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Survival of *Salmonella senftenberg* 775 W to current liquid whole egg pasteurization treatments

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Abstract

The effect of heat shocks at constant temperature (isothermal heat shocks) or at constantly raising temperatures (anisothermal heat shocks) on the thermotolerance in liquid whole egg of the heat-resistant strain *Salmonella senftenberg* 775 W was investigated. The thermotolerance of *Salmonella senftenberg* 775 W increased with isothermal shock temperature. The $D_{63^\circ C}$ in liquid whole egg raised from 1.2 to 3.1 min after 1 h of isothermal heat shock at 54°C. Anisothermal heating lag phases also increased the thermotolerance of *S. senftenberg* 775 W in liquid whole egg to a higher extent, the higher the final temperature reached. Heating rate did not affect the heat resistance of *S. senftenberg* along the isothermal phase. Industrial pasteurization treatment for liquid whole egg at 60°C for 3.5 min, 64°C for 2.5 min and 70°C for 1.5 min would only attain <1, <2 and <4 log cycles reduction, respectively, in the population of this heat-resistant *Salmonella* strain.

Keywords: Pasteurization treatments; *Salmonella senftenberg*; Heat shocks

1. Introduction

During the last three decades the number of food poisoning outbreaks in which different *Salmonella* serotypes have been involved has increased steadily (Tauxe, 1991; Todd, 1996; Wallace et al., 2000). Eggs and egg products have been the most frequently involved foods.

According to heat resistance data in literature, current liquid whole egg heat pasteurization treatment at 60°C for 3.5 min (USA) or at 64°C for 2.5 min (UK) should provide, in liquid whole egg, 5–9 log cycles reduction in the number of the most frequent *Salmonella* serotypes: *S. typhimurium* and *S. enteritidis* (D'Aoust et al., 1987; Humphrey et al., 1990; Shah et al., 1991; Doyle and Mazzota, 2000). However, also other very high heat-resistant serotypes, such as *S. senftenberg* 775 W, have been isolated from foods (Anellis et al., 1955; Davidson et al., 1966; Baird-Parker et al., 1970; Bersani and Cantoni, 1983). Traditional pasteurization treatments would not provide more than 1–4 log cycles reduction in the population of *S. senftenberg* 775 W, as deduced from published heat resistance data (Osborne et al., 1954; Anellis et al., 1955; Goepfert and Biggie, 1968; Ng et al., 1969; Humphrey et al., 1990; Kornacki and Marth, 1993; Orta-Ramirez et al., 1997). Therefore, a lower degree of safety for pasteurized liquid egg contaminated with *S. senftenberg* 775 W would be expected.

In 1987, Ball et al. (1987) designed a new high-intensity ultrapasteurization treatment at 70°C (1.5 min) for liquid whole egg. These authors observed that the ultrapasteurization treatment allowed to obtain liquid whole egg with high functional and microbiological qualities. This treatment should provide in liquid whole egg more than 12 log cycles reduction in the population of salmonellae. However, the real lethal effects of the ultrapasteurization treatments on *S. senftenberg* 775 W have not yet been evaluated.

On the other hand, the high thermal sensitivity of liquid egg components limits the temperature at which the product can be heated. Some soluble proteins began to precipitate at temperatures as low as 57°C, and at 73°C liquid egg coagulates (Hamid-Samimi et al., 1984; Hamid-Samimi and Swartzel, 1984; Herald and Smith, 1989). This fact limits the maximum temperature of the heating fluid that may be used in the pasteurizers

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(Alkskog, 1993). Furthermore, liquid egg has a high viscosity, and therefore, the pasteurization treatments of liquid whole egg have much longer anisothermal heating lag phases as compared with those of other liquid foods (Alkskog, 1993).

Any temperature above the optimum for microbial growth is supposed to exert some lethal effect. However, it has been shown that in most microbial species slow heating, or heating for short periods of time at temperatures above the optimum for growth (heat shocks) may induce a higher heat resistance (Yamamori, 1982; Mackey and Derrick, 1986; Humphrey et al., 1993; Shenoy and Murano, 1996; Xavier and Ingham, 1997). It has been demonstrated that anisothermal heating can also increase bacterial heat resistance (Thompson et al., 1979; Tsuchido et al., 1982; Mackey and Derrick, 1987a, b), and that death and development of thermotolerance may happen simultaneously in heated cell populations (Pagán et al., 1997). In food products requiring long heating lag phases, such as egg products, bacterial pathogens might respond to heat shock and increase their thermotolerance, and as a consequence, they might survive the heat treatment (Thompson et al., 1979; Mackey and Derrick, 1987a, b; Pagán et al., 1997). The effect of heat shocks on the thermotolerance of *S. senftenberg* 775 W has not previously been investigated.

