Predicting thermal inactivation in media of different pH of Salmonella grown at different temperatures

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Abstract

The influence of the growth temperature and the pH of the heating medium on the heat resistance at different temperatures of Salmonella typhimurium ATCC 13311 was studied and described mathematically. The shift of the growth temperature from 10 to 37 °C increased heat resistance of S. typhimurium fourfold. The pH of the heating medium at which heat resistance was maximum was pH 6 for cells grown at 37 °C, but changed with growth temperature. The alkalinization of the heating medium from pH 6 to pH 7.7 decreased the heat resistance of cells grown at 37 °C by a factor of 3. Neither the growth temperature nor the pH modified the z values significantly (4.9 °C). The decimal reduction times at different treatment temperatures, in buffers of different pH of cells of S. typhimurium grown at different temperatures, were accurately described by a mathematical equation (correlation coefficient of 0.97). This equation was also tested for Salmonella senftenberg 775W (ATCC 43845) and Salmonella enteritidis ATCC 13076, strains in which the correlation coefficients between the observed and the theoretically calculated values were 0.91 and 0.98, respectively.

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1. Introduction

The development in recent years of Predictive Microbiology has increased the interest in the description of bacterial behavior (McMeekin et al., 2002). Most of this work has been focused on bacterial growth, whereas the inactivation has received much less attention. However, thermal death kinetics is one of the few areas where mathematical models have been used traditionally to predict food safety margins. The most commonly used model to describe microbial thermal inactivation, that assumes a first order kinetic, permits to establish adequate thermal treatments by the calculation of the decimal reduction times (Dₜ) and the z values. This model has been used by the food industry for the last 50 years. It has been reported that in some conditions there are deviations from first order inactivation kinetics (Cerf, 1977; Humpheson et al., 1998) and several alternative models have been suggested. However, the first order inactivation model is still the most widely accepted.
The inactivation of non-spore forming pathogen microorganisms by a heat treatment is essential in the safe preparation of many foods. Salmonella is one of the microorganisms most frequently implicated in food-poisoning outbreaks (Tauxe, 1991; Todd, 1996), some of them caused by an insufficient heating (D’Aoust, 1989). For this reason, there have been many studies on the heat resistance of different strains of this genus (D’Aoust et al., 1987; D’Aoust, 1989; Doyle and Mazzota, 2000). However, most of these works study the influence of single factors (treatment temperature, water activity of the heating medium, etc.) (Osborne et al., 1954; Anellis et al., 1955; Ng et al., 1969; Gibson, 1973; Corry, 1974; Humphrey et al., 1990; Mañas et al., 2001), and the combined effect of various factors has received much less attention. Blackburn et al. (1997) studied the combined effect of pH, sodium chloride and treatment temperature on Salmonella and Escherichia coli heat resistance. This interaction has been modelized and included in the application software Food MicroModel (http://www.arerc.gov/mfs/inactivation_models.htm). However, apart from those factors that modify heat resistance during the treatment, there are others acting before, or even after, the heat treatment. Factors that act before the heat treatment, e.g. growth temperature, growth phase or heat shocks, may modify bacterial cell’s structure and composition, and hence, not only the bacterial resistance to subsequent heat treatments, but also the effect of other factors such as the pH or the water activity of the heating media (Pagan et al., 1999). Among the factors acting during the heat treatment, the pH of the food is one of the most important. Whereas other characteristics, as water activity or salt concentration, do not vary too much in most foods in which Salmonella may represent a health hazard, the pH is more variable, ranging from alkalinity (e.g. liquid whole egg) to acidity, as in salad dressings as mayonnaise.

The objective of this work was to study the influence of the growth temperature on the heat resistance of Salmonella typhimurium at different heating temperatures in media with different pHs, and to describe this interaction mathematically. In an attempt to detect different responses within this genus, two other different serotypes of Salmonella were used: Salmonella enteritidis and the heat resistant strain Salmonella senftenberg 775W.

