Quantifying the effects of heating temperature, and combined effects of heating medium pH and recovery medium pH on the heat resistance of Salmonella typhimurium

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3 **pH and recovery medium pH on the heat resistance of *Salmonella typhimurium***

4

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12 Abstract

13 The influence of heating treatment temperature, pH of heating and recovery medium on the

14 survival kinetics of *Salmonella typhimurium* ATCC 13311 are studied and quantified. From

15 each non log linear survival curve, Weibull model parameters were estimated. An average

16 shape parameter value of 1.67 was found, which is characteristic of downward concavity

17 curves and is in agreement with values estimated from other *Salmonella typhimurium* strains.

18 Bigelow type models quantifying the heating temperature, heating and recovery medium pH

19 influences, are fitted on scale parameter δ data, (time of first decimal reduction), which

20 reflects the bacterial heat resistance. The estimate of z_T (4.64°C) is in the range of values

21 given in literature for this species. The influence of pH of the heating medium on the scale

22 parameter (z_{pH}: 8.25) is lower than that of the recovery pH medium influence (z'_{pH}: 3.65)

23 **Key words:** Weibull, heating medium, recovery medium, pH, *Salmonella typhimurium*

24 **Nomenclature**

25 N_0: initial number of cells .

26 N: number of surviving cells after the heating time t.

27 δ: scale parameter : first decimal reduction of surviving spores or cells from N_0 to $N_0/10$.

28 p: shape parameter.

29 T: heating temperature.

30 pH: heating medium pH.

31 pH': recovery medium pH.

32 T^*: reference temperature fixed at 60°C.

33 pH^*: reference pH of the heating medium fixed to 7 .

34 pH'_{opt} : recovery medium:pH corresponding to the maximal apparent bacterial heat resistance.

35 z_T: distance of T from T^* which leads to a ten fold reduction in δ-value z_T quantifies the

36 influence of the heating temperature on the bacterial heat resistance.

37 z_{pH} : distance of pH from pH^* which leads to a ten fold reduction in δ-value z_{pH} quantifies the

38 influence of the pH of the heating medium on the bacterial heat resistance.

39 z'_{pH} : distance of pH' from pH'_{opt}, which leads a ten fold reduction in apparent δ -value. z'_{pH}

40 characterises the influence of the pH on the recovery of the micro-organism after a heat

41 treatment .

42 δ^* :estimated δ value corresponding to T*, pH^* and pH'_{opt} conditions.

43

44

Introduction

Salmonellae continue to be a major public health setting a great problem to the food industry. These bacterial species appear in a wide variety of foods and food ingredients. Various heat treatments implemented by the food processing industry are generally effective for destroying the vegetative bacteria. Taking into account not only temperature but other environmental factors is likely to allow significant reduction of heat treatment intensity with the same microbial safety and minimize damage of heat sensitive food. It is commonly agreed that the pH decrease of the heating medium is the main environmental factor after temperature, which reduces the bacterial heat resistance of spores (Bigelow and Esty, 1920) or vegetative form (White, 1963; Blackbrun, Curtis, Humpheson, Billon & McClure, 1997). The same effect was observed for *Salmonella enteritidis*, (Casadei, Ingram, Hitchings, Archer & Gaze, 2001) and for *Salmonella typhimurium* (Mazzotta, 2001).

Moreover the pH of the recovery medium highly influences the apparent heat resistance of bacterial spores for the same heat treatment condition. The *D* values decrease when pH values shift from an optimum (Cook and Brown, 1965). However, as far as we know, the influence of the recovery medium pH on the apparent heat resistance of Salmonella species has never been described. A decrease of pH of the heating medium or of the recovery medium both reduces the bacterial heat resistance (Couvert, Leguérinel & Mafart, 1999). Theses influences can be taken into account for reducing heat treatments. Such a cumulative effect is more or less specific of pH and is not observed for water activity where, oppositely, the protective effect of a low water activity of the heating medium tends to be balanced by the selectivity of a low water activity of the recovery medium towards injured cells (Coroller, Leguérinel & Mafart, 2001).

68 The aim of this study was to quantitatively characterise the impact of heating temperature, pH

69 of the heating medium and pH of the recovery medium on the heat resistance of *Salmonella*

70 *typhimurium* from relevant parameters of appropriated primary and secondary models

71

72 **Materials and methods**

73 *Strain and culturing conditions*

74 The studied strain was *Salmonella typhimurium* ATCC 13311 (NCTC 74). Cultures were

75 stored in cryotube in mixing nutrient broth 50% glycerol 50% at -70°C.

76 The basic heating medium was tryptone salt broth (10g/l tryptone USP (Biokar Diagnostics,

77 A1401HA) and 10g/l NaCl). The pH was adjusted with addition of H_2SO_4 and sterilised by

78 filtration through 0.22µm porosity filter.