The objective of this work was to investigate the effect of pasteurization treatments with different heating lag phases on the survival of *S. senftenberg* 775 W in liquid whole egg. The effect of isothermal heat shocks on the heat resistance of *S. senftenberg* 775 W was also studied as a reference.

2. Materials and methods

2.1. Bacterial culture and media

S. senftenberg 775 W (ATCC 43845) was supplied by the Spanish-Type Culture Collection. During this investigation it was maintained on slants of Nutrient Agar (NA) (Biolife, Milano, Italy) at 2–4°C.

A broth subculture was prepared by inoculating, with one single colony from a plate with NA, a test tube containing 5 ml of sterile Nutrient Broth (NB) (Biolife). After inoculation, this tube was incubated overnight at 37°C. With this subculture, 250 ml Erlenmeyer flasks containing 50 ml of sterile NB were inoculated to an initial concentration of 10^6 cells/ml. These flasks were incubated under agitation (130 rpm; Selecta; Rotabit; Spain) at 37°C. Once the cultures had attained the stationary growth phase and maximum heat resistance (after 29 h of incubation, data not shown), the flasks were stored at 4°C until use. Storage did not affect the heat resistance of *S. senftenberg* throughout the time this investigation was carried out (data not shown). Many environmental factors can affect bacterial heat resistance, some of them still not well known. The storage of the bacterial suspension avoids the use of different cultures and misinterpretations when all data are compared.

2.2. Heat resistance determinations

McIlvaine citrate phosphate buffer of pH 7.7 and pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain) were used as heating media.

Heat treatments were carried out in a specially designed resistometer as already described (Raso et al., 1998). Once temperature had attained stability, 0.2 ml of an adequately diluted cell suspension, native or heat shocked, were injected into the 23-ml treatment chamber containing the heating medium. After inoculation, 0.2-ml samples were collected at different heating times and immediately pour plated using NA as recovery medium. No statistically significant differences ($P<0.01$) were detected (data not shown) among survival curves obtained in NA and in this media with 50 ml/l of sterile liquid whole egg added. For each heat resistance determination, 8–15 samples were taken at different heating times. Plates were incubated for 24 h at 37°C. Previous experiments showed that longer incubation times did not influence survivor counts. After incubation, colony forming units (cfu) were counted with an improved Image Analyzer Automatic Counter (Protos, Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Condón et al., 1996).

2.3. Heat shocks

Isothermic heat shocks were carried out by inoculating 0.2 ml of the bacterial suspension in test tubes containing 2 ml of liquid whole egg, that had been previously stabilized in a water-bath at the desired temperature (48°C, 52°C or 54°C). At preset time intervals, samples were taken for the heat resistance determinations. Anisothermic heat shocks were carried out in the resistometer, using liquid whole egg as the treatment medium. Once the initial temperature was attained, 0.2 ml of the bacterial suspension was injected inside the treatment chamber. The heating rate (0.4–6.6°C/min), from 54°C to the final treatment temperature (60°C, 64°C or 70°C), was set with electrical pulses of different length as it has been previously described (Condón et al., 1989). At least 10 samples were taken along the come-up time and the isothermic treatment, and the number of surviving micro-organisms was estimated as described above.

2.4. Heat resistance parameters

Survival curves were drawn by plotting the logarithm of the number of surviving micro-organisms vs heating time and D_t values (time in minutes for a 10 fold reduction in survival counts) were calculated. The decimal reduction times curves (DRTC) were represented by plotting the log of the D_t values vs treatment temperature, and z values (°C increase necessary to reduce D_t value a log cycle) were calculated. The 95% confidence limits (CL) were calculated with the appropriate statistical package (Statview 512;D. Feldman and J. Gagnon, BrainPower Inc., Calabasas, California, USA). The statistical significance of differences ($P<0.01$) between D_t and z values was tested as described by Steel and Torrie (1960). The theoretical survival curves corresponding to anisothermic treatments were calculated by integrating the lethal effect of the different temperatures for each treatment time with the Excel 5.0 package (Microsoft, Seattle, Washington, USA). For the integration D values interpolated from the corresponding DRTC, obtained under isothermic treatment conditions, were used.

