Influences of Physicochemical Stresses on Injury and Inactivation Behaviour of *Listeria Innocua*

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Abstract

Many minimally processed foods depend on a combination of inhibitory factors to reduce the hazard of foodborne illness. Therefore, inactivation of *Listeria innocua* was studied according to a 2^4 factorial experiment designed to draw conclusions about temperature (52.5 °C and 65.0 °C), pH (4.5 and 7.5), water activity (a_w=0.95 and 0.99) and solute type (NaCl and glycerol) effects. Three different recovery media were used to assess injured cells. Survival data were fitted with a Gompertz-based model and kinetic parameters (shoulder, maximum inactivation rate – k_{max}, and tail) were estimated. Results showed that shoulder was affected by temperature, pH and combined effects; k_{max} was influenced by all factors and their combinations; and tail was affected by a_w, temperature and a_w/pH combination. Results demonstrated the potential occurrence of microbial cross-protection survival techniques between the various stresses, e.g. heat and osmolarity. Indeed, this work clearly established that, to avoid hazards, *Listeria* inactivation must be evaluated with a maximum of environmental factors that undergo alterations. Only thus, appropriate food preservation treatments can be developed and consequently, the safety of food products can be assured.

Keywords: *Listeria*; Microbial thermal inactivation; pH; a_w; Injury

1 Introduction

Pathogenic bacteria in food may encounter different stressing conditions such as high or low temperature, pH and water activity (a_w) values. *Listeria monocytogenes*, the cause of listeriosis, is widespread in the environment and has been found in raw and ready-to-eat vegetables, meat, poultry, seafood and dairy products (Mena et al., 2004; Wang et al., 2013). *L. monocytogenes* can grow at refrigerated temperatures, has the ability to tolerate acidic conditions and can survive on dry surfaces (Norrung, 2000). Consequently,

control of this bacterium is a major challenge for the food producers, since it can adapt to food environments, which normally restrict bacterial growth.

A non-pathogenic species of *Listeria* is *Listeria innocua*. This bacterium is often used as a thermal processing surrogate for *L. monocytogenes* (Kondjoyan & Portanguen, 2008; Char, Guerrero, & Alzamora, 2009), since they share similar characteristics and environments. Besides the criticism of some researchers in assuming the parallelism of both species in a variety of foods and processing conditions, there are a considerable

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number of works that validate *L. innocua* as a primary non-pathogenic indicator of *L. monocytogenes* (Zhang, Ma, Oyarzabal, & Doyle, 2007; Lecompte, Kondjoyan, Sarter, Portanguen, & Collignan, 2008). The similarity of growth properties of both species in a wide range of temperatures, pH and water activity values re-enforces the choice of working with a non-pathogenic species (Begot, Lebert, & Lebert, 1997). The majority of *L. innocua* strains are more heat resistant than *L. monocytogenes*, thus providing a margin of safety in the development/design of new thermal processes involving these microorganisms (Friedly et al., 2008).

The extreme resistance of *Listeria* spp. to many food preservation conditions leads to the application of hurdle combinations that allow milder processing conditions, but still effective in bacteria control (Barker & Park, 2001). In foods, microorganisms are exposed to multiple physicochemical stresses that may, individually or simultaneously, affect their behaviour. However, due to the importance of heat processing in the food industry, researcher's attention has been given to temperature effects. Until recently, few studies had given importance to the combination of other stressful factors, such as pH and a_w, and their effect on microbial stress-adaptation and cross-protection.

Although it has been widely assumed that combined hurdles have additive or synergistic mode of action, some studies (Casey & Condon, 2002; Lee & Kang, 2009) reported antagonistic effects. Therefore, it is crucial to study the effects of several relevant stress factors on microbial inactivation to avoid extended survival problems.

Some of the main important hurdles used in food preservation are temperature, pH and a_w. When a thermal process is applied, the heat resistance of microorganisms is influenced by the physicochemical conditions during heating. Among them, a_w is one of the most important. Data available in literature revealed that when a_w decreases, a marked increase in microbial thermal resistance is often observed. Results also suggested that the heat resistance of bacteria depends both on strain and type of solute chosen (Mazas, Martinez, Lopez, Alvarez, & Martin, 1999; Coroller, Leguerinel, & Mafart, 2001). The pH of the heating media is also an important factor that influences the thermal microbial inactivation. As pH decreases, the microbial heat resistance decreases, so less severe heat treatments may be used to achieve safe products (Miller, Ramos, et al., 2009).