79 The recovery medium was nutrient agar (Biokar Diagnostics, BK021HA). The pH were

80 adjusted with H_2SO_4 prior autoclaving at 121°C for 15 minutes. The pH values were checked

81 after autoclaving.

82 *Preparation of cells suspension*

83 Nutrient broth (Biokar Diagnostics, BK003HA) in 200ml flask was inoculated with

84 *Salmonella typhimurium* and incubated at 37°C for 24 hours under agitation (150 rpm). The

85 culture (40ml) was centrifuged (2000g 15min at 20°C) and re-suspended in 3 ml of heating

86 medium.

87 *Thermal treatment of bacterial suspension and recovery conditions*

88 Capillary tubes of 200 µl (Ringcaps® Duran®) were filled with 100µl of sample and

89 submitted to a thermal treatment in a thermostated water bath. After heating, the tubes were

90 cooled in water/ice bath. After rinsing, the ends were flamed with ethanol. The capillary tubes

91 were broken at both ends and their contents poured into a tube containing 9 ml sterile tryptone

92 salt broth (Biokar Diagnostics, BK014HA) by rinsing with 0.9 ml tryptone salt broth.

93   Viable cells were counted by duplicate plating in ajusted pH nutrient agar (Biokar

94   Diagnostics, BK021HA) and incubated at 37°C for 48h.

95   *Experimental design*

96   To determine survival kinetic parameters, bacteria, for each sample corresponding to different

97   heating times, were counted on nutrient agar plates.

98   Heating temperatures applied were 53, 55, 57 and 59°C (heating and recovery media pH equal

99   7). For studying the effect of pH, a complete factorial design was implemented according to

100   the following levels of pH of the heating medium: (7, 6.5, 6, 5.3, 5, 4.4 and 3.8) and to the

101   following levels of pH of the recovery medium (denoted pH'):7, 6.5, 6, 5.5 and 5 (controlled

102   pH values are given table3) at 55°C.

103   *Data from literature*

104   Data taken from figures in literature were scanned and digitized using the software program

105   DigXY 1.2 (Thunderhead Engineering, Manhattan, USA).

106   *Primary and secondary models*

107   Different authors considered the survival curve as a cumulative form of temporary

108   distribution of lethality event frequency (Cunhan, Oliveira & Oliveira, 1998; Peleg & Cole,

109   1998; Fernandez, Salmeron, Fernandez & Martinez, 1999). In 2002, Mafart, Couvert,

110   Gaillard, and Leguerinel proposed a new presentation of the Weibull frequency distribution

111   model.

112   $$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \quad \text{Eq1}$$

113   Parameter *p* characterises the shape of the curve: concave curves *p*<1, convex curves *p*>1 and

114   linear curves *p*=1, in this case δ value corresponds to classical *D* value. This equation was

115   taken up during the IFT summit in January 2003 (Heldman and Newsome, 2003), was used

116   by different authors (Mafart et al., 2002; Gómez, García, Álvarez, Raso & Condón, 2005;

117   Geeraerd, Valdramidis & Van Impe, 2005; Virto, Sanz, Álvarez, Condón & Raso, 2005;

118   Carlin et al., 2006) and was implemented in this work as primary model.

119   The influences of environmental factors on the Weibull model parameter estimated from

120   survival kinetics related to bacterial spores or vegetative cells, were studied (Fernandez et al.,

121   1999; Fernandez, Collado, Cunhan, Ocio & Martinez, 2002; van Boekel, 2002; Couvert,

122   Gaillard, Savy, Mafart & Leguerinel 2005).These studies showed that the shape parameter

123   was practically independent of the heating temperature and the pH of the heating medium.

124   The conclusion of these studies leads us to determine a single average shape parameter value

125   for a set of kinetics.

126   The δ value, first decimal reduction time, is highly influenced by heating temperature. The

127   classical Bigelow model was used to describe the influence of heating temperature on the δ

128   with the conventional z_T value.

129   The effect of the pH of the heating medium and the pH' of the recovery medium on the heat

130   resistance was described according to the following equation (Leguérinel, Spegagne, Couvert,

131   Gaillard & Mafart, 2005) :

132

$$\log \delta = \log \delta * - \left| \frac{pH - pH*}{z_{pH}} \right| - \left(\frac{pH' - pH'_{opt}}{z'_{pH}} \right)^2 \quad \text{Eq 2}$$

133   *Curve fitting.*

134   In a first time, N_0, δ and p values are estimated from each survival curve to assess the

135   influence of environmental factors on these parameters.

136   In a second time a single shape parameter p value was estimated from the corresponding

137   whole set of experimental kinetics and from set of kinetics taken from the literature. Scale

138   parameter δ values and $\log N_0$ values were determined for each curve.

