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Ozone Inactivation of Acid Stressed *Listeria Monocytogenes* and *Listeria Innocua* in Orange Juice Using a Bubble Column

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1 Title: “**Ozone inactivation of acid stressed *Listeria***
2 ***monocytogenes and Listeria innocua* in orange juice using a**
3 **bubble column”**

4

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25 **Abstract**

26 Orange juice inoculated with *Listeria monocytogenes* strains ATCC 7644, NCTC 11994
27 and *Listeria innocua* NCTC 11288 (10^6 CFU/ml) as challenge microorganisms was
28 treated with direct ozone at 0.098mg/min/ml for different time periods (0-8 min) using an
29 ozone bubble column. Ozone treatment of mild acid stressed and mild acid stress-
30 habituated (pH 5.5) cells of *L. monocytogenes* resulted in higher inactivation times
31 compared to control non-acid stressed cells. Additionally acid stressed cells habituated in
32 orange juice (ATCC 7644 & NCTC 11288), showed higher inactivation times during
33 ozonation by comparison with the control as well as the mild-acid stressed cells. Overall
34 the gaseous ozone treatment applied to orange juice resulted in a population reduction of
35 5 log cycles within a time range that varied between 5 to 9 min.

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37 **Key words: *Listeria monocytogenes*, ozone, bubble column, non-thermal inactivation,**
38 **acid stress, orange juice, microbial kinetics**

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48 **1 Introduction**

49 *Listeria monocytogenes* is a Gram positive, psychrotrophic pathogen ubiquitous in the
50 environment and has been found in fruits and vegetables. *L. monocytogenes* is capable of
51 growing at refrigeration temperatures in high salt and acid foods. *L. innocua* is often
52 selected for inactivation studies because it is non pathogenic but still closely related to *L.*
53 *monocytogenes* (Picart, Dumay & Cheftel, 2002). No outbreaks involving *L.*
54 *monocytogenes* in fruit juices have been reported; however this pathogen has been
55 isolated from unpasteurised apple juice (pH 3.78) and apple-raspberry juice blend (pH
56 3.75) after 1 day storage at 5 °C (Sado, Jinneman, Husby, Sorg & Omiecinsky, 1998).
57 This pathogen is a vehicle of human listeriosis which survived well beyond the normal
58 shelf life of unsterile orange juices (Ryser & Marth, 1991). Oyarzábal, Nogueira and
59 Gombas (2003) studied the survival of *L. monocytogenes* and other foodborne pathogens
60 in apple, orange, pineapple, and white grape juice concentrates and showed that these
61 pathogens were recoverable from all concentrates through 12 weeks of storage at -23 °C.
62 The low pH of fruit juices plays an important role in survival of food borne pathogens.
63 The ability of *L. monocytogenes* to respond to low pH conditions plays an integral role in
64 its survival and resistance to acidic foods (Cotter, Gahan & Hill, 2000), thus affecting the
65 food processing and preservation protocols. The organism can become highly resistant to
66 even extremely acidic conditions due to stress hardening (Lou & Yousef, 1997). Some
67 studies have shown that Acid Tolerance Response (ATR) of *L. monocytogenes*, as a
68 consequence of stress hardening, can result in its increased thermal tolerance in apple,
69 orange and white grape juice (Mazzotta, 2001). Strategies to meet consumer demands for
70 better quality food products include minimal processing, which could introduce potential

71 for pathogen survival. Caggia, Ombretta, Restuccia and Randazzo (2009) reported that
72 orange juice and minimally processed orange juice slices can support the growth of acid
73 adapted *L. monocytogenes*. In food processing technologies, there is an extensive use of
74 low pH environments (decontamination by acetic acid in beef processing, fermentation
75 etc.) which can result in the alteration of the cellular physiology of the pathogen either by
76 *de novo* protein synthesis or by changes in the fatty acid composition of the cell
77 membrane (Foster 1991, Phan-Thanh, Mahouin, & Alige, 2000). This can lead to
78 enhanced resistance to any further or subsequent acid stress which may be part of a
79 processing treatment. This acid tolerance is also termed as acid habituation which is the
80 increased resistance to extreme pH conditions after adaptation to sublethal acidic
81 environments (Koutsoumanis & Sofos, 2004). *L. monocytogenes* is more resistant than
82 many foodborne pathogens to organic acids and can be difficult to control in food
83 processing facilities (Johnson, 2003), therefore it is necessary to evaluate responses of
84 *Listeria* cells exposed to different acidic conditions.