Exposure to a combination of these stressing factors may also induce sublethal physiological and structural changes in cells. These are called injured cells and may have the potential to recover when favourable conditions occur. The majority of techniques for enumerating *L. monocytogenes* requires culturing with selective media. If media do not support the growth of injured cells, the quantitative detection of viable cells can be compromised (Bremer, Osborne, Kemp, & Smith, 1998).

According to the treatment conditions used, the microbial inactivation behaviour may not follow a linear tendency (McKellar & Lu, 2003; Huang, 2009) and initial lags and/or final tailing tendencies may precede and/or follow the maximum inactivation rate period (i.e. sigmoidal kinetics). Therefore, there are several mathematical models that describe non-linear behaviour (Geeraerd, Herremans, & Van Impe, 2000; Albert & Mafart, 2005; Gil, Brandao, & Silva, 2006).

Thus, the objectives of this study were: (i) to evaluate the influence of temperature, pH, a_w and type of solute (NaCl or glycerol) on the inactivation behaviour of *L. innocua*, and (ii) to determine the incidence of injured cells after each treatment using three platting media.

2 Materials and Methods

2.1 The experimental design

A 2^4 factorial design (Box, Hunter, & Hunter, 1978) was applied to assess the effect of (i) temperature, (ii) pH, (iii) water activity and (iv) type of solute used to vary a_w, on the log reduction of *L. innocua* evaluated by shoulder, k_{max} and tail parameters of the Gompertz-based model (equation 1, section 2.3.). The levels assumed for the variables were: (i) 52.5 and 65.0 °C for temperature, (ii) 4.5 and 7.5 for pH, (iii) 0.95 and 0.99 for water activity and (iv) NaCl and glycerol for the type of solute used (totalling 16 cases). Three replicates of all the combinations

were performed.

2.2 Experimental procedures

Cultures

Listeria 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 20h to yield stationary phase cultures. This cell growth phase was chosen due to its higher stress resistance than exponential phase cells (Miller, Gil, Brandao, Teixeira, & Silva, 2009). Cells in each cellular suspension were enumerated by plating appropriate dilutions, in duplicate, on the three solid media studied. The initial *Listeria* concentration was then calculated for each cellular suspension.

Log reduction experiments in broth

TSBYE was used as the basal medium for all experiments. Combinations of temperature (52.5 and 65.0 °C), pH (4.5 and 7.5), a_w (0.95 and 0.99) and type of solute (NaCl and glycerol - Merck) were studied according to the experimental design. The pH was always adjusted with lactic acid (0.5M) 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 medium pH or a_w.
Heat treatments were carried out in a thermostatized water bath. A covered Erlenmeyer flask containing ninety-nine milliliters of stirred TSBYE, adjusted to the specified pH and a_w, was immersed in the water bath. Once the heating medium temperature had attained stability (measured with a thermometer), it was inoculated with 1 mL of cell suspension. Samples

were removed from the stirred TSBYE at different time intervals and immediately placed in a mixture of ice-water.
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 and (iii) Palcam Agar plus selective supplement (Miller, Brandao, Teixeira, & Silva, 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.

2.3 Modelling Procedures

The inactivation model

Assuming that the microbial thermal inactivation follows a sigmoidal behaviour, experimental data can be mathematically described by a Gompertz-based model (Linton, Carter, Pierson, & Hackney, 1995; Gil et al., 2006):

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

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 versatility of fitting linear data and those that contain shoulder and/or tailing effects makes Gompertz an attractive model (Gil, Miller, Brandao, & Silva, 2011).

Data analysis

The parameters of the Gompertz inactivation model, i.e. L, k_{max} and $log(N_{res}/N_0)$, were estimated by non-linear regression analysis, fitting equation 1 to experimental data.
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 standardised half width (SHW) at 95%, i.e. halved

confidence interval divided by the estimate = (confidence interval$_{95\%}$/2) x (1/estimate) x 100. Results from the 2^4 factorial experimental design were analysed by ANOVA procedures.