3. Results

Isothermal survival curves obtained in this investigation followed first-order inactivation kinetics. No high heat-resistant subpopulations were detected among 99.9% of the bacterial cells.

Fig. 1 shows the DRTCs of *S. senftenberg* 775 W heated in liquid whole egg and in citrate phosphate McIlvaine buffer of the same pH (7.7). Our strain showed a decimal reduction time value at 63°C in pH 7.7 buffer of 0.20 min. The heat resistance in liquid whole egg was approximately 10 fold higher than in buffer, and the thermal protective effect was the same at all temperatures tested. No statistically significant differences ($P<0.01$) were found between z values obtained in buffer and in liquid egg ($z = 5.2$°C). The heat resistance observed was within the range of most published data for this strain (Doyle and Mazzota, 2000).

Fig. 2 shows the relationship between the shock temperature for 1 h in liquid whole egg and the decimal reduction time of our micro-organisms at 63°C in the same medium. As shown by the figure, the $D_{63°C}$ value increased with the shock temperature. The $D_{63°C}$ in

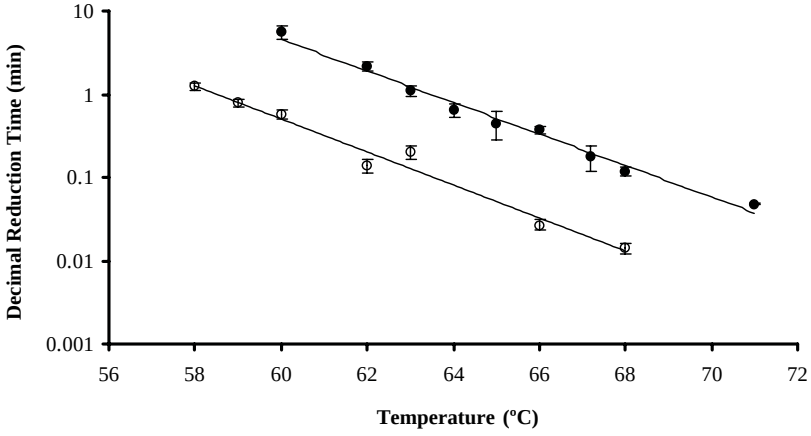

Fig. 1. Decimal reduction time curves of *S. senftenberg* 775 W in liquid whole egg (●) and in pH 7.7 citrate phosphate buffer (○).

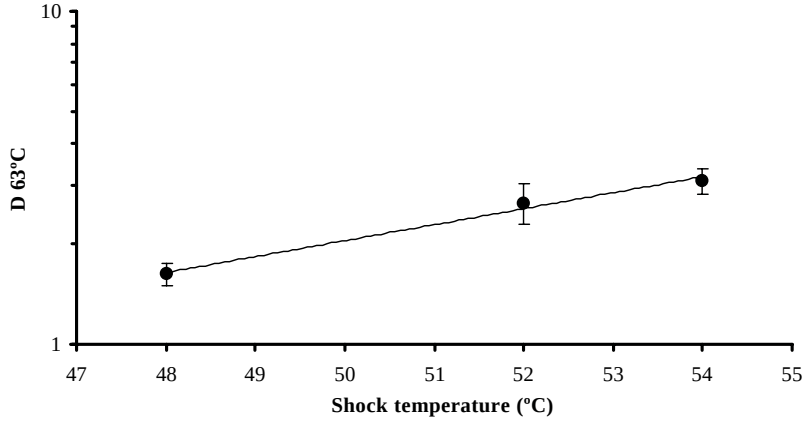

Fig. 2. Heat resistance at 63°C in liquid whole egg of *S. senftenberg* 775 W cells heat shocked for 1 h at different temperatures.

liquid whole egg raised from 1.2 to 3.1 min after 1 h of isothermal heat shock at 54°C

Fig. 3A–C shows the inactivation rate in liquid whole egg of *S. senftenberg* 775 W during a 5 min anisothermal heating lag phase and a subsequent isothermal treatment at 60°C, 64°C or 70°C, respectively. The evolution of the temperature and the theoretical inactivation curve calculated from data in Fig. 1 are also included as reference. The higher the temperature, the greater the differences between the experimental and theoretical values in the isothermal phase.