85 The US Food and Drug Administration (US FDA) issued a final rule requiring fruit and
86 vegetable juice producers to apply a 5-log pathogen reduction process (US FDA, 2004_a).
87 In recent years consumers have increasingly sought ready to use 'fresh-like' products,
88 which are usually refrigerated. This has led the food industry to develop alternative
89 processing technologies, to produce foods with a minimum of nutritional,
90 physicochemical, or organoleptic changes induced by these technologies (Esteve &
91 Frigola, 2007), whilst maintaining safety profiles with respect to pathogens of concern.
92 The FDA's approval of ozone as a direct additive to food in 2001 triggered interest in
93 ozone applications, with a number of commercial fruit juice processors in the US and

94 Europe employing ozone for pasteurization, resulting in industry guidelines being issued
95 by the FDA (USFDA, 2004_b). Ozone is a triatomic allotrope of oxygen and is
96 characterized by a high oxidation potential that conveys bactericidal and viricidal
97 properties (Burlison, Murray & Polard, 1975; Kim, Yousef & Dave, 1999). Ozone
98 inactivates microorganisms through oxidization and residual ozone decomposes to
99 nontoxic products (i.e. oxygen) making it an environmentally friendly antimicrobial agent
100 for use in the food industry (Kim et al., 1999). Ozone as an oxidant is used in natural
101 water treatment, washing and disinfecting of fruits and vegetables, and juice processing
102 to inactivate pathogenic and spoilage microorganisms (Muthukumarappan, Halaweish &
103 Naidu, 2000). In a gas or aqueous phase, ozone has been used to inactivate
104 microorganisms and decontaminate meat, poultry, eggs, fish, fruits, vegetables and dry
105 foods (Fan, Song, McRae, Walker & Sharpe, 2007). Tiwari, Muthukumarappan,
106 O'Donnell and Cullen (2008, 2009_a) and Tiwari, O'Donnell, Patras, Brunton and Cullen
107 (2009_b) recently highlighted that nutritional quality depends on the ozone control
108 parameters of concentration and gas flow rate. Achieving rapid microbial inactivation
109 using optimized control parameters while retaining the nutritional quality is of overall
110 importance.

111 The objectives of this study were to investigate (i) the efficacy of gaseous ozone
112 treatment for reduction of *L. monocytogenes* and *L. innocua* at ambient temperature in
113 orange juice, (ii) ozone treatment efficacy in orange juice inoculated with the acid
114 stressed *Listeria* population, using a range of acid stress conditions, namely mild acid
115 stressed, mild acid stress-habituated and acid stressed but habituated in orange juice.

116 **2. Materials and Methods**

117 **2.1 Bacterial strains**

118 Three strains of *Listeria* were used in this study. *L. monocytogenes* ATCC 7644, *L.*
119 *monocytogenes* NCTC 11994, and *L. innocua* NCTC 11288 obtained from microbiology
120 stock culture, School of Food Science and Environmental Health, Dublin Institute of
121 Technology. Strains were maintained as frozen stocks at -70 °C in the form of protective
122 beads, which were plated onto tryptic soy agar (TSA, Barcelona, Scharlau Chemie) and
123 incubated overnight at 37 °C to obtain single colonies before storage at 4 °C.

124 **2.2 Preparation of orange juice**

125 Oranges (variety: Navalate, Peru) were purchased from a local market and squeezed with
126 a fruit juicer (Rowenta PA4002NEO). The fresh orange juice was then submitted to a
127 finishing process by passing through a sieve (Laboratory test sieve, Retsch, Germany) of
128 1mm diameter (mesh no. 18) to reduce the pulp content (Patil, Bourke, Frias, Tiwari &
129 Cullen, 2009_a). All juice preparations were stored at 4 °C. The pH was measured using a
130 pH meter with a glass electrode (Orion Model, England) and was found to be in the range
131 of 3.5-3.7.

132 **2.3 Experimental design**

133 In order to investigate the efficacy of ozone against *L. monocytogenes* and *L. innocua*
134 microbial populations, four different conditions were investigated;

135 a) To obtain a non acid stressed control *Listeria* population, cells were grown in TSB
136 without glucose (TSB-G). TSB-G was used as the basic medium for obtaining control
137 cells as presence of glucose in the medium results in mild acid stress of cells by reducing
138 the pH of TSB to 4.9.

139 b) To obtain mild acid stressed *Listeria* population, cells were grown in TSB with glucose
140 (TSB+G, 0.25%).

141 c) To obtain 1 h mild acid stress-habituated *Listeria* population, cells were grown in
142 TSB+G, 0.25% and then habituated at pH 5.5 (adjusted using 80% lactic acid) for 1 h and
143 to obtain 18 h mild acid stress-habituated *Listeria* population, cells were grown in
144 TSB+G, 0.25% (pH 5.5).

145 d) To obtain a *Listeria* population habituated in orange juice, cells were grown in
146 TSB+G, 1.25% leading to acid stressed cells which were then habituated in orange juice
147 for 90 min at 37 °C. Cells prepared under these different conditions were then treated
148 with ozone in orange juice.