139

140   Bigelow parameter z_T was estimated from scale parameter δ values obtained from the

141   temperature mono factorial design. Equation 2 parameter values: z_{pH} and z'_{pH} were estimated

142   from scale parameter δ values obtained from pH factorial design.

143   The parameter values and their associated confidence interval were estimated by using a non-

144   linear module (nlinfit and nlparci Matlab 6.1, Statistical Toolbox, The Mathworks).

145

146   **Results and discussion**

147   Survival kinetics curves of *Salmonella typhimurium* showed a clear downward concavity. The

148   same pattern of curves was elsewhere observed for different *Salmonella typhimurium* strains

149   (Garibaldi, Ljichi & Bayne, 1969; Mackey and Derrick, 1986; Jäckle, Geiges & Schmidt-

150   Lorenz, 1987). Such non linear curves were fitted according to the Weibull model.

151   The influences of the heating temperature, the pH of the heating and of the recovery medium

152   on the shape parameter *p* are shown Figure 1. *p* values appear to be not clearly influenced by

153   these environmental factors. This observation is in agreement with those of Fernandez *et al*.

154   (2002), Collado, Fernandez, Rodrigo, Camats and Martinez Lopez. (2003) regarding *Bacillus*

155   *cereus*, Couvert *et al*. (2005) ,and *Bacillus pumilus* and van Boekel's (2002). These authors

156   did not observe any significant influence of the temperature on the shape parameter *p*. The

157   presented results (Figure 1) show no clear influence of the heating medium or the recovery pH

158   on estimates of *p* for the lower pH. However structural correlation between parameters *p* and

159   δ could explain the variability of *p* values (Couvert *et al*., 2005).

160   Then, a single average *p* value was estimated, regardless of the heating temperature, the pH of

161   the heating and of the recovery medium. The three parameters (N_0, δ and *p*) were globally

162   estimated from the whole set of data by using the least square regression method (nlinfit

163   Matlab 6.1). The single *p* value estimated from our set of data (1.677 ± 0.065) is close to the p

164   value estimated from other sets of data from literature for the same Salmonellae species:

165   1.648 ± 0.313 (Jäckle *et al.*,1987), 1.538 ± 0.187 (Garibaldi *et al.,* 1969), 1.429 ± 0.295

166   (Mackey & Derrick, 1986). *log N₀* , the scale parameters δ and their confidence interval

167   coefficients as functions of different pH and temperature conditions, are presented in Table 1

168   and 2. The scale parameter δ values are influenced by environmental factors: heating

169   temperature, pH of the heating and the recovery medium. Within the investigated temperature

170   range (53°C -59°C) the classical Bigelow relationship was kept to quantify the effect of

171   temperature on δ value. The corresponding z_T value 4.64°C (Table 3) is lower than z_T values

172   determined from Jäckle *et al*. (1987) data for the same bacterial strain but are in agreement

173   with z_T value estimated from Mackey and Derrick data (1986) and with other values found in

174   literature. Doyle and Mazzotta (2000) reviewed z_T values concerning different *Salmonella*

175   *typhimurium* strains heated in different media. These z_T values, ranging from 3.24°C to 9.5°C

176   with a mean of 5.56°C, illustrates the large variability of z_T values reported in literature. For

177   the same *Salmonella typhimurium* strain ATCC13311 Casadei *et al.* (2001) reported a z_T-

178   value of 4.6°C (Table 4).

179   Both the pH of the heating and the recovery medium affect the heat resistance of *Salmonella*

180   *typhimurium* as shown Figure 2. A decrease of the pH of the heating medium reduces the

181   salmonella heat resistance. This observation has been reported by Blackburn *et al*. (1997) and

182   Casadei *et al.* (2001). Concerning the influence of the recovery medium, low pH reduces heat

183   resistance parameter value δ of *Salmonella typhimurium*. A similar effect was observed for

184   the thermal inactivation of bacterial spore (Cook and Brown, 1965; Lopez, Gonzalez, Mazas,

185   Gonzalez, Martin & Bernardo, 1997) but never for vegetative bacteria cells.

186   Equation 2 was used to describe the effect of the heating medium pH and the recovery

187   medium pH on δ values. This model does not take interactions between these two

188   environmental factors into account. To estimate the weight of the possible interaction, a

189   variance analysis was performed. For the studied pH range, the weight of heating and

190 recovery medium pH represent 62.9% and 34.7% respectively, while the unexplained

191 variance comprising interactions represented only 2.4% of the total variability. This

192 observation concerning *Salmonella typhimurium* is in agreement with Couvert et al. (1999)

193 results related to *Bacillus cereus* spores. Such observations led us to neglect interactions and

194 to retain the simple Equation 2 without crossed term. Model parameters (Eq 2) determined for

195 *Salmonella typhimurium* (Table 5) were estimated from the whole set of δ values, according

196 to factorial design.