Fig. 4 shows the survival curves at 64°C in liquid whole egg of *S. senftenberg* after different anisothermal heating up rates: 0.55°C/min, 2°C/min and 4°C/min. No statistically significant ($P<0.01$) differences could be detected among the slopes of the three survival curves. Therefore, it could be deduced that the heating rate did not significantly modify, in the range explored, the heat resistance at 64°C.

Fig. 5 shows the number of survivors of *S. senftenberg* to industrial liquid egg pasteurization treatments at 60°C for 3.5 min, 64°C for 2.5 min and 70°C for 1.5 min. Data in the figure are the mean of at least three counts with different heating rates that had a negligible influence on the lethal effect. The inactivating effect of the pasteurization treatments

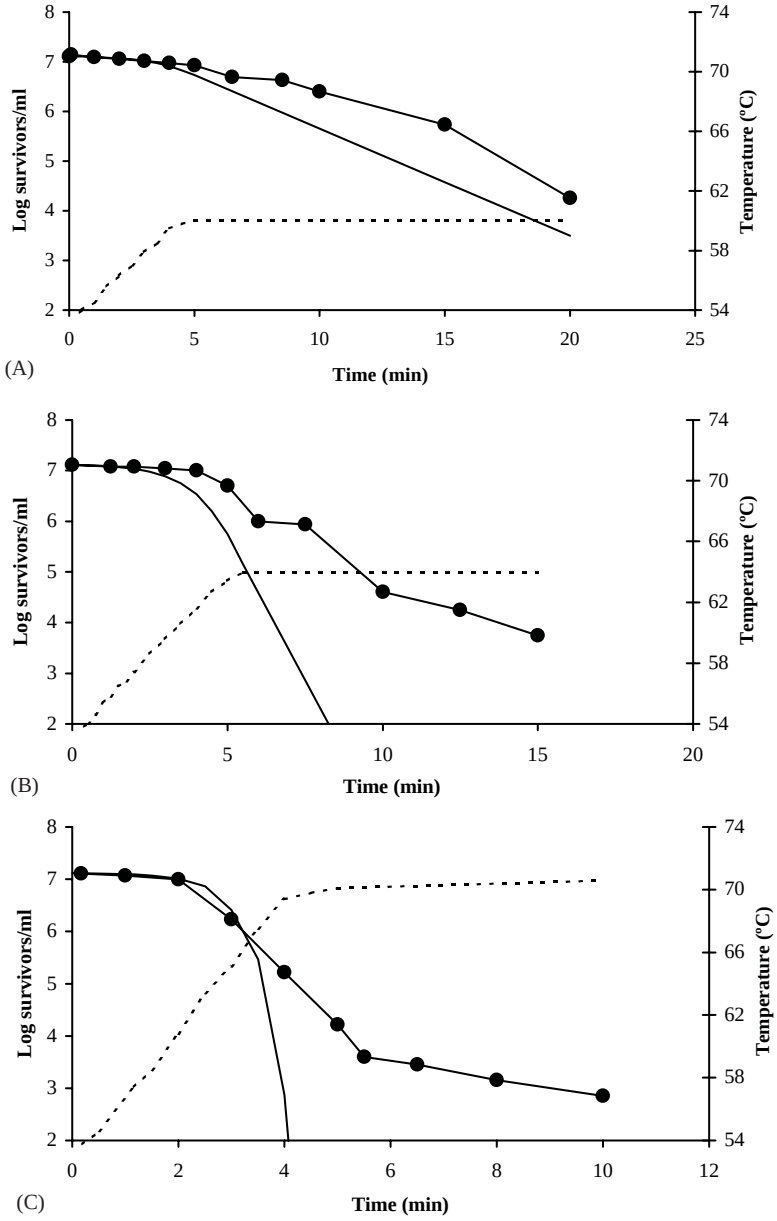

Fig. 3. Inactivation rate of *S. senftenberg* 775 W along industrial liquid whole egg pasteurization treatments at 60°C (A), 64°C (B) and 70°C (C). Initial temperature 54°C. Anisothermal heating-up phase for 5 min (•) Experimental data; (—) theoretical inactivation rate calculated from D_t and z values obtained under isothermal heat treatments; (…) time–temperature curve of the liquid egg.

