar	$\log(D) = 0.078 - \left(\frac{T-60}{5.59}\right) - \left(\frac{pH-7.5}{3.57}\right)^2$ $R^2 = 0.98$
Log *L*	TSAYE	$\log(L) = 0.063 - \left(\frac{T-60}{3.99}\right) - \left(\frac{pH-7.5}{2.60}\right)^2$ $R^2 = 0.84$
	Palcam Agar	$\log(L) = -0.268 - \left(\frac{T-60}{4.02}\right) - \left(\frac{pH-7.5}{2.87}\right)^2$ $R^2 = 0.88$

L with pH and temperature can be observed in Fig. 3 (for TSAYE medium). For the other media, the quality of the predictions was similar.

These models allowed insightful information concerning the sensitivity of the microbial response in relation to temperature (assessed by z_T and z_T^*) and in relation to pH (assessed by z_{pH} and z_{pH}^*). These parameters (and their confidence intervals at 95%) are included in Fig. 4.

It can be concluded that *L. innocua* is more sensitive to temperature variations than to pH variations. The values estimated for z_T's were higher than the ones estimated for z_{pH}'s. Different z_T values (related to log *D*) were obtained when different media are used. If TSAYE is chosen as plating medium, results showed that *L. innocua* was less heat sensitive. The media did not affect z_{pH}'s values.

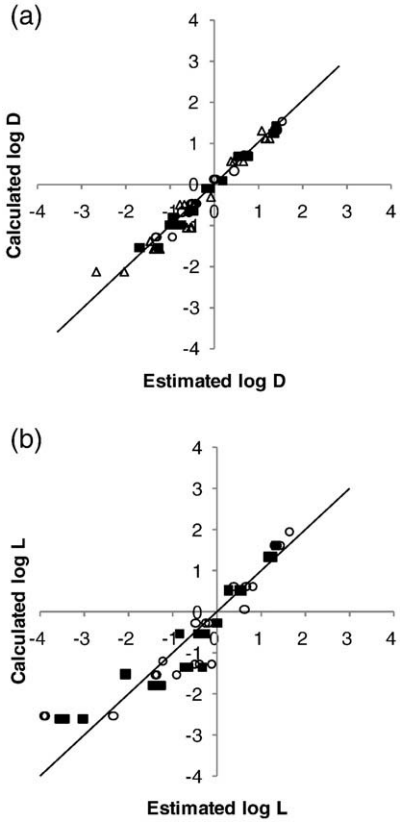

Fig. 2. Comparison between the calculated and estimated log *D* (a) and log *L* (b) values for all the experimental data, using TSAYE (○), TSAYE + 5%NaCl (Δ) and Palcam Agar (■) as the recovery media. The lines represent the diagonals.

Results obtained in this work are important information to be used in the development of efficient experimental designs when the pathogenic specie (*L. monocytogenes*) would be the target.

Conclusions

To achieve accurate results concerning microbial thermal inactivation predictions, it is crucial to account for the highest number of influencing factors. In the case of *L. innocua* inactivation, temperature, pH, enumeration media and their combinations affected significantly the shoulder and maximum inactivation rate. The tail was only influenced by temperature and recover media. Although the type of acid effect was not significant in all kinetic parameters, hydrochloric acid was the least inhibitory one. Minimal differences were observed between the organic acids, lactic and acetic, and between these and the strong inorganic acid.

Care must be taken with the plating media used, since selective media may be inadequate for the recovery of injured *Listeria* cells. If only selective media is employed for the detection and enumeration of stressed microorganisms, injured cells may not recover and underestimated values may be predicted. To overcome this problem, a non-selective agar overlay technique can be used.

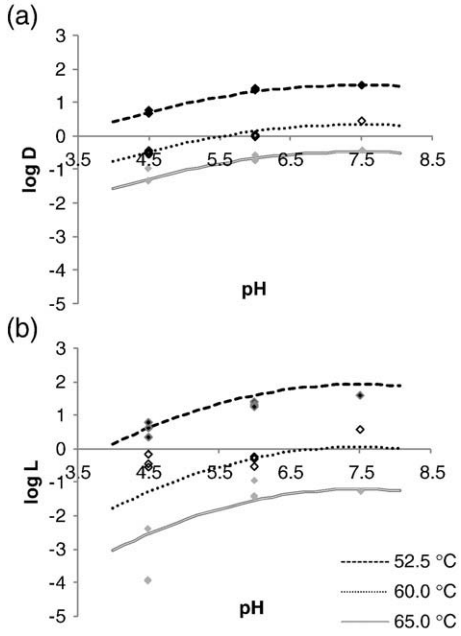

Fig. 3. Combined influence of temperature and pH on log *D* (a) and log *L* (b) for TSAYE medium. The lines represent model fits (Eqs. (2) and (3)).

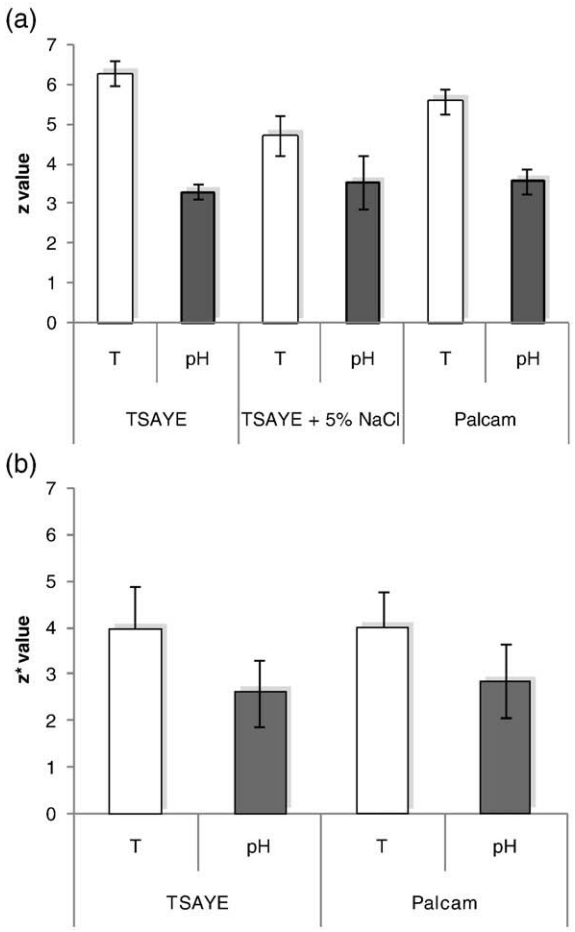

Fig. 4. Temperature and pH sensitivity of log *D* (a) and log *L* (b) expressed by *z*- and *z**-values, for the enumeration media used. The bars represent the confidence intervals at 95% of *z*- and *z**-values.

Bigelow-inspired models that include the pH and temperature effects on the kinetic parameters, allowed accurate predictions.

According to estimated *z*-values, *L. innocua* is less sensitive to variations of pH than to temperature variations.

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