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Inactivation of *Salmonella enterica* Serovar Enteritidis by Ultrasonic Waves under Pressure at Different Water Activities

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The inactivation of *Salmonella enterica* serovar Enteritidis by ultrasonic waves (20 kHz; 117-μm wavelength) under pressure (175 kPa) at nonlethal temperatures (manosonication [MS]) and lethal temperatures (manothermosonication [MTS]) in media of different water activities has been investigated. Heat decimal reduction time values increased 30 times when the water activity was decreased from nearly 1 to 0.96, but the MS resistance was increased only twofold. The inactivation of *Salmonella* serovar Enteritidis by ultrasound under pressure at low water activities was a phenomenon of the "all-or-nothing" type. A synergistic lethal effect was observed between heat and ultrasound in media with reduced water activity; the lower the water activity, the greater the synergistic effect. This work could be useful for improving sanitation and preservation treatments of foods, especially those which are sensitive to temperature and those in which components protect microorganisms to heat. It also contributes to our knowledge of microbial inactivation mechanisms by MS and MTS treatments.

During the last 3 decades, the number of food poisoning outbreaks in which different *Salmonella enterica* serotypes were involved has increased in industrialized countries (34, 35). In the late 1980s, the frequency of isolation of *Salmonella enterica* serotype Enteritidis from eggs and egg products has increased steadily (25, 34). The influence of different environmental factors on the survival of other *Salmonella* serotypes has been investigated repeatedly (12, 13, 15, 37), but little is known about their effect on *Salmonella* serovar Enteritidis.

It is well-known that the heat resistance of microorganisms is influenced by many environmental factors. The water activity (a_w) of the heating medium is one of the most important factors (5, 7, 10, 16, 36). The thermal protective effect of media with reduced a_w is very high (100-fold) (14, 33). The use of high-intensity heat treatments to obtain the required microbial inactivation would impair food quality. Therefore, perhaps the new nonthermal methods of bacterial inactivation would be good alternative methods for the preservation and sanitation of products with reduced a_w.

Microbial inactivation by high-power ultrasound under pressure at nonlethal temperatures (manosonication [MS]) and lethal temperatures (manothermosonication [MTS]) was first reported by Sala et al. in 1995 (29). Recently, Raso et al. (27) reported the influence of temperature, pressure, and amplitude of ultrasonic waves on the lethal effect of ultrasound. Pagán et al. (24) studied the effects of MS and MTS on gram-positive and gram-negative bacterial species, and Mañas et al. (20) studied the lethal effect of MS and MTS on several *Salmonella* serotypes suspended in buffer and in liquid whole egg. However, no specific investigations have been performed on *Salmonella* bacterial inactivation by ultrasonic waves under pressure in media with reduced water activity. From published data (20), it could be deduced that the eventual advantages of MS or MTS for sanitation and/or food preservation purposes will be higher in temperature-sensitive foods (e.g., when raw materials are contaminated with very heat-resistant bacterial species or when food components protect microorganisms against heat).

The purpose of this work was to investigate the inactivation of *Salmonella* serovar Enteritidis by ultrasonic waves under pressure at different temperatures in media of different water activities. The heat resistance of this microorganism in the same medium was also studied as a control.

Bacterial culture and media. The *Salmonella* serovar Enteritidis strain (ATCC 13076) used in this investigation was supplied by the Spanish Type Culture Collection. Erlenmeyer flasks containing 50 ml of sterile nutrient broth (Biolife, Milan, Italy) were inoculated to a final concentration of 10^6 cells ml^{-1} and incubated at 37°C under agitation (130 rpm) (Selecta; Rotabit, Barcelona, Spain). When the stationary growth phase was reached (after 24 h of incubation), suspensions were stored at 4°C until use. This storage did not change cell resistance to heat or ultrasonic waves for the time in which this investigation was performed ($P < 0.05$) (data not shown).