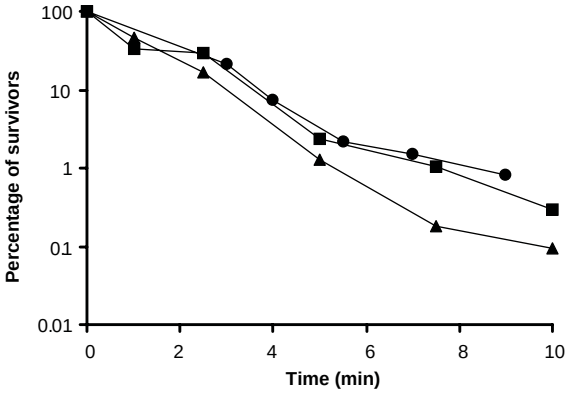

Fig. 4. Survival curves at 64°C in liquid whole egg of *S. senftenberg* 775 W after different anisothermal heating-up rates: (●) 0.55°C/min, (■) 2°C/min and (▲) 4°C/min.

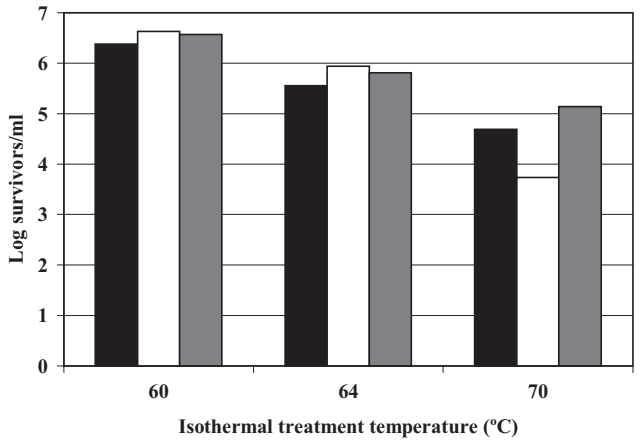

Fig. 5. Number of *S. senftenberg* 775 W survivors in liquid whole egg after pasteurization treatments of 60°C for 3.5 min, 64°C for 2.5 min and 70°C for 1.5. Initial number: 10^7 cfu/ml. Each column represents the mean of at least three counts with anisothermal heating-up phases for (■) 2 min, (□) 5 min and (▨) 16 min.

increased with the temperature of the isothermal phase.

4. Discussion

One of the biggest problems in the design of a pasteurization process for any food product is the important difference, sometimes reported, between the heat resistance data obtained in laboratory media, e.g. buffers, and in real foods. The heat resistance of our strain of *S. senftenberg* in citrate phosphate buffer of the same pH of the liquid whole egg (pH 7.7) was much lower than that observed in the food (Fig. 1). From the decimal reduction time values at 60°C, 64°C and 70°C estimated in buffer, it would be deduced that current minimum egg pasteurization treatments in USA and UK, and the ultrapasteurization treatments would provide 7, 34 and 319 log cycle reduction, respectively, on the population of *S. senftenberg* 775 W. Apparently, even the less intense treatment (60°C for 3.5 min.) would provide a reasonable safety margin with respect to this micro-organism. However, as shown by Fig. 1, liquid whole egg strongly protected the micro-organism against heat inactivation ($D_{60}=5.5$; $D_{64}=0.65$, $D_{70}=0.082$), and consequently, the same pasteurization treatments would provide 0.6, 3.8 and more than 15 log cycle reduction on *S. senftenberg* 775 W populations. From these data it would be concluded that Public Health would only be guaranteed by ultrapasteurization treatments at 70°C, when liquid whole egg is contaminated with the high heat-resistant *S. senftenberg* 775 W.

4.1. Isothermal shocks

The capability of bacterial cells to develop higher heat resistance as a consequence of heat shocks at sublethal temperatures was reported for the first time in *Escherichia coli* (Tsuchido et al., 1982; Yamamori, 1982). Further studies have demonstrated the existence of this protective mechanism on most bacterial species. The effect of heat shocks on some *Salmonella* serotypes, such as *S. typhimurium*, *S. thompson* and *S enteritidis*, has already been published (Mackey and Derrick, 1986, 1987a, b, 1990; Bunning et al., 1990; Shah et al., 1991; Humphrey et al., 1993; Xavier and Ingham, 1997). But, to the best of our knowledge, the effect on the heat-resistant strain *S. senftenberg* 775 W has not previously been investigated.