The heat resistance of microorganisms has been traditionally expressed in terms of D-values (decimal reduction time, or time required to inactivate 90% of the population). These values are calculated assuming that microbial inactivation follows first order kinetics and can be calculated using data of the maximum inactivation rate period, where the D-value is the reciprocal of k_{max}.

$$D = \frac{1}{k_{max}} \qquad (2)$$

Statistica$^{©}$ 6.0 (StatSoft, USA) and Microsoft$^{®}$ Excel 2000 (Microsoft Corporation, USA) were used for all calculations and statistical analysis.

3 Results and Discussion

3.1 Thermal inactivation behaviour of *L. innocua*

This study aimed at determining the influence and interactions of temperature, pH, a$_w$ and type of solute (used to change a$_w$) on the inactivation of *L. innocua* 10528. Although some works have proven the significant influence of temperature, pH and a$_w$ on microbial inactivation (Blackburn, Curtis, Humpheson, Billon, & McClure, 1997; Juneja & Eblen, 1999; Jagannath, Nakamura, & Tsuchido, 2003), studies of their effects on the features of non-linear inactivation kinetics (this meaning initial shoulders, maximum inactivation rate and tail) are scarce. Therefore, the effect of these factors on kinetic parameters was now studied using the conditions defined according to the 2^4 experimental design. The Gompertz-based model was fitted to experimental inactivation data, and kinetic parameters were estimated for all situations considered. These results are included in Tables 1 and 2, respectively for a$_w$=0.95 and 0.99. The kinetic behaviour of *L. innocua* for all conditions tested can be visualized in Fig. 1. The initial concentration of *L. innocua* was determined to be approximately 10^7 cfu mL^{-1} for all conditions tested. The adequacy of model fits was assessed by residual analysis

(i.e. residuals were random, and normally distributed with mean equal to zero and constant variance). Values of the coefficient of determination, R^2, were in all cases above 0.92, revealing model adequacy in data description.

In relation to the precision of the kinetic parameters, shoulder was estimated with the lowest precision (assessed by the standardized half width at 95%). The experimental design used may explain this lack of precision, since less experimental points were gathered in the initial inactivation period. This was evident in the cases where higher temperatures and lower pH were imposed (the shoulder does not occur).

Results of the experimental design analysis showed that, with the exception of the type of solute, all factors significantly affected the maximum inactivation rate (at a significance level of 5%). Although the major effect was due to temperature, the following main effects and combinations had also a significant influence on k$_{max}$: pH, temperature/pH, a$_w$, a$_w$/pH, a$_w$/temperature, a$_w$/pH/temperature.

For shoulder parameter, the temperature, pH and the combination temperature/pH were the factors that significantly affected this kinetic parameter (at a significance level of 13%). The occurrence of a shoulder can be attributed to differences in cell's resistance. While some treated cells may be totally injured, avoiding their culturability/survival, others might have sublethal damage that makes them more sensitive to the subsequent treatment exposure. In cases where *L. innocua* was treated at high temperature and subjected to lactic acid, the shoulder became almost inexistent, which means the instantly unviability of the majority of *Listeria* cells. The combination of these two factors was very effective in microbial inactivation, which is indicative of their synergistic effect. This behaviour has also been observed in other studies (Juneja & Eblen, 1999; Lee & Kang, 2009). Although it has been reported that organic acids' effectiveness increases with temperature increase, this fact can be affected by other factors such as the type of acidifier and microorganism.

In relation to tail parameter, a$_w$ was the most significant factor, followed by temperature and the combined effect of a$_w$/pH (at a significance level of 30%). The tail suggests an apparently

increased resistance of *L. innocua* in the later stages of the treatment exposure. As pointed out by several researchers (Booth, 1998; Rowan, 1999), these survivors are likely to be physiologically protected from the stress exposure. In this case, the lower the a_w, the higher the thermoresistance of *Listeria* cells.

Temperature had a positive effect on k_{max} and tail parameter, which means higher inactivation rates are observed and the tail tendency becomes evident when temperature rises. Concerning the shoulder, the temperature effect is negative. The shoulder period becomes less visible for higher temperatures. These effects revealed that, although temperature is very lethal in microbial reduction, some thermoresistance is obtained in the later stages of the heat treatment.

