Bacterial Resistance to Ultrasonic Waves under Pressure at Nonlethal (Manosonication) and Lethal (Manothermosonication) Temperatures

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The decimal reduction times of Streptococcus faecium, Listeria monocytogenes, Salmonella enteritidis, and Aeromonas hydrophila corresponding to heat treatment at 62°C were 7.1, 0.34, 0.024, and 0.0096 min, and those corresponding to manosonication treatment (40°C, 200 kPa, 117 μm) were 4.0, 1.5, 0.86, and 0.90 min, respectively.

In this research, the resistance of Streptococcus faecium, L. monocytogenes, Salmonella enteritidis, and Aeromonas hydrophila to heat, MS, and MTS treatments was investigated and compared. The influence of the amplitude of UW, static pressure, and temperature on the rate of inactivation by MS and MTS was also studied.

Bacterial culture and media. The strains of S. faecium (STCC 410), L. monocytogenes (STCC 4031), S. enteritidis (STCC 4300), and A. hydrophila (STCC 839) used in this investigation were supplied by the Spanish Type Culture Collection. A suspension of each microorganism was prepared by inoculating 250-ml Erlenmeyer flasks containing 50 ml of sterile tryptic soy broth (Biolife, Milan, Italy) with 0.6% yeast extract (Biolife) added to a final concentration of 10^6 cells ml^-1. These flasks were incubated at 37°C until the culture reached the stationary growth phase and maximum heat resistance (data not shown).

Determination of resistance to heat, MS, and MTS. Resistance to heat, MS, and MTS was determined with a specially designed resistometer as already described (13). Once the treatment temperature had attained stability, 0.2 ml of an adequately diluted cell suspension was injected into the 23-ml treatment chamber containing McIlvaine citrate-phosphate buffer at pH 7 (4). At least five 0.1-ml samples were collected at preset intervals in test tubes containing melted, sterile tryptic soy agar-yeast extract medium (Biolife). The tubes were immediately plated and incubated at 37°C for 48 h (S. faecium, S. enteritidis, and L. monocytogenes) or at 30°C for 24 h (A. hydrophila). Previous experiments showed that longer incubation times did not influence survivor counts (data not shown). CFU were counted with an improved Image Analyser Automatic Counter (Protos, Analytical Measuring Systems, Cambridge, United Kingdom) as previously described (3). The inactivation rate was measured by determining the decimal reduction time...
TABLE 1. Resistance of S. faecium, S. enteritidis, L. monocytogenes, and A. hydrophila to heat and MS treatments.*

<table>
<thead>
<tr>
<th>Species</th>
<th>$D_{62}^c$ (95% CL) (min)</th>
<th>$z$ (°C)</th>
<th>$D_{MS}$ (95% CL) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. faecium</td>
<td>7.1 (6.1–8.6)</td>
<td>6.0 (4.9–7.7)</td>
<td>4.0 (3.9–4.2)</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>0.024 (0.021–0.028)</td>
<td>4.4 (3.9–5.0)</td>
<td>0.86 (0.76–0.98)</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>0.34 (0.31–0.37)</td>
<td>5.9 (5.5–6.1)</td>
<td>1.5 (1.5–1.6)</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>0.0096 (0.0080–0.012)</td>
<td>5.9 (5.6–6.1)</td>
<td>0.90 (0.78–1.1)</td>
</tr>
</tbody>
</table>

* MS conditions: 40°C, 200 kPa, 117 μm. 
** Survival curves corresponding to these data always showed $r_0 \geq 0.98$. 
*** $D_{62}$, D value for heat treatment at 62°C.