2. Material and methods

2.1. Bacterial culture and media

The strains of S. typhimurium (ATCC 13311), S. enteritidis (ATCC 13076) and S. senftenberg (ATCC 43845) used in this investigation were supplied by the Spanish Type Culture Collection. During this investigation they were maintained at 2–4 °C on slants of Nutrient Agar (NA) (Biolife, Milan, Italy).

A broth subculture was prepared by inoculating with one single colony from a plate, a test tube containing 5 ml of sterile Nutrient Broth (NB) (Biolife). After inoculation, this tube was incubated overnight at 37 °C. Erlenmeyer flasks (250 ml) with 50 ml of sterile NB were inoculated with this subculture to an initial concentration of 10^6 cells/ml approx. The flasks were then incubated under agitation (125 rpm) (Selecta, mod. Rotabit, Spain) for 24 h at 40 °C, for 29 h at 37 °C, 60 h at 20 °C and 9 days at 10 °C. After these times, cells had attained stationary growth phase and maximum thermostolerance (data not shown). Cells suspensions were stored at 4 °C until use. No variations in heat resistance were observed during storage (up to 30 days, data not shown).

2.2. Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC as described elsewhere (Condón et al., 1993). Twenty-five milliliters of McIlvaine citrate–phosphate buffers of different pHs (from 4.0 to 7.7) were used as heating media. Once the heating medium temperature had attained stability (T ± 0.05 °C), it was inoculated with 0.2 ml of an adequately diluted cell suspension. After different heating times, 0.1 ml samples were collected and immediately pour-plated in NA. At least eight different heating times were used for each heat resistance determination.

2.3. Incubation of heated samples and survival counting

After heat treatments, the incubation of plates for survival counting was carried out at 37 °C for 24 h. Previous experiments showed that longer incubation times did not increase survivor counts (data not shown). Colony forming units (CFU) were counted.
2.4. Heat resistance parameters and fitting of data

$D_t$ values (time in minutes for survival count to drop 1 log cycle) were calculated from the slope of the straight portion of survival curves. Survival curves were drawn by plotting log of number of survivors vs. their corresponding heating times. Decimal reduction time curves (DRTC) were obtained by plotting log $D$ values vs. their corresponding treatment temperatures. $z$ values ($^\circ$C increase in temperature for $D_t$ value to drop 1 log cycle) were calculated from the slope of the corresponding DRTC. Correlation coefficients ($r_0$) and 95% confidence limits (CL) were calculated by the appropriate statistical package (StatView SE+Graphics™, Abacus Concepts, Berkeley, CA). The statistical significance ($p \leq 0.05$) of differences between the $D$ and $z$ values were tested as described by Steel and Torrie (1960). Equations to describe the influence of the growth temperature and pH on heat resistance, and correlation between observed and theoretically predicted $D_t$ values, were calculated with the Excel 5.0 software (Microsoft, Seattle, USA).

3. Results and discussion

3.1. Influence of growth temperature on $S$. typhimurium heat resistance

Growth temperature between 10 and 37 $^\circ$C modified the heat resistance of $S$. typhimurium (Fig. 1). The heat resistance of our strain grown at 37 $^\circ$C ($D_{58} = 0.33$ min) was similar to that reported by other authors in laboratory media (Doyle and Mazzota, 2000). As shown in the figure, a shift in growth temperature from 10 to 37 $^\circ$C increased the $D_{58}$ value of $S$. typhimurium from 0.092 to 0.33 min. This magnitude of the increase in heat resistance due to changes in the growth temperature was similar to that described by Ng et al. (1969) and by Dega et al. (1972). However, cells grown at 40 $^\circ$C showed the same heat resistance as those grown at 37 $^\circ$C (Fig. 1). This may be due to a lesser induction of the protective responses in cells grown at temperatures above the optimum for growth.