197 The determination coefficient between experimental and calculated values (R² :0.958) and

198 Figures 3a and 3b illustrate the goodness of fit of the model. The high z_{pH} parameter value

199 8.25, indicates a poor influence of the heating medium pH on the heat resistance for this

200 Salmonella strain. For other salmonella strains few data allow to evaluate this z_{pH} parameter.

201 From Blackburn et al. (1997), Casadei et al. (2001) and Mañas, Pagán, Raso and Condón

202 (2003) data, an optimal pH value appears to be variable and often lower than 7. This

203 observation leads to replace a fixed reference *pH**, equal to 7, by a variable optimal pH

204 parameter (*pH$_{opt}$*) to be estimated Eq 3.

205
$$\log \delta = \log \delta_{opt} - \left(\frac{T - T*}{z_T} \right) - \left| \frac{pH - pH_{opt}}{z_{pH}} \right| \quad \text{Eq 3}$$

206 Parameters z_{pH}, pH_{opt} and z_T estimated from published values are shown Table 4. Regarding

207 the studied bacterial strain and different bacterial species, z_{pH} values estimated from data of

208 literature and our own data are lower than z_{pH} obtained in this work, even for the same

209 *Salmonella typhimurium* strain ATCC 11331. This difference could be explained by errors on

210 *D* value estimates due to the fitting of concave curves by linear regressions or growth

211 condition and physiology state of bacteria which can have produced stress protein.

212 Concerning the effect of the pH of the recovery medium, the z'_{pH} value (3.6) indicates a

213 higher influence of this factor than that of the heating medium pH. It is generally accepted

214   that the pH of the recovery medium exerts a large influence on the apparent heat resistance of

215   spores: D-values decrease as pH is reduced (Cook and Brown, 1965; Yokoya and York, 1965;

216   Cook and Gilbert, 1968; Mallidis and Scholefield, 1986; Santos and Zarzo, 1996; Lopez et al.,

217   1997). Observed z'_{pH} values, which characterized the influence of the recovery medium pH,

218   for *Salmonella typhimurium* cannot be compared with values regarding other Salmonella

219   strains or species. As far as we know, no data which could be compared to our results, are

220   available from literature.

221   The primary model derived from the Weibull distribution and describing non linear survival

222   kinetics associated with Bigelow type secondary models, can be easily used to optimise heat

223   treatment process calculations taking the heating and recovery pH influences into account. For

224   example, compared to a heat treatment in food at pH 7, a heat treatment for *Salmonella*

225   *typhimurium* in food at pH 5, could reduce the heating time to a 3.5 ratio, or, with the same

226   heating time, could reduce the heating temperature of 2.25°C with the same lethal efficiency.

227   In practice, to ensure safety of acid foods, heating pH is the pH of food before heat treatment.

228   Because foods represent both the heating and the recovery medium, the input recovery pH is

229   likely to keep the value of the heating pH. However, it frequently occurs that a decrease of

230   food pH is observed during the heat treatment. In this case, for safety reasons, it is

231   recommended to retain the value of the pH which is measured immediately after the heat

232   treatment.

233   This work confirms the impact of low recovery medium pH on the apparent heat resistance.

234   This influence allows reducing heat treatments with the same safety objective to keep better

235   nutritional and sensory quality of foods. This approach could be extended to other vegetative

236   strains and species which would require further data: p and z parameter values, related to

237   heating temperatures and heating and recovery medium pH. From these data, standard values

238   of z_{pH} and z'_{pH} could be defined according to a similar approach to the one that was

239 implemented for the standard z_T value equal to 7°C for most resistant vegetative cells and

240 input in pasteurisation process calculations.

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332   death rate" of endospores of aerobic, thermophilic bacteria. Applied Microbioloy 13, 993-

333   999.

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338

339 **Table legends**

340 Table1

341 log N_0 and δ estimates and their confidence interval coefficients as functions of the heating

342 temperature.

343

344 Table 2

345 log N_0 and δ estimates and their confidence interval coefficients as functions of the heating

346 and the recovery medium pH.