149 **2.4 Preparation of cell suspensions and culture conditions**

150 For the first (a) and second investigation (b), a single isolated colony of each strain was
151 inoculated separately either in TSB-G or in TSB+G, 0.25% to produce non acid stressed
152 cells (control sample) and mild acid stressed cells, respectively. Cultures were then
153 incubated overnight at 37 °C and were then harvested by centrifugation (SIGMA 2K15,
154 Bench Top Refrigerated Ultracentrifuge, AGB scientific LTD.) at 10,000 rpm for 10min
155 at 4 °C. The cell pellet was washed twice with sterile phosphate buffered saline (PBS,
156 Oxoid LTD, UK). The pellet was re-suspended in PBS and the bacterial density was
157 determined by measuring absorbance at 550nm using McFarland standard (BioMérieux,
158 Marcy -l'Etoile, France). The inoculum was then diluted in maximum recovery diluent
159 (MRD, Scharlau Chemie) to obtain approximately 10^7 cells/ml. For each investigation, the
160 cell concentration was further diluted in orange juice to yield a final concentration of 10^6
161 cells/ml and then ozone treatment was applied.

162 For the third investigation (c), two acid stress-habituation conditions were imposed, i.e.,
163 1 hour and 18 hours. For the 1 hour habituation environment, working cultures were
164 grown overnight in TSB+G, 0.25% at 37 °C (thus creating a mild acid stress
165 environment). Cells were then harvested by centrifugation at 10,000 rpm for 10min at 4
166 °C. The cell pellet was washed twice with sterile PBS, re-suspended in 10 ml TSB
167 adjusted to pH 5.5, and incubated at 37 °C for 1h (Cheng, Yu & Chou, 2003; Caggia et
168 al., 2009). To prepare 18 h habituated cells, bacterial strains were grown directly in
169 TSB+G, 0.25% (pH 5.5) at 37 °C. The mild acid stress-habituated cells were diluted in
170 MRD (pH 5.5) to yield approximately 10^7 cells/ml, with further dilution in orange juice
171 (pH 3.5-3.7) to a final concentration of 10^6 cells/ml and then ozone treatment was applied.
172 For the fourth investigation (d) the working cultures were incubated overnight in TSB+G,
173 1.25% at 37 °C. This was performed to produce a more acid stressed population, as
174 described by Buchanan and Edelson (1996) with some modifications. The pH of the
175 culture following overnight incubation was measured using a pH meter with a glass
176 electrode and was found to be in the range of 4.4-4.6. Cultures were then centrifuged as
177 described above and cell pellet was resuspended directly in 10ml orange juice (pH 3.5-
178 3.7) and incubated at 37 °C for 90 min. Cultures were further diluted in orange juice to
179 yield an approximate final concentration of $10^6 - 10^7$ cells/ml and then ozone treatment
180 was applied.

181 **2.5 Ozone treatment**

182 Ozone gas was generated using an ozone generator (Model OL80, Ozone services,
183 Burton, Canada, Fig. 1). Ozone was produced by a corona discharge generator. Pure
184 oxygen was supplied via an oxygen cylinder (Air Products Ltd., Dublin, Ireland) and the

185 flow rate was controlled using an oxygen flow regulator. A previously determined
186 optimum flow rate of 0.12L/min with an ozone concentration of 0.098mg/min/ml was
187 applied for each treatment (Patil, Cullen, Kelly, Frias & Bourke, 2009_b). Excess ozone
188 was destroyed by an ozone destroyer unit. To prevent excess foaming, 20 µl sterile anti-
189 foaming agent (Antifoam B emulsion, Sigma Aldrich, Ireland Ltd.) was added before
190 each ozone treatment. The treatment of all orange juice samples previously inoculated
191 with *Listeria* strains (as described in section 2.4) was carried out for 7-8 minutes with
192 sampling intervals of 1 min. All experiments were performed in duplicate and replicated
193 at least twice.

194 **2.6 Microbiological analysis**

195 The efficacy of treatment was determined in terms of reduction in viable counts over
196 time. Populations of challenge organism were determined by plating onto TSA and
197 selective media (Palcam), respectively. Samples (1ml aliquots) were withdrawn from
198 treated juice at specific time intervals, serially diluted in MRD and 0.1ml aliquots of
199 appropriate dilutions were surface plated on TSA and Palcam agar. Plates were incubated
200 at 37 °C for 48 h and then colony forming units were counted. Results were reported as
201 Log₁₀CFU/ml. Data were pooled and average values and standard deviations were
202 determined. Means were compared using ANOVA followed by LSD testing at $p < 0.05$
203 level (SPSS, version 15.0).