The mechanisms by which growth temperature affects heat resistance are not fully understood. However, it is known that bacterial cells are able to modify the lipidic composition of their cytoplasmic membrane to maintain a constant degree of fluidity, and hence, its functionality as a permeable barrier. As a consequence, any increase in growth temperature increases the saturation degree of the membrane fatty acids. This fact has been related to a higher bacterial heat resistance (Beuchat and Worthington, 1976; Katsui et al., 1981). Moreover, in $S$. typhimurium it has also been observed an increase in heat resistance as a consequence of a higher saturation degree of membrane lipids due to the addition to the culture medium of chemical compounds as sodium benzoate (Tomlins et al., 1982). Other cell structures, such as the cell wall or the outer membrane, also change their functionality with changes in the growth temperature (Pagan et al., 1999). Table 1 shows the $z$ values in pH 7 citrate–phosphate buffer of $S$. typhimurium grown at different temperatures. The 95% confidence limits and $r_0$ values for each DRTC are also included in the table. No statistically significant differences ($p \leq 0.05$) could be found among the $z$ values determined at the four temperatures. Taken into account the considerations explained above, and as described in Appendix A, we developed the following general equation to describe the effect of growth temperature up to 37 $^\circ$C ($T_g$) and treatment temperature ($T_t$) in $S$. typhimurium heat resistance:

$$\log D(T_t, T_g) = 10.52 - \frac{1}{z}T_t + (0.0243T_g)$$

where a unique $z$ value of 4.9 $^\circ$C was estimated from the whole set of data. This equation permits the
calculation of any \( D \) value of cells grown and heated at any temperature \( (r^2 = 0.99) \), within the range used in this investigation.

### 3.2. Influence of the pH of the heating medium on heat resistance of \( S. \) typhimurium

Fig. 2 shows the effect of the pH of the treatment medium on \( D_{56} \) values of \( S. \) typhimurium grown at its optimum temperature \( (37 \, ^\circ C) \). As can be seen in the figure, the heat resistance was maximum at pH 6.0, and the acidification of the heating medium to pH 4.0 decreased the decimal reduction time value from 2.2 to 0.89 min. The magnitude of the effect of the change of the pH of the heating medium was similar to that observed previously by other authors for several strains of \( S. \) typhimurium \( (Anellis et al., 1955; Blackburn et al., 1997) \) and also for other vegetative cells as \( Listeria \) monocytogenes \( (Págán et al., 1998) \) or \( Yersinia \) enterocolitica \( (Págán et al., 1999) \). It has been also reported by other authors that the maximum heat resistance of salmonellae is obtained at slightly acidified media \( (Osborne et al., 1954; Anellis et al., 1955; Blackburn et al., 1997) \). Although the mechanisms by which the pH of the heating medium influences the heat resistance are not fully known, cell envelopes seem to have an important role. Some authors suggest that alkalinity produces the solubilization of proteins and saponification of lipids of the cytoplasmic membrane \( (Teo et al., 1996) \). According to other authors, the higher thermal sensitivity of bacterial cells in acidic media could be due to the displacement, by hydrogen ions, of divalent cations, responsible for the stability of the outer membrane and also of other cellular structures, such as the ribosomes \( (Bender and Marquis, 1985) \).

The magnitude of the influence of pH on the heat resistance of \( S. \) typhimurium did not change with treatment temperature. The DRTC of \( S. \) typhimurium at an alkaline (pH 7.7) and an acidic pH (pH 6.0) are represented in Fig. 3. As can be observed in the figure, the \( z \) values were the same, and were also equal to those observed for neutral pH. Consequently, the ratio between the \( D_t \) values at the two pHs tested was constant all over the

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**Table 1**

\( z \) values in pH 7 citrate–phosphate buffer of \( S. \) typhimurium ATCC 13311, \( S. \) enteritidis ATCC 13076 and \( S. \) senftenberg ATCC 43845 grown at different temperatures