Heat, MS, and MTS treatments. Heat, MS, and MTS treatments were performed in a specially designed resistometer as described previously (27). The resistometer, a mixing method that avoids the heating lag phases, allowed us to obtain survival curves to heat and ultrasound treatments at different temperatures, pressures, and ultrasonic wave amplitudes. Once the temperature, pressure, and amplitude of ultrasonic waves were stabilized, the cell suspension (0.2 ml) was injected into the 23-ml treatment chamber containing the treatment medium. Before the injection, cells were allowed to adapt to a solution with the same a_w as the treatment medium for 5 min. Longer adaptation times did not change the survival curve profiles (data not shown). After injection and at preset intervals, 0.1-ml samples for each treatment time were directly collected into

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test tubes of melted sterile nutrient agar (NA) (Biolife) and immediately plated. Survival curves were plotted, with 6 to 15 separate samples collected over time. NA plates were incubated for 24 h at 37°C. Previous experiments showed that longer incubation times did not influence survivor counts. When damage and repair mechanisms were investigated, NA with 3% (wt/vol) sodium chloride (Panreac, Barcelona, Spain) added (NA-SC) was used as the recovery medium. This medium did not affect the viability of undamaged cells (data not shown). The NA-SC plates were incubated for 48 h at 37°C. After incubation, the CFU were counted with an Image Analyzer Automatic Counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) as described elsewhere (3).

McIlvaine citrate phosphate buffer (pH 7; $a_w > 0.99$) (6) or the same medium with different amounts of sucrose (Azucarera Española, Madrid, Spain) added (according to the data of Robinson and Stokes [28]) was prepared and used as the treatment media. The final a_w of the treatment media (0.98 and 0.96) was measured at room temperature with a specially designed instrument (Water Activity System model CX-1; Decagon Devices, Inc., Pullman, Wash.).

Heat, MS, and MTS resistance parameters. The inactivation rate was measured by determining the decimal reduction time value (D_t for heat, D_{MS} for MS, and D_{MTS} for MTS treatments) from the slope of the regression line of the survival curves. Decimal reduction time curves (DRTC) were obtained by plotting $\log_{10}$ D values versus their corresponding heating temperatures, and z values (increase in temperature [in degrees Celsius] for the D value to decrease by one $\log_{10}$ cycle) were calculated. The coefficient of determination (r^2) of survival curves and the 95% confidence limits of D and z values were calculated by using the Excel package (Microsoft, Seattle, Wash.).

The individual contributions of heat and ultrasound under pressure to the lethal effect of MTS treatments at different temperatures were evaluated by determining how well experimental values matched the theoretical DRTC values. Theoretical D_{MTS} values were calculated, as Raso et al. (27) described, with the equation $D_{MTS} = (D_t \times D_{MS})/(D_t + D_{MS})$.

Heat resistance. The survival curves obtained in this investigation followed first-order inactivation kinetics, at least for 99.9% of the cell population. Neither temperature adaptation phenomena nor subpopulations resistant to high heat or ultrasound under pressure were detected. As a consequence, D values were a useful parameter for resistance comparison purposes.

Table 1 shows the decimal reduction time values at different temperatures of *Salmonella* serovar Enteritidis heated in media with different a_ws. The 95% confidence limits and the r^2 values of the corresponding survival curves have also been included to illustrate the precision of the results. Table 1 also includes the z values, their 95% confidence limits, and the corresponding r^2 values. The temperature resistance values for *Salmonella* serovar Enteritidis treated in McIlvaine buffer of pH 7 ($a_w > 0.99$) found in this study were consistent with the little data published (2, 20) on this serotype and were similar to those obtained for other temperature-sensitive serotypes of *Salmonella* (8, 9, 20). Also, the z value was similar to that reported for most vegetative cells investigated (17, 20, 22, 30).