347

348 Table 3

349 Parameters z_T estimated (from log δ values determined) from our own (data) and published

350 data for different *Salmonella* strains

351

352 Table 4

353 z_T, z_{pH} and pH_{opt} estimates from published classical D values for different *Salmonella* species

354

355 Table 5

356 z_{pH}, z'_{pH}, pH'_{opt} estimates for *Salmonella Typhimurium* ATCC 13311 (log δ values)

357

Figure legends

Figure 1

Weibull shape parameter values and their confidence interval 95% evaluated for each kinetic

of heating temperature, and heating and recovery medium pH

Figure 2

Survival kinetics experimental data and fitted curves, with p–value equal1.67, at different

heating medium pH for different recovery pH (pH 7 ●, pH 6.5 □, pH6▲, pH5.5 ▽, pH 5 ◆)

and at different temperatures (59°C7 ●, 57°C □, 55°C ▲, 53°C ▽) heating and recovery pH
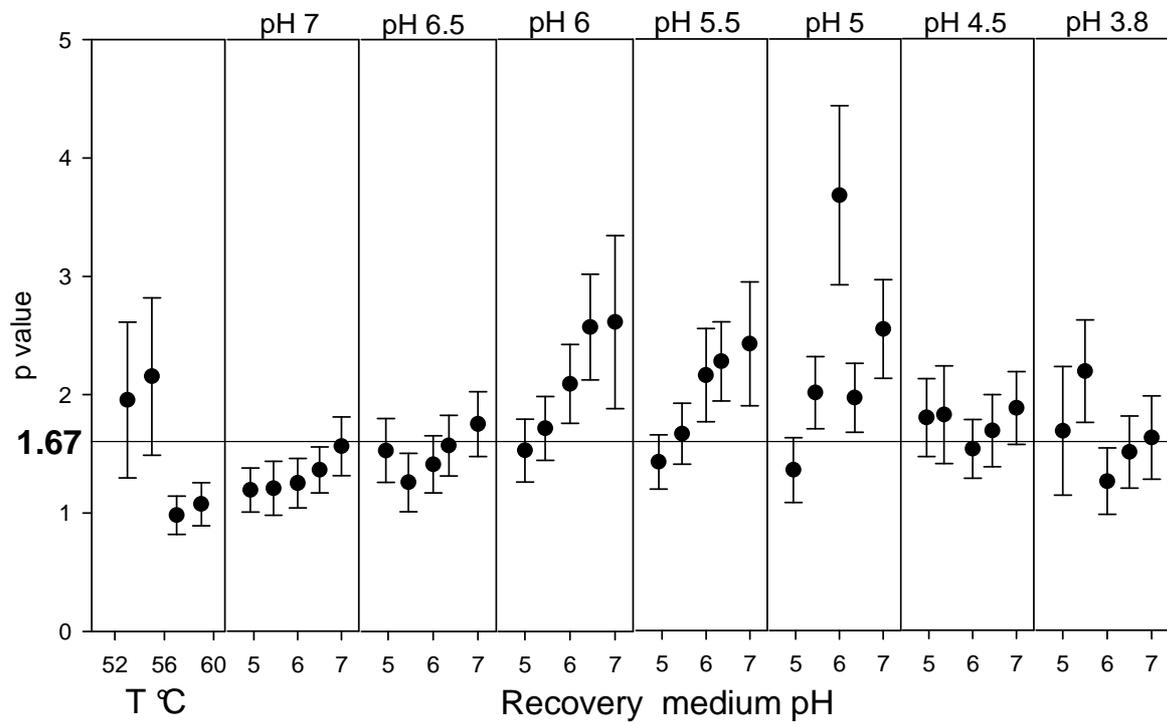
fixed at 7