<table>
<thead>
<tr>
<th>Strain</th>
<th>( T_g ) (°C)</th>
<th>( z )</th>
<th>C.L. (95%)</th>
<th>C.L. (95%+)</th>
<th>( r_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S. ) typhimurium</td>
<td>40</td>
<td>5.0</td>
<td>4.7</td>
<td>5.4</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4.6</td>
<td>4.0</td>
<td>5.6</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.7</td>
<td>4.1</td>
<td>5.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.9</td>
<td>4.3</td>
<td>5.7</td>
<td>0.99</td>
</tr>
<tr>
<td>( S. ) enteritidis</td>
<td>40</td>
<td>4.7</td>
<td>4.1</td>
<td>5.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>5.0</td>
<td>4.6</td>
<td>5.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.4</td>
<td>4.2</td>
<td>4.6</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.5</td>
<td>4.6</td>
<td>6.9</td>
<td>0.99</td>
</tr>
<tr>
<td>( S. ) senftenberg</td>
<td>40</td>
<td>4.9</td>
<td>4.2</td>
<td>5.8</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>5.0</td>
<td>4.6</td>
<td>5.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.5</td>
<td>4.3</td>
<td>4.6</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.0</td>
<td>4.6</td>
<td>5.4</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\( T_g \): growth temperature; C.L.: confidence limits; \( r_0 \): correlation coefficient.

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**Fig. 2.** Influence of the pH of the heating medium on the decimal reduction time value at \( 56 \, ^\circ C \) of \( S. \) typhimurium grown at \( 37 \, ^\circ C \).

**Fig. 3.** Decimal reduction times curves (DRTC) in citrate–phosphate buffer of pH 6 (●) and pH 7.7 (○) of \( S. \) typhimurium ATCC 13311 grown at \( 37 \, ^\circ C \).
range of temperatures studied. These results are in agreement with those of Anellis et al. (1955) and with those of Blackburn et al. (1997) for several strains of *Salmonella*.

As other authors have previously suggested (Blackburn et al., 1997), the relationship between pH, within the range used in this study, and heat resistance at a constant temperature could be described by a second-degree polynomial equation (Fig. 2).

### 3.3. Influence of growth temperature on the effect of pH of the heating medium on the heat resistance of *S. typhimurium*

The interaction between pH of the treatment medium and growth temperature on *S. typhimurium* heat resistance is shown in Fig. 4. As can be observed in the figure, the magnitude of the influence of the pH on heat resistance decreased as the growth temperature lowered. Whereas the decimal reduction time of *S. typhimurium* grown at 40 °C decreased fourfold when alkalinizing the pH of the treatment medium from pH 6.0 to pH 7.7, in cells grown at 10 °C it only decreased twofold. Furthermore, a decrease in growth temperature shifted the pH of maximum heat resistance to the neutral zone. This interaction, related to the heat resistance at neutral pH, was described by the following expression, for *S. typhimurium* (Eq. (6), Appendix A):

\[
\log D(T_t, T_g, \text{pH}) = (-0.0039T_g - 0.035) \\
\times (7.0 - \text{pH})^2 + (0.0079T_g) \\
+ 0.0575 \times (7.0 - \text{pH}) \\
+ (10.52 - 0.204T_t) \\
+ (0.0243T_g)
\]

The correlation coefficient \(r_0\) between the experimentally determined and the theoretically calculated \(D\) values was 0.97.

As the mechanisms by which growth temperature and pH modify heat resistance are unclear, no definitive conclusions can be taken about the interaction between the two factors. However, from our results, we can conclude that the changes that growth temperature produces in bacterial cells (possibly in their envelopes), render cells with a different sensitivity to changes in pH of the treatment medium. Pagán et al. (1999) demonstrated with *Y. enterocolitica*, that the outer membranes of cells grown at 4 and at 37 °C were different, since only those cells grown at 37 °C were sensitized to lysozyme and nisin after a heat treatment. As it is known, in Gram negative bacteria,
the outer membrane acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Nikaido and Vaara, 1985). The integrity of the lipopolysaccharide layer (LPS) in this outer membrane is maintained by the presence of divalent cations as magnesium. If these cations are replaced from their positions, some LPS molecules can be released, and the outer membrane becomes more permeable to lysozyme, nisin, bile salts and some antibiotics (Nikaido and Vaara, 1985). If the acidic pH of the heating medium sensitizes bacterial cells through a displacement of divalent cations, and if the outer membrane of the cells grown at higher temperatures is more easily disrupted, as suggested in Y. enterocolitica by Pagan et al. (1999), it seems logical that the pH of the heating medium influenced the heat resistance in a higher degree in those cells grown at higher temperatures.