204 **2.7 Microbial inactivation kinetics**

205 The GInaFiT tool was employed to perform the regression analysis of the microbial
206 inactivation data (Geeraerd, Valdramidis & Van Impe, 2005). The Weibull model
207 (Mafart, Couvert, Gaillard & Leguerinel, 2002) was used to analyze the data:

208
$$\log_{10}(N) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

209 where N (CFU/ml) is the number of microorganisms at time t , N_0 (CFU/ml) is the initial
 210 number of microorganisms, δ [min] (time for the first decimal reduction) and p [-] are
 211 parameters related to the scale and shape of the inactivation curve, respectively. The
 212 Weibull distribution corresponds to a concave upward survival curve if $p < 1$ and concave
 213 downward if $p > 1$ (van Boekel, 2002).

214 The numerical estimates of δ and p were used to calculate a desired log reduction. The
 215 time required to obtain a 5 log reduction (t_{xd}) was calculated using equation 3. For this
 216 case study x was equal to 5

217
$$t_{xd} = \delta \times (x)^{\frac{1}{p}} \quad (2)$$

218 **2.8 Determination of degree of injury and recovery index**

219 The non-selective medium TSA was expected to support the growth of both uninjured
 220 and ozone injured cells whereas the selective medium, Palcam agar was expected to
 221 support growth of uninjured populations. The difference from selective to non-selective
 222 media gives an indication of cell injury during the ozone treatment. Percent injury was
 223 calculated by using equation 3 (Hansen & Knochel, 2001). It was calculated by choosing
 224 the time intervals of samples which resulted in colony formation on both the media used.

225

226

227
$$\% \text{ injured cells} = \frac{\text{cfu/ml on TSA} - \text{cfu/ml on Palcam}}{\text{cfu/ml on TSA}} \times 100 \quad (3)$$

228

229 A recovery index was defined as the t_{5d} (time required to obtain a 5 log reduction)
230 determined from the counts on the Palcam divided by t_{5d} determined from the counts on
231 TSA (Hansen & Knochel, 2001).

232 **3. Results**

233 The inactivation kinetics of *Listeria* in orange juice were fitted using the Weibull model,
234 which provided estimations of microbial inactivation parameters in terms of the
235 processing times required. The Weibull parameters δ and p are shown in Table 1. The
236 shape parameter p , gave downward concavity for the kinetic curves of all the *Listeria*
237 strains (Figs. 2, 3 and 4). p values of >1 indicates a greater susceptibility of
238 microorganisms to the treatment (van Boekel, 2002).

239 **3.1 Inactivation of *Listeria monocytogenes* NCTC 11994**

240 The inactivation curves of *L. monocytogenes* NCTC 11994 are shown in Fig. 2. Ozone
241 treatment of mild acid stressed population required a longer treatment time to achieve
242 reduction by 5 log cycles (t_{5d}) compared to control non acid-stressed cells. For these test
243 conditions, significant differences were observed for recovery index as well as for t_{5d}
244 ($p<0.05$) (Table 1). Ozone treatment of 18 h acid stress-habituated population recorded
245 the highest time required for achieving t_{5d} compared to other test conditions investigated
246 (Table 1). Recovery index and t_{5d} values for acid stress-habituated cells showed
247 significant difference compared to the other test conditions ($p<0.05$). In the case of acid
248 stressed cells habituated in orange juice, t_{5d} was achieved in comparatively less time than
249 that required for mild acid stressed and 1 h or 18 h acid stress-habituated cells (Table 1).
250 In the case of cells habituated in orange juice, lower % injury was obtained (Table 1) and
251 for the precise estimation of the uninjured vs. the injured population, counts on Palcam

252 agar were recorded for up to 6 min of ozone treatment by which time the detection limit
253 was not reached for both media used.

254 **3.2 Inactivation of *Listeria monocytogenes* ATCC 7644**

255 Survivor curves for *Listeria* strain ATCC 7644 following ozone treatments are presented
256 in Fig. 3. In the case of control non acid-stressed, mild acid stressed and acid stressed
257 cells habituated in orange juice, t_{5d} was achieved in less than 6 min of ozone treatment
258 with no significant differences obtained with the recovery index for any of the test
259 conditions studied (Table 1).

260 In the case of acid stress-habituated populations (1 h and 18 h), a significant difference
261 was observed in t_{5d} values compared to the three other test conditions investigated
262 ($p < 0.05$). At all test conditions where acid stress was applied, $\geq 97.4\%$ injury was
263 observed indicating the efficacy of ozone in conjunction with applied acid stress
264 conditions (Table 1). However, for the control non acid stressed cells, a smaller % injury
265 was observed.

266 **3.3 Inactivation of *Listeria innocua* NCTC 11288**

267 Ozone inactivation curves of *L. innocua* cells for different test conditions are shown in
268 Fig. 4. The control non acid-stressed and mild acid stressed cells were reduced by 5 log
269 cycles in short treatment times (Table 1).

270 Mild acid stress-habituation of cells for the longer duration (18h) followed by
271 ozone treatment resulted in significantly higher t_{5d} value compared to other test
272 conditions investigated (Table 1). However, a significant difference was observed in t_{5d}
273 values for orange juice habituated cells, compared with mild acid stressed cells and
274 control non acid-stressed cells ($p < 0.05$).