Figure 3a 3b

Observed and calculated log δ values for different conditions of heating and recovery medium
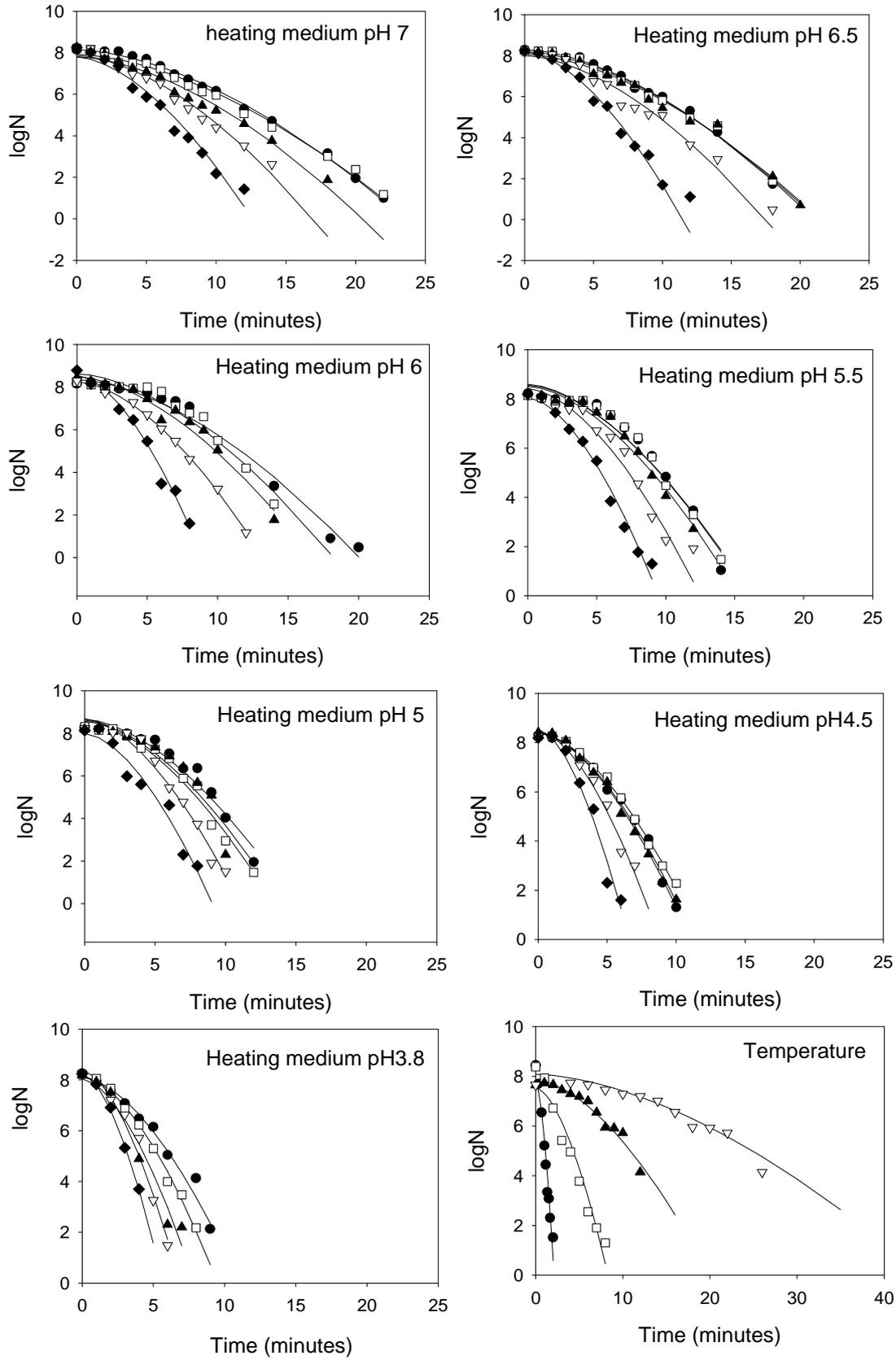
pH