3.4. Validation of the equation for S. enteritidis and S. senftenberg 775W

Fig. 5 shows the the decimal reduction times at 58 °C in pH 7.0 buffer, of S. typhimurium, S. enteritidis and S. senftenberg 775W grown at 10, 20 and 37 °C. The heat resistance of S. typhimurium and S. senftenberg 775W grown at 37 °C (D58 = 0.33 and 2.8 min, respectively) was similar to that reported by other authors for these serotypes in laboratory media (Doyle and Mazzota, 2000). This could be due to the use by other authors of wild-type strains, which are generally more resistant to environmental stresses (Shah et al., 1991; Humphrey et al., 1995). As can be seen in the figure, S. senftenberg 775W was, as expected, more heat-resistant than the other two strains. Its decimal reduction times were 7- and 11-fold higher, approximately, than those of S. typhimurium and S. enteritidis, respectively, for cells grown at 37 °C. These differences were constant for all heating temperatures tested, as no statistically significant differences (p ≤ 0.05) could be found between the three z values (average z value = 4.9 °C) (Table 1).

As shown by Fig. 5, S. enteritidis heat resistance was much less affected by growth temperature than in the other two strains. Whereas a shift in growth temperature from 10 to 37 °C increased the D58 value of S. typhimurium and S. senftenberg 775W fourfold, that of S. enteritidis hardly doubled. A different behavior of several strains of the same species grown at different temperatures has already been described for Aeromonas hydrophila (Palumbo et al., 1987).

The pH of the treatment medium exerted a similar effect on the heat resistance of the three strains of Salmonella tested. Changes in pH modified their D values in a similar magnitude, and this effect was the same for the different treatment temperatures (the z values did not change) (data not shown). For these reasons, Eq. (5) described in Appendix A could predict with accuracy the influence of the pH on the heat resistance of the three strains. However, as the heat resistance of the three strains was very different (Fig. 5), and so was also the influence of growth temperature, the value Log D(Ti = 0 °C, Tg = 0 °C) and parameter d in Eq. (5) had to be substituted for each strain. The estimated values for each of these parameters for the three strains are included in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log D(Ti = 0 °C, Tg = 0 °C)</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium</td>
<td>10.52</td>
<td>0.0243</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>10.88</td>
<td>0.0078</td>
</tr>
<tr>
<td>S. senftenberg</td>
<td>11.52</td>
<td>0.0197</td>
</tr>
</tbody>
</table>
The correlation between the observed and the calculated values of the three strains is shown in Fig. 6. The correlation coefficients \( r \) between them, in a linear basis comparison, were 0.97, 0.91 and 0.98 for \( S.\ typhimurium \), \( S.\ senftenberg \) 775W and \( S.\ enteritidis \), respectively.

Although more studies would be useful to determine if every strain of Salmonella follows the same behavior as that observed in this study, our results suggest that with this equations it would be possible to calculate any \( D \) value, within the range of growth temperatures (10–37 °C), heating temperatures (52–68 °C) and pH of the heating medium (4.0–7.7) investigated, from two experimental values: two \( D_t \) values at pH 7 of cells grown at two different temperatures (at or below the optimum for growth). These two values are necessary to calculate the \( D(T_t = 0\ °C, T_g = 0\ °C) \) and the parameter \( d \) of Eq. (5), parameters that were strongly strain-dependent.