The pH affected k_{max} and shoulder, but not the tail parameter. Lowering pH value implies higher inactivation rates and narrow shoulders, which indicates that acidic pH is very effective on *Listeria* inactivation. Water activity positively influenced k_{max} and tail, which means lower inactivation rates are observed and the tail tends to disappear when a_w decreases. In this case, some microbial stress adaptative response is occurring, which increases the time of the bacterium survival. However, once energy consumption is required to activate microbial repair mechanisms, *L. innocua* cells may become metabolically exhausted and die sometime after.

By observing the results shown in Table 1 and Fig. 1, it is possible to conclude that shoulder times were higher when glycerol was used (instead of NaCl) and for lower temperatures, higher pH values and rich media (i.e. TSAYE). For lower temperatures, lower pH values and when glycerol was used, higher shoulders and lower inactivation rates were observed. Consequently, the overall heat resistance was higher when compared to NaCl results. The influence of glycerol on the initial shoulder can be explained by the immediate intracellular access of glycerol as a result of direct passage through the cell membrane. The non-permeability of the cell membrane to NaCl may lead to the differences observed when both solutes are used.

3.2 Physicochemical stresses

As already said, and when dealing with an analysis of variance, the temperature (followed by pH and a_w) had the major significant impact in the inactivation behaviour. These great effects made the influence of the solute type negligible. However, if the temperature and pH effects were not considered, *L. innocua* inactivation may be influenced by the solute used to change a_w. This can be better understood with the results presented in Fig. 2, where D-values are represented for a_w of 0.95 (with NaCl and glycerol used as solutes) and a_w of 0.99 (without solute addition). For pH media of 7.5, *L. innocua* was more heat resistant when NaCl was used to change a_w. This was observed for the lower and higher temperatures tested. However, for media pH of 4.5 and for the lower temperature, an inverted tendency was observed. In this case, *L. innocua* became more heat resistant if glycerol had been used.

Several studies demonstrated that the water activity effect depends on the treatment temperature. Coroller et al. (2001) and Chiewchan, Pakdee, and Devahastin (2007) concluded that a_w effect was more pronounced at higher heating temperatures. Mattick et al. (2001), when studying the heat tolerance of *Salmonella* spp., demonstrated that for temperatures equal or higher than 70 ˚C the bacterial cells were more heat tolerant at low a_w than those at higher a_w. At temperatures below 65 ˚C the reverse was observed, which is in agreement with our results. The high-temperature target(s) appears to be protected by low a_w, perhaps through improved stability of proteins, reduced mobility of water, or the direct effects of solutes, whereas the lower-temperature target(s) is clearly not protected by low a_w. In this work, the combination of high temperature with low a_w, also increased the survival of *L. innocua* (antagonistic effect) in the majority of the situations tested. However, this was not the case for conditions (i) T=52.5 ˚C and pH=4.5 using NaCl and (ii) T=65.0 ˚C and pH=7.5 using glycerol, where *L. innocua* cells were more susceptible to heat (Fig. 1). These different results are probably due to the combination of several stressful conditions. A synergistic effect can be achieved when different stresses reach different targets within the microbial cells (e.g., cell
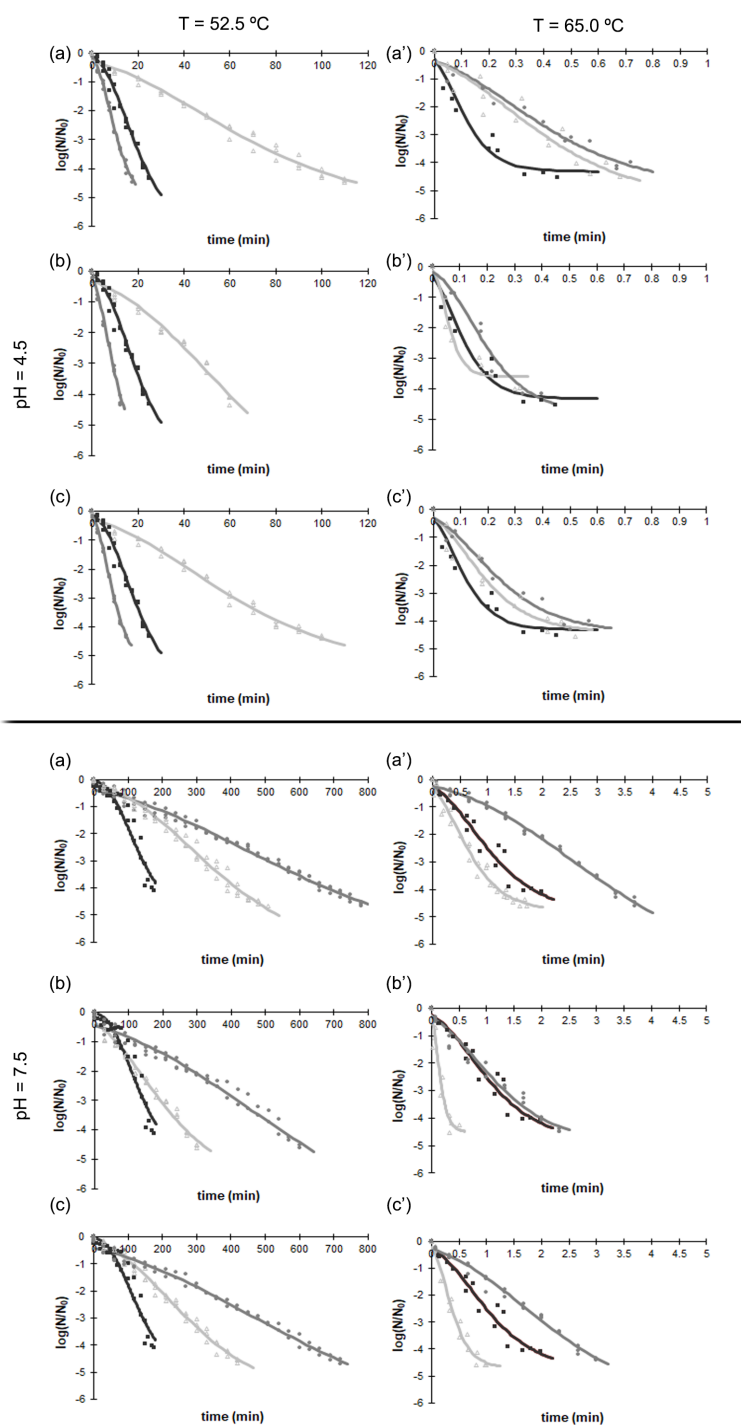
T = 52.5 ºC   T = 65.0 ºC