375

376 T°C   Recovery medium pH

377
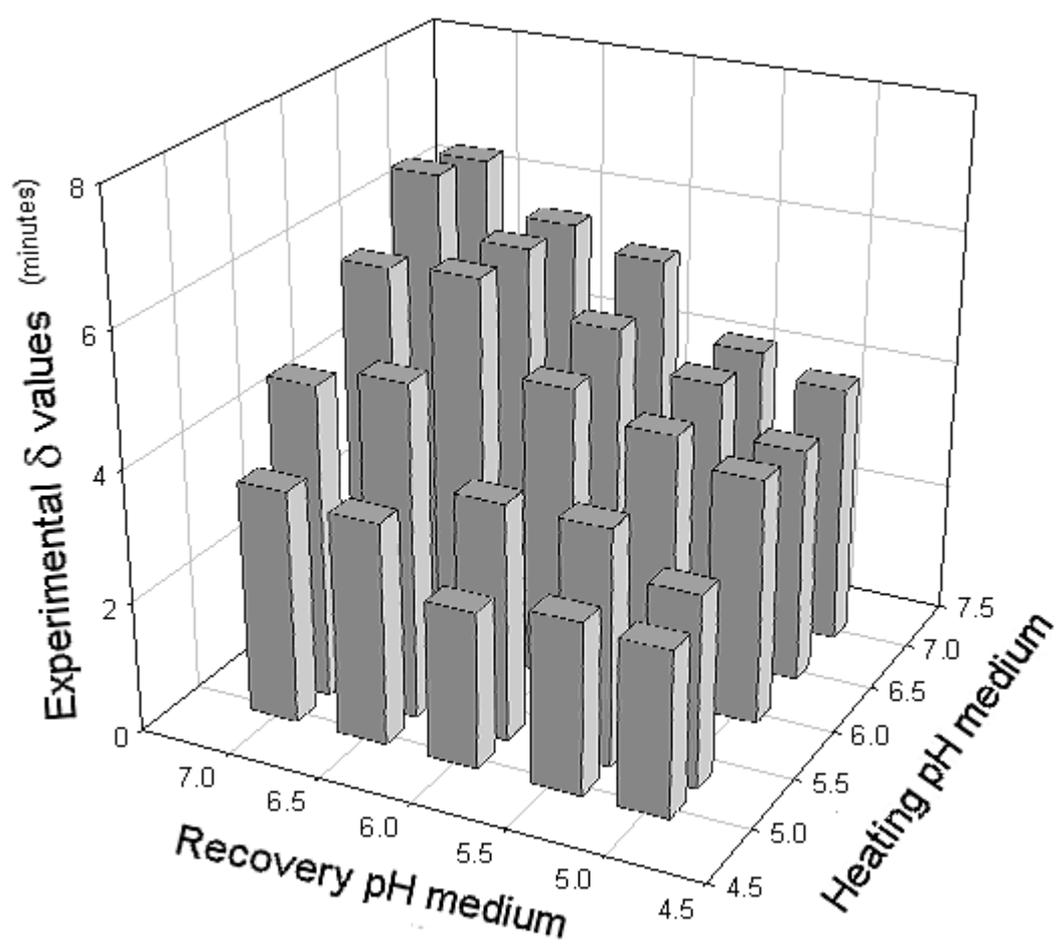
378 Figure 1

379
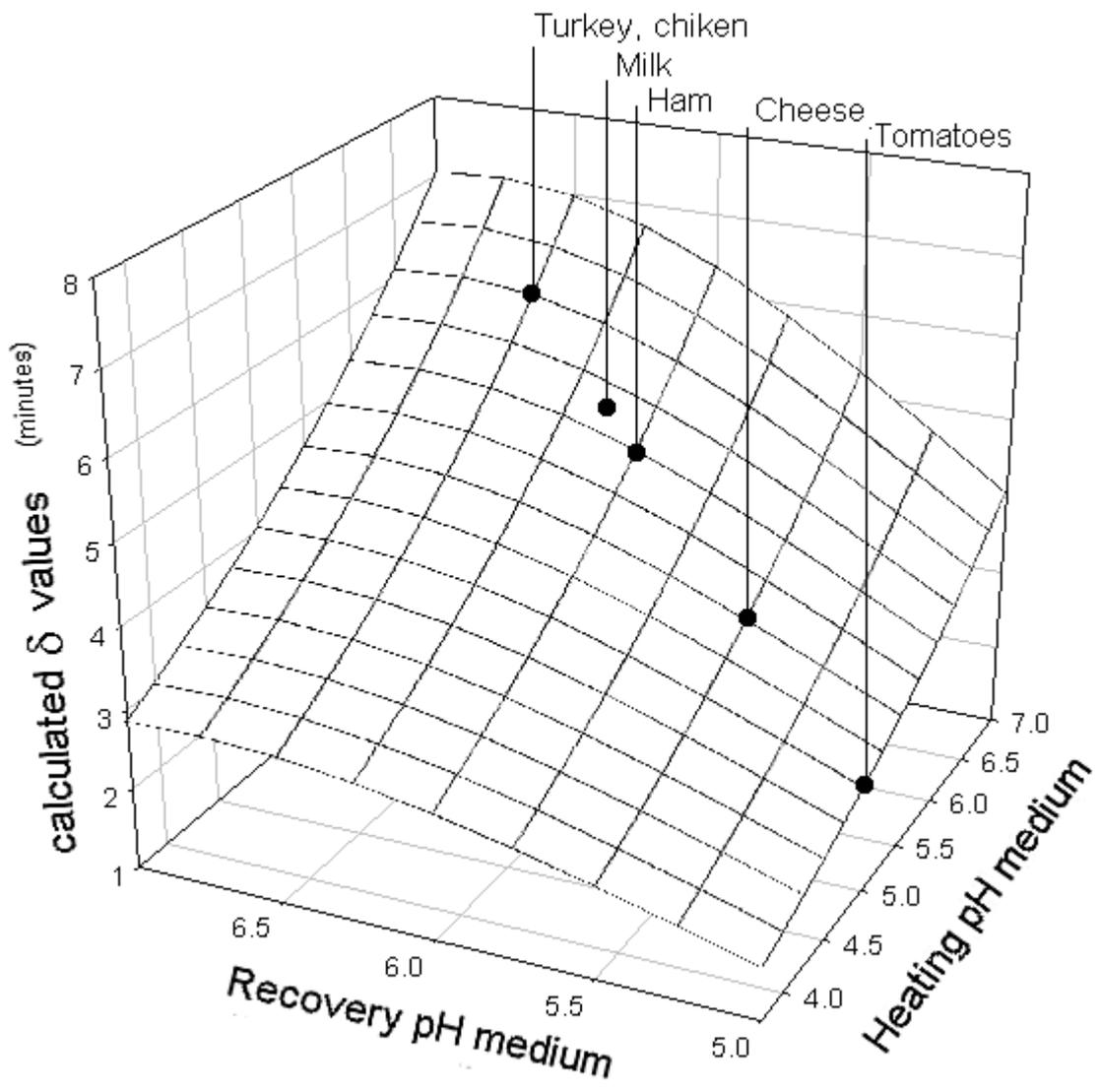
380

381   Figure2

382
383   figure 3a
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385
386
387   Figure 3 b
388