(D value; $D_T$ for heat, $D_{MS}$ for MS, and $D_{MTS}$ for MTS) calculated from the slope of the straight portion of the survival curve. DRTC curves were obtained by plotting log D values versus the corresponding treatment temperatures. For heat treatments, z values (°C increase in temperature required for the $D_T$ value to drop 1 log cycle) were calculated from the slope of the corresponding DRTC. Correlation coefficients ($r_o$) and 95% confidence limits (CL) were calculated by the appropriate statistical package (StatView SE Graphics, Abacus Concepts Inc., Berkeley, Calif.). The statistical significance ($P \leq 0.05$) of differences between the D and z values was tested as described by Steel and Torrie (18). Regression lines of the influence of static pressure and UW amplitude were fitted and parameters were derived by using the Excel 5.0 package (Microsoft, Seattle, Wash.). The individual contributions of heat and UW to the lethal effect of MTS treatment at different temperatures was evaluated by determining how experimental values matched the theoretical DRTC. Theoretical $D_{MTS}$ values were calculated as described by Raso et al. (13), with the equation $D_{MTS} = (D_T \times D_{MS})/(D_T + D_{MS})$.

Resistance to heat and MS treatments. The $D_T$ and z values of our strains were in the range of most published data. For the most heat-resistant species (S. faecium), the D value for heat treatment at 62°C was approximately 700 times that of the last thermotolerant (A. hydrophila) (Table 1). Therefore, the intensity of a heat treatment designed to sterilize a given product contaminated with these species could vary up to 1,000-fold. On the contrary, resistance of the same species to MS treatment only varied approximately fivefold (Table 1) and the intensities of treatment required should not be very different.

As shown by Table 1, gram-positive and coccal forms were, as found with heat (21) and UW (1, 2) inactivation, the most MS-resistant microorganisms. The greater the bacterial heat resistance, the lower the ratio of the heat inactivation rate to the MS inactivation rate.

Effect of amplitude on MS inactivation rate. At 200 kPa and 40°C, the $D_{MS}$ values of all of the species investigated decreased exponentially with UW amplitude increases between 62 and 150 μm (Fig. 1). No statistically significant differences ($P \leq 0.05$) were found among the slopes of the regression lines shown in Fig. 1. For all of the species investigated, the $D_{MS}$ value decreased by one-sixth when the amplitude was increased 100 μm. Therefore, in the range of UW amplitudes investigated, this relationship followed the general equation $\log D_{MS} = \log D_0 - 0.0091 \times (A - 62)$, where $D_{MS}$ is the decimal reduction time for each MS treatment, $D_0$ is the decimal reduction time of MS treatments at an amplitude of 62 μm, and A is the UW amplitude. The goodness of fit of this general equation for the four sets of experimental data is demonstrated by the high correlation between the theoretical and experimental values ($r^2 = 0.984$). The inactivation of microorganisms suspended in a liquid medium by UW is thought to be due to cavitation (9). Bacterial inactivation by UW seems to be due to the very high pressures developed during cavitation (5, 17) and/or the release of free radicals in the medium (8, 15). Raso et al. (13) demonstrated that addition of free-radical scavengers to the medium did not influence the rate of Y. enterocolitica inactivation and concluded that vegetative cells were probably inactivated as a consequence of the mechanical disruption of the cell membranes. The higher inactivation rate at greater amplitudes could be due to an increase in the number of bubbles liable to implode per unit of time in a given volume and/or to an increase in the volume of liquid in which cavitation is liable to occur (20). The magnitude of the influence of UW amplitude on S. faecium, L. monocytogenes, S. enteritidis, and A. hydrophila was the same, independently of the individual resistance to MS treatment. These results indicated that differences in cell wall structure between the differ-
ent species investigated did not modify the influence of the UW amplitude.