The influence of 1 h shocks at different temperatures on *S. senftenberg* 775 W heat resistance is shown in Fig. 2. Temperatures over 54°C could not be used because of their high lethal effect on the bacterial population. Our results agree with those of other authors (Mackey and Derrick, 1986; Shah et al., 1991; Pagán et al., 1997) who have observed that the developed heat resistance increased with the preadaptation temperature. Pagán et al. (1997) observed that the effect of heat shocks depended on the heating time and shock temperature. As shown by Fig. 2, the D_{63} of our strain in liquid whole egg increased with the preadaptation temperature between 48°C and 54°C. It has been demonstrated that some bacterial cells can produce 12–14 different types of heat shock proteins (HSPs) as a response to heat shocks (Jorgensen et al., 1996). If the thermotolerance increases were due to these HSPs, the differences in the final thermotolerance at each temperature could be due either to differences in the amount of HSPs synthesized or to differences in the HSPs synthesized at different temperatures. From our data (Fig. 2) an exponential relationship between heat resistance and heat-shock temperatures could be deduced, which could be in agreement with the first hypothesis.

Other authors have reported increases of D_t values in different heat-sensitive serotypes of salmonella after shocks at temperatures as low as 42°C (Shah et al., 1991). *S. senftenberg* 775 W is a very heat-resistant serotype and it seems logical that the minimum shock temperature might be related to the inherent microorganism heat resistance.

Overall, the D_{63} of *S. senftenberg* 775 W after a heat shock at 54°C for 1 h increased from 1.1 to 3.1 min. Pagán et al. (1997) demonstrated that the increase in thermotolerance due to a heat shock remained constant independent of the treatment temperature. The z value of the heat-shocked cells was the same as that of the native cells. Therefore, it could be estimated that USA and UK current ultrahigh liquid whole egg pasteurization treatments would not provide more than 0.25, 1.5 and 11 log cycles inactivation of a population of *S. senftenberg* 775 W that had been heat shocked under these conditions.

4.2. Anisothermal shocks

The existence of isothermal shocks in industrial practice is unusual. However, most food processes include anisothermal heating up phases that, in some cases, may act as heat shocks. Thompson et al. (1979) observed that the survival of several salmonella serotypes in meat heated at rising temperatures was higher than that calculated from theoretical D_t and z values. Also Tsuchido et al. (1982) demonstrated that several bacterial species anisothermally heated showed heat resistances up to ninefold higher than expected. Mackey and Derrick (1987a, b) observed the heat resistance of *S. typhimurium* increased with anisothermal shocks, although the magnitude of the protective effect did not attain that of isothermal shocks.

To investigate the effect of anisothermal heating lag phases on the survival of *S. senftenberg* 775 W, we simulated some industrial liquid egg pasteurization processes at different temperatures with anisothermal heating-up phases (from 54°C to either 60°C, 64°C or 70°C) for different times (16, 5, and 2.5 min). The initial temperature (54°C) was chosen because it is commonly used in industry as a homogenization temperature (Martínez and Maurer, 1975), before the final heating-up phase and subsequent heat treatment. Fig. 3A–C summarizes the results obtained in treatments with anisothermal lag phases of 5 min, followed by isothermal heating at 60°C, 64°C and 70°C. Curves obtained with longer (16 min) or shorter (2.5 min) heating-up phases (data not shown) lead to the same conclusions. The figure also includes the temperature curve and the theoretical survival curve calculated from decimal reduction times and z values obtained in liquid whole egg with instantaneous heating (data of Fig. 1). As shown by Fig. 3, heating lag phases protected *S. senftenberg* 775 W against the subsequent treatment at isothermal temperature. The greater the differences between theoretical and experimental survivors counts, the higher the final isothermal treatment temperature.