Figure 1: Thermal inactivation data of *L. innocua* at all conditions of pH and temperature, using the three recovery media: (a) and (a') TSAYE; (b) and (b') TSAYE + 5%NaCl; (c) and (c') Palcam Agar. Each plot has a survival curve for a_w of 0.99 (■) and 0.95, this last one adjusted with NaCl (●) or glycerol (△). The lines represent model fits (Eq. 1).

Table 1: Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 at a_w 0.95, for all conditions tested.

Solute	pH	T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
				L (min)	SHW$_{95\%}$	k$_{max}$ (min^{-1})	SHW$_{95\%}$	Log(N$_{res}$/N$_0$)	SHW$_{95\%}$
NaCl	4.5	52.5	TSAYE	1.61	28.0	0.32	6.8	-5.3	8.6
			TSAYE + 5% NaCl	0.94	62.9	0.37	8.5	-6.1	24.9
			Palcam Agar	1.34	23.9	0.36	5.4	-5.4	7.6
		65.0	TSAYE	0.00	*	6.74	12.1	-5.0	23.9
			TSAYE + 5% NaCl	0.03	165.4	15.30	33.3	-4.8	28.5
			Palcam Agar	0.00	2540.7	10.54	27.8	-4.4	17.6
	7.5	52.5	TSAYE	32.29	52.5	0.01	3.7	-6.9	13.7
			TSAYE + 5% NaCl	41.22	102.9	0.01	9.5	-9.1	35.2
			Palcam Agar	33.04	59.4	0.01	4.0	-7.7	17.3
		65.0	TSAYE	0.48	24.7	1.43	4.9	-7.7	19.7
			TSAYE + 5% NaCl	0.00	*	2.31	9.2	-5.0	15.0
			Palcam Agar	0.20	49.9	1.65	6.3	-6.3	18.4
Glycerol	4.5	52.5	TSAYE	3.69	94.6	0.05	7.7	-5.5	11.6
			TSAYE + 5% NaCl	8.82	98.5	0.08	20.5	-9.1	63.3
			Palcam Agar	4.10	76.9	0.05	7.6	-5.5	11.7
		65.0	TSAYE	0.00	*	7.61	16.5	-5.4	37.6
			TSAYE + 5% NaCl	0.01	362.4	37.45	80.5	-3.6	13.0
			Palcam Agar	0.00	8046.8	13.30	23.9	-4.4	17.0
	7.5	52.5	TSAYE	66.65	18.3	0.01	5.3	-6.6	14.4
			TSAYE + 5% NaCl	5.54	224.7	0.02	8.5	-6.0	19.6
			Palcam Agar	45.54	22.7	0.01	5.7	-6.1	12.1
		65.0	TSAYE	0.00	*	3.98	9.4	-4.8	7.3
			TSAYE + 5% NaCl	0.00	1609.1	16.45	48.0	-4.5	22.1
			Palcam Agar	0.03	157.9	7.49	18.7	-4.7	10.0