275 The lower % injury observed for acid stressed cells habituated in orange juice after 7 min
276 ozone treatment underlines the importance of investigating the efficacy of ozone in real
277 product formulations in addition to simulated stress conditions in model media.

278 **4. Discussion**

279 The direct application of ozone was found to be effective for the inactivation of *Listeria*
280 in orange juice (Figs. 2, 3, and 4). However, there were some significant effects of
281 bacterial cell pre-treatment and condition observed on inactivation efficacy. The pre-
282 treatments and conditions employed were designed to mimic the environment that a
283 contaminating population could be exposed to in orange juice and other food processing
284 scenarios. Literature studies on the efficiency of ozone for inactivating *Listeria* in food
285 products vary (Olmez & Akbas, 2009; Rodgers, Cash, Siddiq & Ryser, 2004; Vaz-Velho,
286 Silva, Pissao & Gibbs, 2006; Yuk, Yoo, Yoon, Moon, Marshall & Oh, 2006). Olmez &
287 Akbas (2009), stated that the efficiency of ozone treatment can be related to the delivery
288 method.

289 Applying a mild acid stress actually increased the ozone treatment time required
290 for a 5 log reduction for both strains of *L. monocytogenes* by comparison with the control
291 population. However, in the case of *L. innocua*, applying a mild acid stress did not
292 significantly effect the ozone treatment time required by comparison with the control.
293 Leistner (2000) reported that simultaneous exposure of bacteria to different stress factors
294 requires increased energy consumption and leads bacteria to cellular death through
295 metabolic exhaustion.

296 Foodborne bacteria encounter organic and inorganic acids in foods or in the
297 gastrointestinal tract and cells of the host (Yousef & Courtney, 2003). Adaptation of *L.*

298 *monocytogenes* to sublethal stresses has been demonstrated to protect the pathogen to a
299 variety of normally lethal conditions present in certain foods (Lou and Yousef, 1997).
300 The resistance or adaptation of microorganisms to acid conditions can have implications
301 for food safety. In this study, acid stress-habituated *Listeria* cells had an increased
302 resistance to ozone treatment and also recorded the highest time for achieving 5 log (t_{5d})
303 reductions. Similar findings of significantly increased resistance of *L. monocytogenes* to
304 heat were reported by Mazzotta (2001) after acid adaptation of *Listeria* in single strength
305 apple, orange and white grape juices adjusted to pH 3.9. Caggia et al. (2009) recorded the
306 highest acid tolerance response of *L. monocytogenes* OML 45 strain, after 3h treatment in
307 TSB adjusted to pH 5.7, thus concluding that cells adapted to acidic environments can
308 grow in normally lethal pH conditions.

309 It has been reported that the heat and acid resistance of *L. monocytogenes* are strain
310 dependant (Skandamis, Yoon, Stopforth, Kendall & Sofos, 2008). Phan-Thanh et al.
311 (2000) reported the lowest pH value which *L. monocytogenes* could resist was dependant
312 on the strain and the kind of acid used. Our results also showed that the extent of
313 increased acid resistance varied with the bacterial strain and acid stress conditions. Strain
314 NCTC 11994 was the most resistant strain independent of the applied conditions.

315 In orange juice production, low acidic conditions are present before the pasteurization
316 process and may induce an ATR that can result in increased thermal tolerance (Caggia et
317 al., 2009). The exposure to sequential acid stressors such as a prior acid stress followed
318 by an acid environment in the product may result in cross protection to a subsequent
319 processing treatment as observed here. In the case of all 18 h acid stress-habituated
320 populations, the highest t_{5d} values were estimated, however, lower recovery indices were

321 reported, where greater recovery of cells was evident on non-selective media by
322 comparison with selective media (Table 1). The applied acid stress did not promote
323 recovery on selective medium (Palcam) at the same rate of the recovery on non-selective
324 medium (TSA), however the injured sub-population may have a greater resistance to
325 ozone. Therefore, to mimic the stresses encountered in food processing environments,
326 conditions like acid stress-habituation and habituation in actual orange juice should be
327 considered for determining inactivation parameters (e.g., t_{xd} , %injury, recovery index)
328 and process design in foods.

329 From the present study and based on the different inactivation responses to ozone
330 treatment it was also observed that inactivation responses of *L. innocua* NCTC 11288
331 were closer to those of *L. monocytogenes* ATCC 7644 than *L. monocytogenes* NCTC
332 11994.