Heat resistance developed by isothermal heat shocks in liquid egg (Fig. 2) could not explain the survival of *S. senftenberg* 775 W after anisothermal treatments. Data in Fig. 3 demonstrated that the protective effect of anisothermal heat shock increased with the maximum temperature attained, being the highest at 70°C in the range explored. However, isothermal shocks at temperatures over 54°C could not be applied, because they produced a fast inactivation of bacterial cells. Both data seemed to be in contradiction. Pagán et al. (1997) observed that heat inactivation and acquisition of heat resistance through heat shocks were two simultaneous phenomena that occurred during thermal treatments of *L. monocytogenes* cell populations. Our results seemed to confirm these observations. It was likely that shocks at low-temperature protected cells against successive higher temperatures, thus acting as a new heat shock that increased the heat resistance again. This could explain the survival of *S. senftenberg* even at 70°C. However, the development of thermotolerance should arise very quickly, before the heat inactivation of cells occurred ($D_{70} = 0.082$; Fig. 1). The induction of protein production in *Pseudomonas aeruginosa* as soon as 1 min after the cells had attained the shock temperature (45°C) has been reported (Allan et al., 1988).

Overall, the existence of different mechanisms of development of thermal tolerance, for isothermal and anisothermal heat shocks, cannot be discarded. Humphrey et al. (1993) demonstrated the existence of two different protecting mechanisms initiated by thermal shocks on *S. enteritidis*. One was slow and related with protein synthesis, whereas the other was faster and, according to the authors, possibly related with changes in cell envelopes. Moreover, it is well known that the bacterial heat-shock response is regulated by several parallel control mechanisms that can be triggered by different heat stresses, e.g. extreme vs mild heat shock (Brissette et al., 1990; Gross, 1996).

When heat shocked cells are heat treated some deviations of the first-order inactivation kinetic have been observed by different authors (Pagán et al., 1997). Fig. 3C shows the inactivation rate of *S. senftenberg* 775 W during an anisothermal heating lag phase and a subsequent isothermal treatment at 70°C. After the heating lag phase, a first-order inactivation kinetic should be expected. However, after approximately 6 min of treatment a change in the slope of the survival curve was observed. This could be due to a heterogeneous distribution of the heat resistance in the native cell population, but also to a different rate response to heat shock of the bacterial cells. Pagán et al. (1997) demonstrated that the percentage of high heat-resistant

cells in the suspension increased with the shocking time. This could explain why we observed the most pronounced tail at the faster heating rate (3.3°C/min; Fig. 3C). Overall, Fig. 3C demonstrated that anisothermal heating from 54°C to 70°C, at a rate of 3.3°C/min, allowed obtaining the higher heat resistance of *S. senftenberg* 775 W in liquid whole egg ever published.

Fig. 4 shows the survival curves at 64°C of *S. senftenberg* after anisothermal lag phases at different heating rates: 0.55°C/min, 2°C/min and 4°C/min. The statistical analysis of our data indicated that the heating rate did not significantly influence ($P<0.01$) the profile of survival curves of *S. senftenberg* 775 W cells to pasteurization treatments. Perhaps, the behavior of this serotype is different from that observed for other bacterial species (Mackey and Derrick, 1987a, b; Stephens et al., 1994), or the heating rate range explored (0.4–6.6°C/min) was not wide enough. However, anisothermal heating lag phases out of this range (from 2.5 to 16 min) lost practical significance, as they are not applied in the industry. Overall, in our experimental conditions, it was the final isothermal temperature and not the heating rate which was the factor that determined the increase in thermotolerance.

Fig. 5 shows the number of survivors of a population of *S. senftenberg* 775 W suspended in liquid whole egg (initial number: 10^7 UFC/ml) after simulated industrial pasteurization treatments (60°C for 3.5 min, 64°C for 2.5 min and 70°C for 1.5 min) with different anisothermal heating lag phases. We can conclude that the industrial ultrapasteurization treatment at 70°C with long heating up phases, which are applied to maintain the functionality of liquid egg, would only reach 3–4 log cycles reduction on the number of *S. senftenberg* 775 W. This inactivation level is still far from the 9 log cycles recommended by the FDA on the population of *Salmonella* spp. in egg product pasteurization treatments.

Development of heat resistance as a consequence of previous heat shocks is a widespread fact in the microbial world, and our results perhaps explain the eventual isolation of bacterial pathogens from foods that laboratorial heat resistance data might not explain (Tauxe, 1991; Todd, 1996; Wallace et al., 2000). The high thermal sensitivity of liquid egg components will probably prevent the application of more intense heat treatments; therefore only the development of alternative bacterial inactivation methods or, alternatively, more effective heating procedures could provide more adequate sanitized liquid whole egg.

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