**Acknowledgements**

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**Appendix A. Mathematical equations**

The mathematical relationship between growth temperature, up to 37 °C, and heat resistance could be described by the general equation:

\[
\log D(T_t, T_g) = \log D(T_t = 0\ °C, T_g = 0\ °C) + dT_g
\]

where \( D(T_t, T_g) \) is the decimal reduction time value (min) at \( T_t \) temperature (°C) of cells precultured at \( T_g \) (between 10 and 37 °C); \( \log D(T_t = 0\ °C, T_g = 0\ °C) \) is the theoretical decimal reduction time value at this temperature \( T_t \) for cells grown at 0 °C. Parameter \( d \) represents the slope of the regression line correlating growth temperature \( T_g \) (from 10 to 37 °C) and heat resistance (Fig. 1). \( \log D(T_t = 0\ °C) \) value in Eq. (1) can be easily calculated from the corresponding DRTCs, following the general equation:

\[
\log D(T_t = 0\ °C) = \log D(T_t = 0\ °C, T_g = 0\ °C) - T_t/z
\]

where \( \log D(T_t = 0\ °C, T_g = 0\ °C) \) is the theoretical decimal reduction time at 0 °C of a suspension grown at 0 °C, \( z \) is the negative inverse of the slope and \( T_t \) is the treatment temperature (°C).

As the effect of the growth temperature was the same at all heating temperatures tested (Table 1), we developed the following general equation by combining Eqs. (1) and (2):

\[
\log D(T_t, T_g) = \log D(T_t = 0\ °C, T_g = 0\ °C) - T_t/z + (dT_g)
\]

where a unique \( z \) value of 4.9 °C was estimated from the whole set of data. \( \log D(T_t = 0\ °C, T_g = 0\ °C) \) and parameter \( d \) are included in Table 2.

The relationship between pH, within the range used in this study, and heat resistance at a constant temperature could be described by a second-degree polynomial equation (\( \log D_t = a \times pH^2 + b \times pH + c \)), in which parameters \( a \) and \( b \), which were determined stepwise, varied linearly with growth temperature \( (r^2 = 0.88 \text{ for both of them}) \). This interaction, related
to the heat resistance at neutral pH, was described by the equation:

$$\log D(T_t, T_g, \text{pH}) = (-0.0039T_g - 0.035) \times (7.0 - \text{pH})^2 + (0.0079T_g + 0.0575) \times (7.0 - \text{pH})$$

$$+ \log D(T_t, T_g, \text{pH} = 7).$$

(4)

where \(D(T_t, T_g, \text{pH})\) is the decimal reduction time at a \(T_t\) treatment temperature, and pH value between 4 and 7.7, of cells grown at \(T_g\) growth temperature; and \(D(T_t, T_g, \text{pH} = 7)\) is the decimal reduction time at pH 7.0 and at this same temperature \(T_t\).

As treatment temperature did not change the magnitude of the effect of pH on heat resistance (Fig. 4), the value \(D(T_t, T_g, \text{pH} = 7)\) can be easily calculated from growth temperature (between 10 and 37 °C) and treatment temperature with Eq. (3). By combining Eqs. (3) and (4), we obtained the following general expression:

$$\log D(T_t, T_g, \text{pH}) = (-0.0039T_g - 0.035) \times (7.0 - \text{pH})^2$$

$$+ (0.0079T_g + 0.0575) \times (7.0 - \text{pH})$$

$$+ (\log D(T_t = 0 \, ^\circ C, T_g = 0 \, ^\circ C)$$

$$- T_t/2 + (dT_g)).$$

(5)

Which is, for \(S. typhimurium\):

$$\log D(T_t, T_g, \text{pH}) = (-0.0039T_g - 0.035) \times (7.0 - \text{pH})^2 + (0.0079T_g + 0.0575) \times (7.0 - \text{pH})$$

$$+ (10.52 - 0.204T_t$$

$$+ (0.0243T_g).$$

(6)

References