333 **5. Conclusions**

334 This work has shown that direct ozone treatment can be used to inactivate *L.*
335 *monocytogenes* and *L. innocua* in orange juice. The efficacy of ozone treatment was
336 found to be a function of strain and duration of acid stress-habituation conditions. The
337 data also indicate that adaptive stress responses should be taken into account for process
338 design or method development for the inactivation of *L. monocytogenes*. Inactivation
339 times for a 5 log cycle reduction were achieved in between 5.08 and 8.44 min. Therefore,
340 direct ozone diffusion treatment could be used as a potential alternative to traditional
341 thermal pasteurisation for control of *Listeria* populations in fruit juices or other liquid
342 foods.

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453 **Figure captions**

- 454 Figure 1 Schematics of the ozone processing equipment.
- 455 Figure 2 Ozone inactivation of *Listeria monocytogenes* NCTC 11994
- 456 (a) Control non acid-stressed cells
- 457 (b) Mild acid-stressed cells
- 458 (c) 1 h acid stress-habituated cells
- 459 (d) 18 h acid stress-habituated cells
- 460 (e) Habituated cells in orange juice
- 461 Figure 3 Ozone inactivation of *Listeria monocytogenes* ATCC 7644
- 462 (a) Control non acid-stressed cells
- 463 (b) Mild acid-stressed cells
- 464 (c) 1 h acid stress-habituated cells
- 465 (d) 18 h acid stress-habituated cells
- 466 (e) Habituated cells in orange juice
- 467 Figure 4 Ozone inactivation of *Listeria innocua* NCTC 11288
- 468 (a) Control non acid-stressed cells
- 469 (b) Mild acid-stressed cells
- 470 (c) 1 h acid stress-habituated cells
- 471 (d) 18 h acid stress-habituated cells
- 472 (e) Habituated cells in orange juice
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Table 1: Parameters of the Weibull model and the time required to reach a 5 log reduction for *Listeria* strains in orange juice (Different letters indicate a significant difference at the 0.05 level between each type of condition).

Microorganism	Condition	$\delta(\text{min}) \pm \text{STE}$	$p \pm \text{STE}$	R^2	$t_{5d}(\text{min})$	Recovery index	% injury
<i>L. monocytogenes</i> NCTC 11994	Control non-acid stressed	3.48±0.64	3.17±1.04	0.93	5.78 ^a	0.99 ^k	95.9
	mild-acid stressed cells	3.07± 0.55	1.97± 0.41	0.96	6.95 ^b	0.76 ^l	99.7
	1h acid stress-habituation	4.05± 0.40	2.64± 0.38	0.98	7.45 ^c	0.79 ^l	97.8
	18 h acid stress-habituation	4.45± 0.69	2.52± 0.65	0.93	8.44 ^d	0.60 ^{lm}	99.9
	Habituated cells in orange juice	2.96± 0.73	1.97± 0.48	0.94	6.69 ^{ab}	0.89 ^k	76.6
<i>L. monocytogenes</i> ATCC 7644	Control non-acid stressed	2.99±0.47	2.84±0.64	0.94	5.27 ^e	0.98 ⁿ	91.6
	mild-acid stressed cells	3.17± 0.30	2.89± 0.42	0.98	5.53 ^e	1.00 ⁿ	99.8
	1h acid stress-habituation	4.12± 0.90	2.74± 0.89	0.90	7.41 ^f	0.75 ^o	99.3
	18h acid stress-habituation	4.54± 0.52	3.00± 0.60	0.95	7.77 ^f	0.80 ⁿ	99.2
	Habituated cells in orange juice	1.43± 0.56	1.14± 0.24	0.95	5.87 ^e	0.86 ⁿ	97.4
<i>L. innocua</i> NCTC 11288	Control non-acid stressed	2.94±0.66	2.66±0.82	0.91	5.38 ^h	0.96 ^p	74.6
	mild-acid stressed cells	3.44± 0.47	4.14± 1.45	0.94	5.08 ^h	1.0 ^q	99.8
	1h acid stress-habituation	4.17± 0.34	4.33± 0.96	0.97	6.05 ⁱ	0.85 ^{pr}	98.4
	18h acid stress-habituation	4.12± 0.42	2.62± 0.40	0.97	7.60 ^j	0.80 ^r	89.5
	Habituated cells in orange juice	1.82± 0.88	1.30± 0.40	0.91	6.26 ⁱ	0.83 ^f	66.7