Effect of pressure on MS inactivation rate. The rate of vegetative-cell inactivation by MS increased drastically with a rise in static pressure. However, as the pressure was raised, the magnitude of this increase decreased progressively (Fig. 2). This performance is described by the general equation \[ \log D_{MS} = \log D_0 - 0.0026 \times P + 2.2 \times 10^{-5} \times P^2 \], where \( D_{MS} \) is the decimal reduction time corresponding to MS treatments at an amplitude of 117 \( \mu \)m and 40°C, \( D_0 \) is the \( D \) value corresponding to MS treatment at 117 \( \mu \)m and 40°C at ambient pressure, and \( P \) is the static relative pressure. The goodness of fit of this general equation for the four sets of experimental data is demonstrated by the high correlation between the theoretical and experimental values (\( r^2 = 0.989 \)). The magnitude of the effect of static pressure was the same for all of the species investigated. When the pressure was raised from 0 to 100 kPa, the \( D_{MS} \) (117 \( \mu \)m and 40°C) dropped to one-half of its original value. However, a further pressure rise from 300 to 400 kPa only made this value decrease by approximately 20%. Static pressure during ultrasonic treatment increases the intensity of cavitation (19). The increase in the inactivation rate when the pressure was raised was probably due to an increase in bubble implosion intensity. The lower response to static pressure increases at higher pressures was probably due to the reduction in the number of bubbles undergoing cavitation (19). Overall, these physical changes affected all of the species investigated to the same extent.

Effect of temperature on MS and MTS inactivation rates. Figure 3 shows the experimental \( D \) values of MS and MTS treatments at different temperatures for the four bacterial species investigated. The theoretical DRTC corresponding to MTS treatments (dotted line), calculated as described in Materials and Methods, and the DRTC corresponding to heat treatments have also been included. The relationship between the experimental and theoretical MTS values is illustrated for \( S.\ enteritidis \), \( L.\ monocytogenes \), and \( A.\ hydrophila \) in Fig. 4A and for \( S.\ faecium \) in Fig. 4B. In the range of 40 to 68°C, the experimental values of \( S.\ faecium \) did not match the theoretical values (Fig. 4B). Therefore, the rate of \( S.\ faecium \) inactivation by MTS in this range seemed to be a synergistic instead of an additive effect. The magnitude of this synergistic effect at 62°C
is illustrated in Fig. 5, which shows the survival curves of *S. faecium* corresponding to heat (62°C), MS (200 kPa, 117 μm, 40°C), and MTS (200 kPa, 117 μm, 62°C) treatments, as well as the theoretical survival curve (dotted line) that should be obtained if the effect of MTS is additive. The number of survivors after MTS treatment was lower (approximately 1 log cycle after 4 min of treatment) than that of the theoretical survival curve. The rate of inactivation by UW under pressure was independent of temperature until a maximum temperature was reached (MS). This maximum temperature was different for each bacterial species investigated (Fig. 3). At values above these maximum values, the inactivation rate increased drastically with temperature (MTS). Similar behavior was observed in *Y. enterococitica* by Raso et al. (13), who hypothesized that this profile was the result of the addition of the inactivation rate of heat to the inactivation rate of UW under pressure. Our results obtained with *L. monocytogenes*, *S. enteritidis*, and *A. hydrophila* confirmed this hypothesis (Fig. 4A). On the contrary, we observed a disagreement between the theoretical values and experimental values obtained with *S. faecium* (Fig. 4B). As shown by Fig. 3, the experimental rate of *S. faecium* inactivation by MTS was higher than that calculated theoretically between 50 and 68°C. These results demonstrated that in this range of temperatures, the rate of *S. faecium* inactivation by MTS was the result of a synergistic instead of an additive effect (Fig. 5). A similar synergistic effect has also been observed in the inactivation of spores of *B. subtilis* spores (14). Raso et al. (14) suggested that this synergistic effect could be due to disruption of the bacterial spore cortex, causing protoplast rehydration and loss of heat resistance. Perhaps similar damage in the cell wall peptidoglycan could explain this synergistic effect on *S. faecium*.

**Conclusions.** We can conclude that the differences in vegetative cell resistance to MS were much smaller than those observed in resistance to heat treatment. The rate of bacterial cell inactivation by UW increased with increases in amplitude and static pressure, the magnitude of the increase being the same for all of the bacterial species investigated. Therefore, the influence of these factors can be predicted. The rate of *L. monocytogenes*, *S. enteritidis*, and *A. hydrophila* inactivation by MTS was the result of the additive effects of heat and UW under pressure, but a synergistic effect on *S. faecium* was found.

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**REFERENCES**