389
390
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Heating temperature °C	$\log N_0$	CI $_{95\%}$	δ minutes	CI $_{95\%}$
59	7.730	± 0.385	0.619	± 0.047
57	7.522	± 0.352	2.495	± 0.182
55	7.837	± 0.297	5.846	± 0.690
53	8.077	± 0.315	12.741	± 1.547

392   Table 1
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		Global p value		1.677	±0.065
Heating pH	Recovery pH	$\log N_0$	CI $_{95\%}$	δ minutes	CI $_{95\%}$
7.02	7	8.155	±0.244	6.734	±0.475
7.02	6.5	7.925	±0.244	6.922	±0.499
7.02	6	7.812	±0.257	6.010	±0.447
7.02	5.45	7.872	±0.281	4.949	±0.380
7.02	4.92	7.808	±0.302	3.696	±0.258
6.5	7	8.295	±0.257	5.956	±0.440
6.5	6.35	8.197	±0.257	6.059	±0.454
6.5	6	8.067	±0.257	6.178	±0.472
6.5	5.45	7.998	±0.281	5.063	±0.397
6.5	4.95	8.191	±0.321	3.276	±0.236
6	7	8.356	±0.270	5.657	±0.370
6	6.45	8.626	±0.281	5.041	±0.394
6	6	8.489	±0.284	4.683	±0.367
6	5.45	8.261	±0.323	3.760	±0.277
6	5	8.570	±0.352	2.483	±0.181
5.3	7	8.599	±0.283	4.458	±0.314
5.3	6.35	8.559	±0.282	4.506	±0.319
5.3	6	8.514	±0.299	4.273	±0.335
5.3	5.45	8.425	±0.320	3.513	±0.268
5.3	4.92	8.066	±0.336	2.731	±0.191
5	7	8.659	±0.300	4.107	±0.310
5	6.35	8.568	±0.302	3.728	±0.261
5	6	8.613	±0.319	3.857	±0.324
5	5.45	8.684	±0.322	3.047	±0.209
5	4.95	7.990	±0.356	2.628	±0.201
4.4	7	8.414	±0.321	3.171	±0.223
4.4	6.45	8.392	±0.321	3.338	±0.244
4.4	6	8.400	±0.322	3.114	±0.217
4.4	5.35	8.488	±0.352	2.458	±0.178
4.4	4.95	8.548	±0.394	1.836	±0.141
3.8	7	8.117	±0.322	3.170	±0.226
3.8	6.5	8.152	±0.351	2.722	±0.214
3.8	6	8.045	±0.398	2.281	±0.182
3.8	5.5	8.402	±0.394	1.935	±0.155
3.8	5	8.264	±0.455	1.611	±0.183

Table2

Bacterial strain		Heating medium	n*	T°C range	z_T°C	CI95 %	R²
Salmonella typhimurium ATCC13311		Tryptone salt broth	4	51-59	4.64	±0.877	0.989
Salmonella typhimurium ATCC13311	Jäckle *et al.* 1987	Tryptone salt broth	3	58.5-61.5	8.55	±1.796	1.000
Salmonella typhimurium NCBI 10248	Mackey & Derrick 1986	Tryptone salt broth	5	50-59	3.44	±0.510	0.994

403 * n: data number
404 Table3
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Bacterial strain		Heating medium	n	T°C range	$z_T°C$	CI 95%	pH range	z_{pH}	CI 95%	pH_{opt}	R²
Salmonella typhimurium ATCC13311	Manas *et al.* 2003	Citrate buffer	8				4-7.7	3.27	0.969	5.75	0.939
Salmonella enteritidis P167807	Blackburn *et al.* 1997	Tryptone salt broth + HCl /NaOH	8				4.3-9.5	3.92	2.261	6.00	0.724
Salmonella typhimurium NCTC 74	Casadei *et al.* 2001	Nutrient broth + Citric acid	8	48-54	4.61	1.204	7 & 3	2.17	0.203	ND Fixed at 7	0.994

* n: data number

Table 4

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	Parameter values	CI 95%
log $\delta*_{55°C,\ pH7,\ pH'opt}$	0.851	±0.053
z_{pH}	8.254	±1.572
z'_{pH}	3.655	±1.349
pH'_{opt}	6.805	±0.656
R²	0.958	

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426   Table 5
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