TSAYE: Tryptic Soy Agar supplemented with 0.6% yeast extract.
* Considerable high meaningless value.

Table 2: Estimated shoulder, maximum inactivation rate and tail parameters of *L. innocua* 10528 at a_w 0.99, for all conditions tested.

pH	T (°C)	Media	Shoulder		Maximum inactivation rate		Tail	
			L (min)	SHW$_{95\%}$	k$_{max}$ (min^{-1})	SHW$_{95\%}$	Log(N$_{res}$/N$_0$)	SHW $_{95\%}$
4.5	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
	65.0	TSAYE	0.00	9877.6	21.19	29.4	-4.3	13.4
		TSAYE + 5% NaCl	0.00	4490.3	500.93	4424.4	-4.5	9.3
		Palcam Agar	0.00	2087.9	50.25	45.4	-4.2	12.5
7.5	52.5	TSAYE	40.00	32.9	0.03	16.4	-5.2	48.3
		TSAYE + 5% NaCl	0.00	*	0.09	39.9	-3.4	21.9
		Palcam Agar	20.00	111.1	0.04	35.5	-8.2	79.9
	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

TSAYE: Tryptic Soy Agar supplemented with 0.6% yeast extract.
* Considerable high meaningless value.

membrane, DNA, enzyme systems). This situation will require extensive energy-consumption to maintain internal homeostasis which may cause the microorganisms to become metabolically exhausted (Leistner, 2000). Stress reactions due to a particular stress factor might also lead to the microorganism becoming more tolerant to other stressful conditions. This phenomenon is called "cross-tolerance" (Leistner, 2000).

Studies on the combined effects of organic acids and solutes on pathogens survival are scarce. Casey and Condon (2002) showed that NaCl reduced the inhibitory effect of lactic acid on *E. coli* by raising its cytoplasmic pH (pH_i). In contrast with these findings, Fang, Siegumfeldt, Budde, and Jakobsen (2004) and Lebert, Dussap, and Lebert (2004) observed a significant decrease in pH_i following exposure to high concentrations of NaCl. However, when glycerol was added to the medium to decrease a_w, they observed that pH_i remained constant. As recognized, weak acids in their unprotonated form can diffuse into the cell and dissociate, thereby lowering the intracellular pH. In the presence of glycerol, the free H^+ resulted from the acid dissociation will be buffered, which consequently promotes the stability of pH_i. In the presence of NaCl, the concentration of free H^+ will rise, due to the lack of buffering capacity of the salt, and therefore the pH_i decreases.

This fact, together with the capacity of glycerol to diffuse freely across the inner membrane, can explain our results at pH 4.5, where the glycerol had less inhibitory effect than NaCl at 52.5 ˚C. At 65.0 ˚C, the temperature and a_w effects are more notable as is the similarity of the results for the two solutes.