TABLE 1. Heat resistance of *Salmonella* serovar *Enteritidis* in media with different a_ws

a_w	T (°C)[a]	D_t (min)[b]	95% CL[c]	r^2
>0.99	53	2.60	2.47–2.73	0.99
	54	1.73	1.64–1.82	0.99
	56	0.78	0.74–0.82	0.99
	58	0.22	0.21–0.23	0.98
	60	0.10	0.09–0.10	0.99
	63	0.03	0.03–0.03	0.99
	z (°C)[d]	**4.89**	**4.64–5.13**	**0.99**
0.98	56	3.68	3.50–3.86	0.99
	58	1.53	1.45–1.61	0.99
	60	0.88	0.84–0.93	0.99
	62	0.38	0.36–0.40	0.98
	65	0.12	0.11–0.12	0.99
	68	0.04	0.03–0.04	0.99
	z (°C)	**6.03**	**5.73–6.33**	**0.99**
0.96	58	4.56	4.34–4.79	0.98
	60	2.70	2.57–2.84	0.99
	62	1.33	1.26–1.40	0.99
	64	0.68	0.64–0.71	0.99
	67	0.16	0.16–0.17	0.99
	71	0.05	0.05–0.05	0.99
	z (°C)	**6.38**	**6.06–6.70**	**0.99**

[a] Temperature of the treatment.
[b] Decimal reduction time values.
[c] 95% CL, 95% confidence limits.
[d] Increase in temperature for the D_t value to decrease by one $\log_{10}$ cycle. The z values are shown in bold type.

From these data, it could be deduced that current heat pasteurization treatments for liquid food with high a_ws (close to 1) were sufficient to avoid a food poisoning outbreak caused by *Salmonella* serovar Enteritidis.

The a_w of the heating media had a great influence on the heat resistance of this serotype. The D value at 60 min increased from 0.10 to 0.88 and 2.70 min when the a_w was reduced from >0.99 to 0.98 and 0.96, respectively. The z value significantly increased ($P > 0.05$) from 4.9 to 6.4°C when the a_w was decreased from >0.99 to 0.96. Therefore, the thermal protective effect of media with reduced a_w increased with increases in the treatment temperature. Comparing our data with those obtained by other investigators is difficult, since the effect of a_w on microbial inactivation depends on the solute (1, 4, 9, 16, 31). From published data obtained for solutions of sucrose and water, it could be deduced that the effect of a_w on the thermal tolerance of *Salmonella* serovar Enteritidis was among the highest published for *Salmonella* serotypes. It was similar to that observed for serovars Alachua, Anatum, and Montevideo (9) but greater than those reported for serovars Infantis, Typhimurium, and Tennessee (8, 9, 33). For *Salmonella* serovar Senftenberg, the effect of a_w of the heating media was lower ($D_t \times 2$ or $D_t \times 3$) (8, 9, 19). It is generally believed that the thermal protective effect of reduced a_ws is higher for the most heat-sensitive species (1, 8, 9). Our results are in accordance with this hypothesis.

Figure 1 shows the survival curves of *Salmonella* serovar

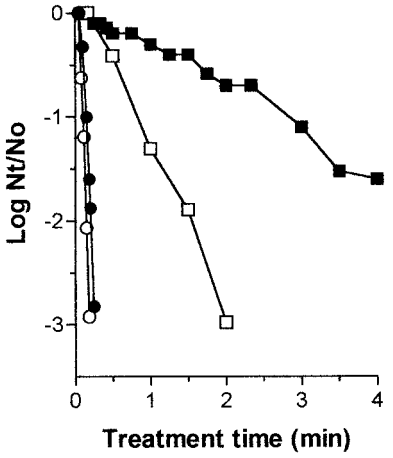

FIG. 1. Survival curves of *Salmonella* serovar Enteritidis treated in media with different a_ws at 60°C. The bacteria were treated in media with an a_w of >0.99 (●, ○) or 0.96 (■, □) and recovered in NA (●, ■) or NA-SC (○, □). Nt/No, number of cells treated/number of original cells.