At neutral pH, opposite results were observed. When NaCl was used as the solute, *L. innocua* was more heat resistant. Mazas et al. (1999) and Stewart et al. (2002) arrived at similar conclusions. *Listeria* spp. is recognized to tolerate high concentrations of NaCl and this adaptability appears to be related to the capacity of the bacteria to accumulate intracellular solutes. The work of Patchett, Kelly, and Kroll (1992) showed that *L. monocytogenes* accumulates K^+, betaine and glutamate to provide compensation for the osmotic stress caused by increased salinity. These findings suggested that not only osmotic stress but also physicochemical properties of the solutes used to control a_w, as well as the biological effects of the solutes on the microorganism, lead to variation of cell behaviour. In fact, the protective influence of a_w decrease could be balanced by a specific antagonistic and toxic effect of glycerol, at neutral pH.

3.3 Effect of plating media

The heat resistance of microorganisms is frequently evaluated in terms of D-value. Such an approach assumes linearity between the logarithmic of the number of viable microorganisms with time (this meaning a linear kinetics). Deviations from linearity can be observed in the results presented in Fig. 1, for the majority of the conditions tested. For all studied conditions, k_{max} values were converted to D-values (Eq. 2). D-values calculated using data obtained in non-selective media (TSAYE) and in selective media (TSAYE+5%NaCl and Palcam Agar) are shown in Fig. 2.

In all cases, D-values using TSAYE were the highest as this medium supports the growth of most cells, including those that can suffer any kind of injury. D-values for selective media are likely to be lower as the growth of injured cells is prevented. In the case of TSAYE+NaCl, a lower survival of *Listeria* means that the osmoregulatory functions of the cytoplasmic membrane has been damaged. Those cells, where some enzymes may be inactivated by the replacement of their cations by Li^+, will be sensitive to LiCl in Palcam Agar. For the conditions of Fig. 2a and 2c (lower temperature), it is possible to note that D-values obtained using TSAYE+NaCl were lower than those determined using TSAYE for a_w of 0.99, but the ones from Palcam Agar are similar to those obtained in non-selective medium. This indicates that under high a_w heating conditions, a great number of bacteria survivors had cytoplasmic membrane damage and that for high and low a_w cell injury in enzymes functionality is not relevant. For the situation presented in Fig. 2d (higher temperature and higher pH), D-values determined using both selective media were lower than those obtained using TSAYE for a_w of 0.99 and when glycerol was utilized
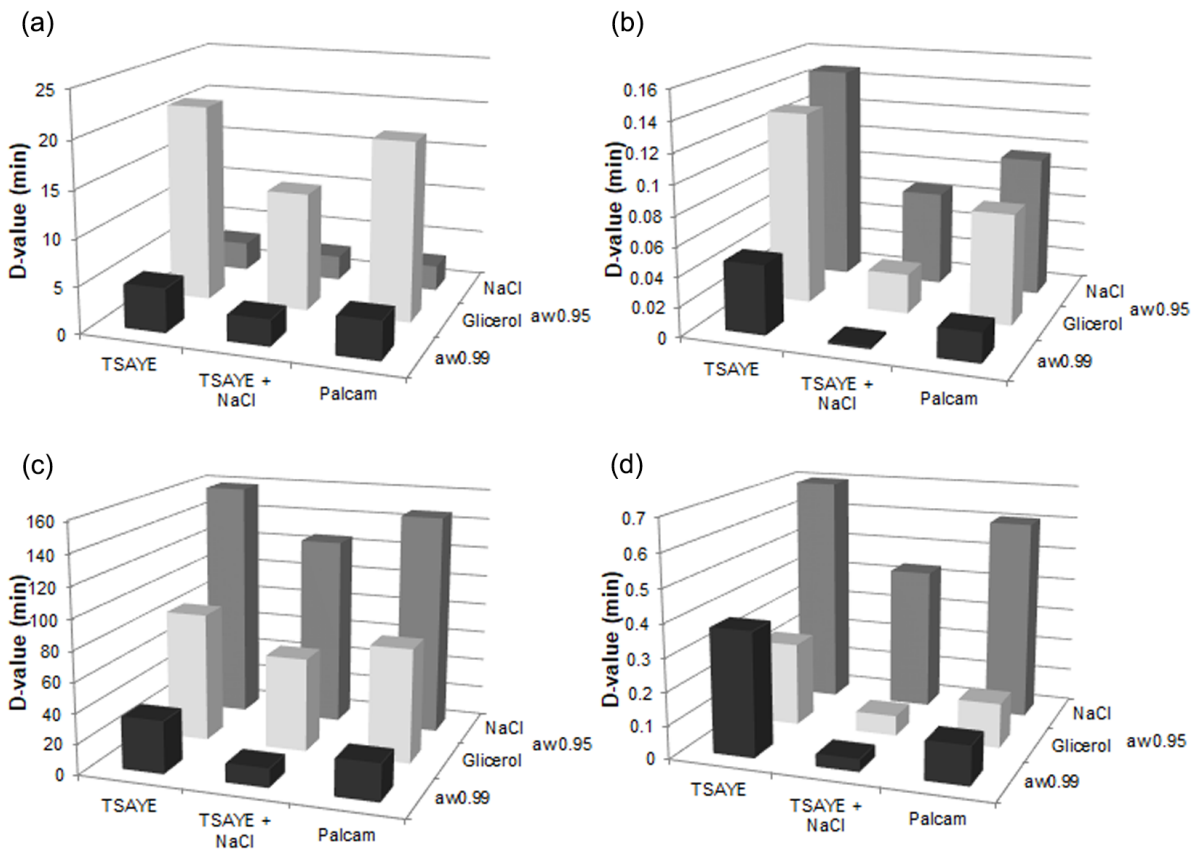
Figure 2: D-values of *L. innocua* treated at (a) pH=4.5 and T=52.5 ˚C, (b) pH=4.5 and T=65.0 ˚C, (c) pH=7.5 and T=52.5 ˚C and (d) pH=7.5 and T=65.0 ˚C, at a_w of 0.99 and 0.95, this last one adjusted with NaCl or glycerol, for three different recovery media.