TABLE 2. Resistance to ultrasonic waves (117-μm wavelength; 20 kHz) under pressure (175 kPa) at several temperatures of *Salmonella* serovar *Enteritidis* suspended in media with different a_ws

a_w	T (°C)[a]	D (min)[b]	95% CL[c]	r^2
>0.99	35	0.89	0.84–0.93	0.99
	50	0.77	0.73–0.81	0.99
	53	0.42	0.40–0.44	0.99
	56	0.25	0.24–0.26	0.99
	59	0.12	0.12–0.13	0.99
	63	0.02	0.02–0.02	0.98
0.98	35	0.85	0.81–0.89	0.98
	50	0.46	0.44–0.48	0.99
	60	0.16	0.15–0.17	0.99
	65	0.08	0.07–0.08	0.99
	68	0.04	0.03–0.04	0.99
0.96	35	1.37	1.30–1.44	0.99
	50	0.87	0.83–0.92	0.99
	56	0.32	0.30–0.33	0.99
	60	0.25	0.24–0.26	0.99
	67	0.09	0.09–0.09	0.99
	71	0.04	0.04–0.04	0.98

[a] Temperature of the treatment.
[b] Decimal reduction time values.
[c] 95% CL, 95% confidence limits.

Enteritidis heated at 60°C in McIlvaine buffer (pH 7) with an a_w of >0.99 and in the same medium with 41% (wt/vol) sucrose added (a_w = 0.96), obtained by growing cells after treatment in NA and NA-SC. As can be observed in Fig. 1, the percentage of damaged cells increased with the treatment time and was greater at reduced a_ws. The medium with reduced a_w protected *Salmonella* serovar Enteritidis by increasing the thermal stability of some bacterial structures to heat. However, a higher injury repair capability also contributed to the high thermal tolerance of cells heated in media with lower a_ws. The D_t value obtained in NA-SC was 1.4 times lower than that obtained in NA after heating in citrate phosphate buffer (0.07 and 0.10 min, respectively) but 4 times lower after heating in medium with an a_w of 0.96 (0.62 and 2.46 min, respectively).

Overall, the thermal inactivation of *Salmonella* serovar Enteritidis in liquid foods with an a_w of 0.96 would require an increase, by approximately 30 times, of the intensity of current treatments designed for the pasteurization of liquid foods with an a_w close to 1. These thermal treatments would probably impair the quality of most liquid foods. Therefore, alternative sanitation processes would be very useful.

MS or MTS resistance. Table 2 shows the decimal reduction time values for ultrasonic waves (117-μm wavelength) under pressure (175 kPa) at different temperatures of *Salmonella* serovar Enteritidis treated in media with different a_ws. The D_{MS} value in citrate phosphate buffer of pH 7 (0.89 min) was similar to that previously reported for this serotype by different researchers (20, 24). While the heat resistance of *Salmonella* serovar Enteritidis increased 30 times when the a_w of the treatment media decreased from >0.99 to 0.96 (Table 1), the D_{MS} value (Table 2) hardly doubled (0.89 and 1.37 min, respectively). This seemed to confirm that the mechanisms by which heat and ultrasound inactivate microorganisms are different. Furthermore, comparison of the survival curves obtained by growing cells in NA and in NA-SC after MS treatments in citrate phosphate buffer of pH 7 showed the lack of any damaged cells (data not shown), which contrasted with the results observed after heat treatments (Fig. 1). This would also indicate that the bacterial inactivation by MS is a phenomenon of the "all-or-nothing" type, probably due to the mechanical disruption of the cell envelopes, as has previously been observed by several researchers (20, 21, 23, 26) in media with high a_ws.

Figure 2 shows the relationship between the temperature and decimal reduction time values to MS and MTS treatments of *Salmonella* serovar Enteritidis obtained in media with a_ws of >0.99 (Fig. 2A), 0.98 (Fig. 2B), and 0.96 (Fig. 2C). The DRTC corresponding to heat treatments and the theoretical DRTC corresponding to MS and MTS treatments have also been included. The theoretical DRTC has been calculated, as proposed by Raso et al. (27), by assuming that heat and ultrasonic waves acted independently and that heat, MS, and MTS destruction of bacterial cells were single reactions ruled by first-order kinetics. As shown in Fig. 2A, experimental data obtained in McIlvaine buffer (pH 7) fitted the theoretical decimal reduction time values with a r^2 of ≥0.98. The D_{MS} value was the same until 50°C. From 50 to 60°C, the rapid decrease in the D value would be due to the exponential increase in the lethality of heat by linear increases in temperature, making the lethality of ultrasound negligible at 60°C. Over this temperature, the D_{MTS} and D_t became equal. The same behavior has been observed in several bacterial species treated at high a_ws (20, 23, 24, 27). These data indicated that the lethality of MTS was the result of adding the inactivation rate due to heat to that due to ultrasound. The individual contributions of heat and ultrasound to the whole lethal effect depended on the temperature.

In contrast to the values observed when *Salmonella* serovar Enteritidis was subjected to MT or MTS treatment in citrate phosphate buffer (pH 7) (Fig. 2A), the theoretical D_{MS} and D_{MTS} values did not fit the experimental values obtained in media with reduced a_ws (Fig. 2B and C). The lower the a_w was, the greater the differences were. The D_{MTS} value at 60°C in

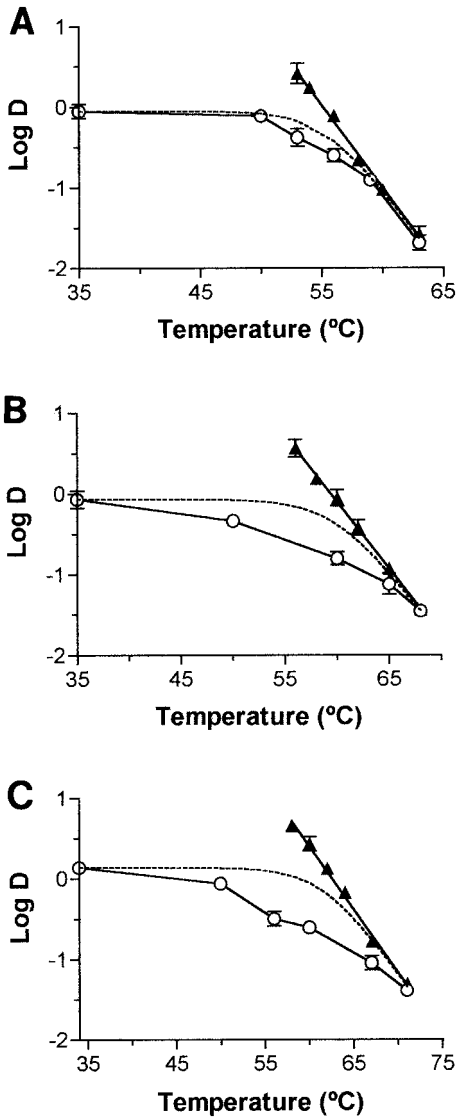

FIG. 2. Resistance of *Salmonella* serovar Enteritidis to ultrasound (117-μm wavelength) under pressure (175 kPa) at different temperatures in media with a_ws of >0.99 (A), 0.98 (B), and 0.96 (C). Decimal reduction time values to ultrasonic waves under pressure (○) and to heat (▲) are shown. The broken line represents the theoretical DRTC to ultrasonic waves under pressure calculated by the equation $D_{MTS} = (D_t \times D_{MS})/(D_t + D_{MS})$.

medium with an a_w of 0.96 was three times lower than was expected. This indicated the existence of a synergistic lethal effect between heat and ultrasound. The existence of a synergistic effect in which the whole lethal effect was higher than the lethal effect of heat added to the lethal effect of ultrasound has been previously reported for bacterial cells resistant to very high heat (21, 24).

Conclusions. The addition of sucrose to the treatment media strongly protected *Salmonella* serovar Enteritidis cells to heat but hardly changed their MS resistance. *Salmonella* serovar Enteritidis inactivation by ultrasonic waves under pressure was a phenomenon of the all-or-nothing type. No sodium chloride-sensitive cells could be detected after MS treatments. The whole lethal effect of MTS in phosphate citrate buffer (pH 7) with an a_w of >0.99 was the result of the lethal effect of heat added to that of ultrasonic waves under pressure. When *Salmonella* serovar Enteritidis cells were treated by MTS in media with reduced a_ws, a synergistic effect was observed. The lower the a_w was, the higher the synergism. The synergistic effect was due to the sensitizing effect of heat, and ultrasound was the ultimate cause of bacterial inactivation.

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