to decrease a_w to 0.95. This implies that under these conditions many survivors may have cytoplasmic membrane damage and enzymes inactivation. For the most severe conditions tested (Fig. 2b; higher temperature and lower pH) no distinct differences were observed for D-values calculated for the three media used. This is indicative that survivors did not suffer any form of injury that can be repaired. All these conclusions were validated by the D-values calculated (lacking associated uncertainty), and the k_{max} estimates and related precision. This allowed significance of the highlighted conclusions. From these results it is possible to conclude that lower tem-

peratures potentiate the inactivation of *Listeria* cells with damaged cytoplasmic membranes. It is also important to note that at 52.5 ˚C and at high a_w, cells with damaged cytoplasmic membranes survive and can repair. However, when they are heated in a medium with low a_w, the additional osmotic stress caused by any solute may result in cell death rather than sublethal injury. As referred by Gabriel (2013) aside from some protection conferred by solutes against heat injury, the decrease in a_w also brings forth physical damage to cellular membranes and provides reduction of intracellular moisture which can negatively affect the function of cellular enzymes. Exposures to

acidic environments are also known to induce disruption of biochemical processes within the cells such as enzymes functions and nutrient transport (Beales, 2004). At higher temperatures, the study of Aljarallah and Adams (2007) showed that ribosomal inactivation was a primary target of cell damage.

4 Conclusions

To achieve accurate results concerning the thermal inactivation of microorganisms, it is crucial to study the influence of relevant factors. Although the results from the present work pointed out that temperature was the main significant factor that influences *L. innocua* survival, pH and a_w (and their combined effects) had also a significant impact. If temperature and pH effects were not considered, *L. innocua* inactivation may also be affected by the solute used to change a_w. For media pH of 7.5, *L. innocua* is more heat resistant when NaCl is used to change a_w. This is observed for the lower and higher temperatures tested. However, for media pH of 4.5 and for the lower temperature, an inverted tendency was observed. In this case, *L. innocua* became more heat resistant if glycerol is used. These results suggest that some physicochemical properties of the solutes used to control a_w may influence the microbial thermal resistance. A further comparison of results, where the a_w effect is considered alone, may lead to restricted conclusions.

Care must be taken with the enumeration media chosen, since selective media may be inadequate for the recovery of injured *Listeria* cells. Comparing results obtained in selective media with the ones obtained in non-selective media, it can be concluded that at lower temperature, *Listeria* inactivation occurred by damage in the cytoplasmic membrane, which can be repaired at high a_w (0.99), but not at low a_w (0.95).

Every attempt should be made to control relevant important processing conditions that affect microbial survival. Special attention must be given to the possible interactions between these factors, as synergistic or antagonistic effects may happen.

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