δ – time for the first decimal reduction

STE - standard error

p - parameters related to the scale and shape of the inactivation curve

R^2 - coefficient of determination

% injury- calculated using equation 1

Recovery index- t_{5d} determined on Palcam divided by t_{5d} determined on TSA

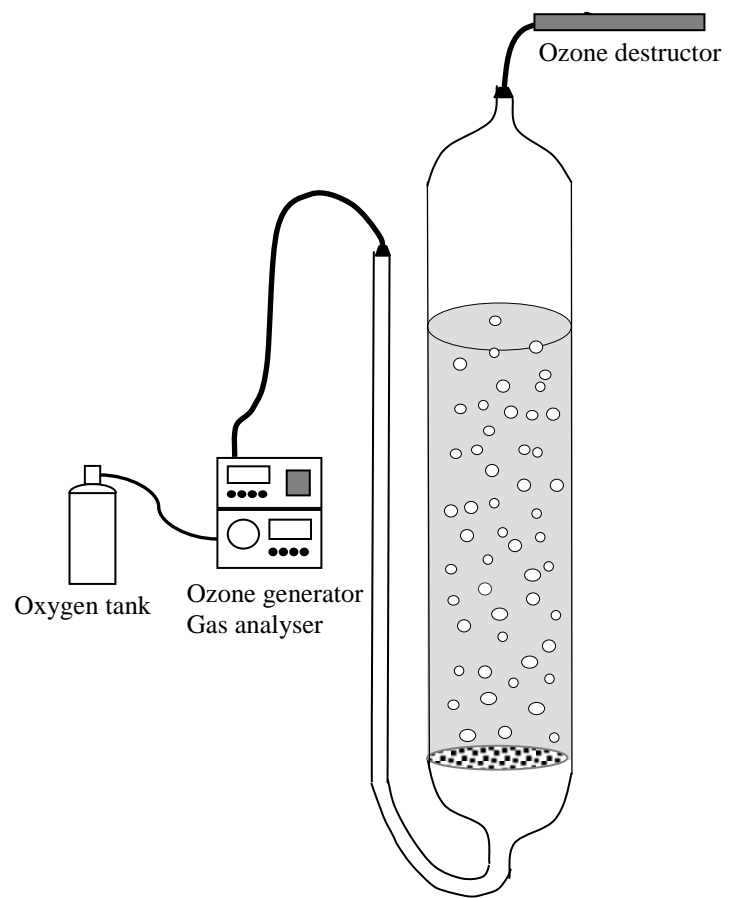


Fig. 1

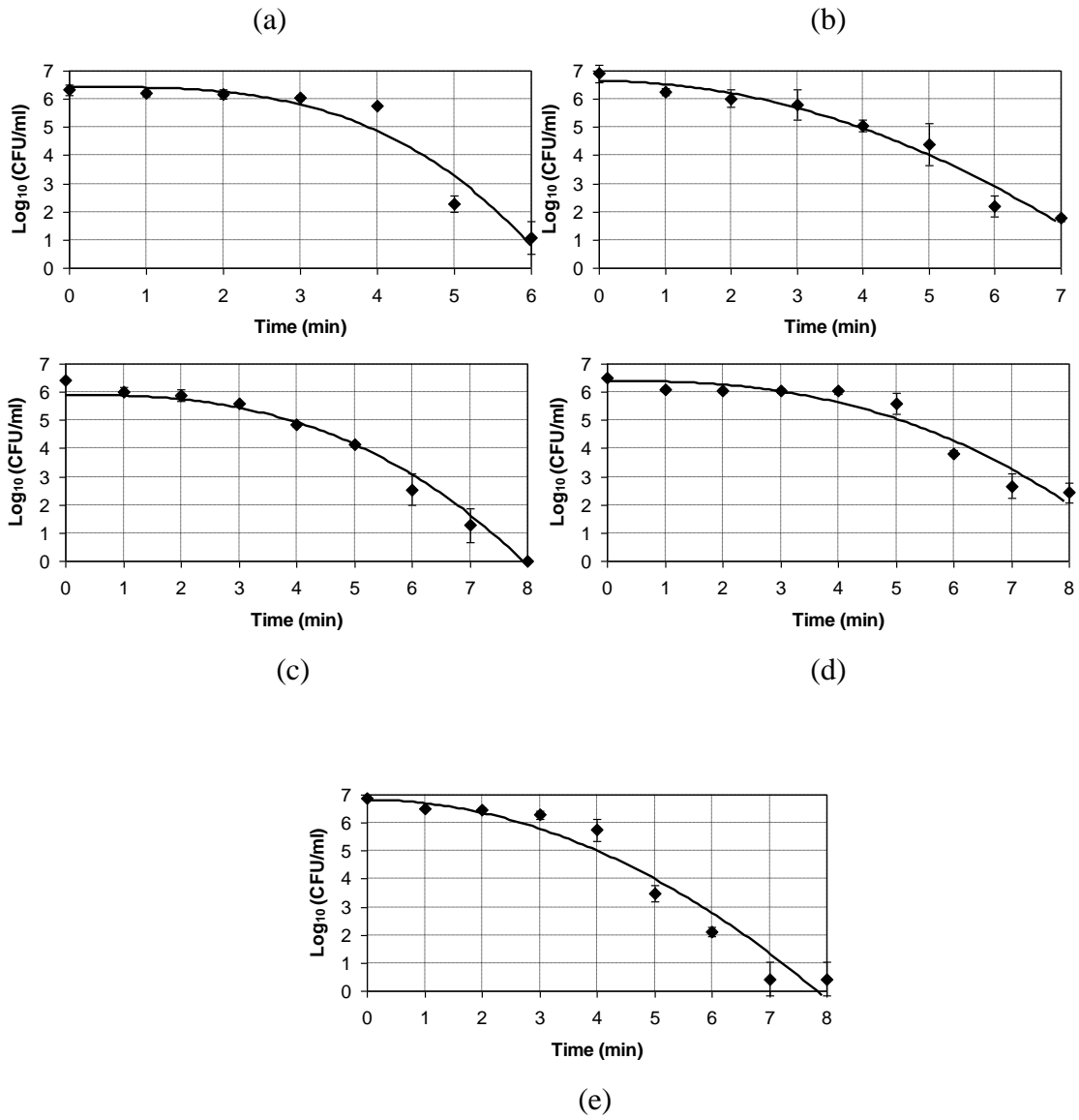


Fig. 2

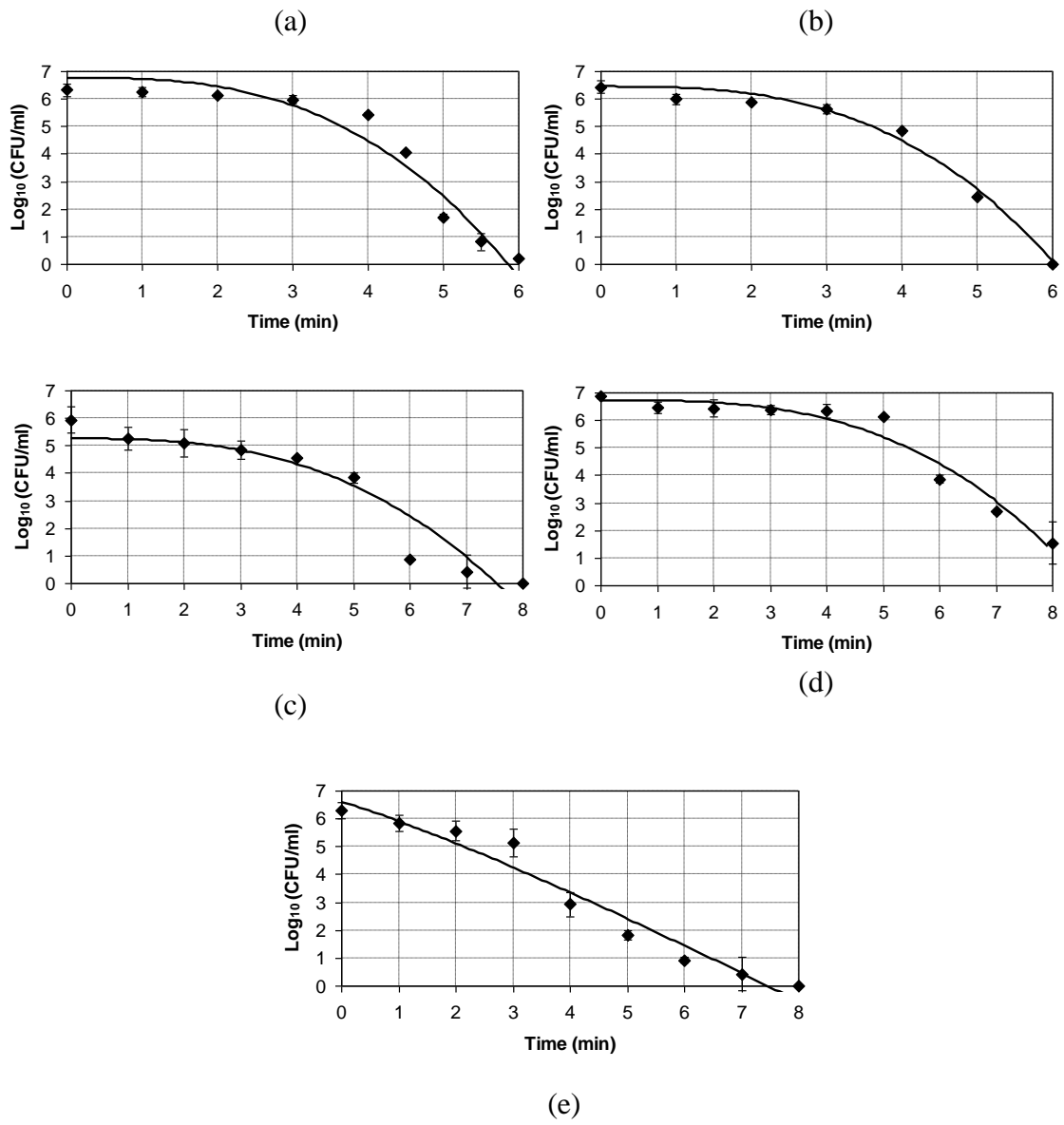


Fig. 3

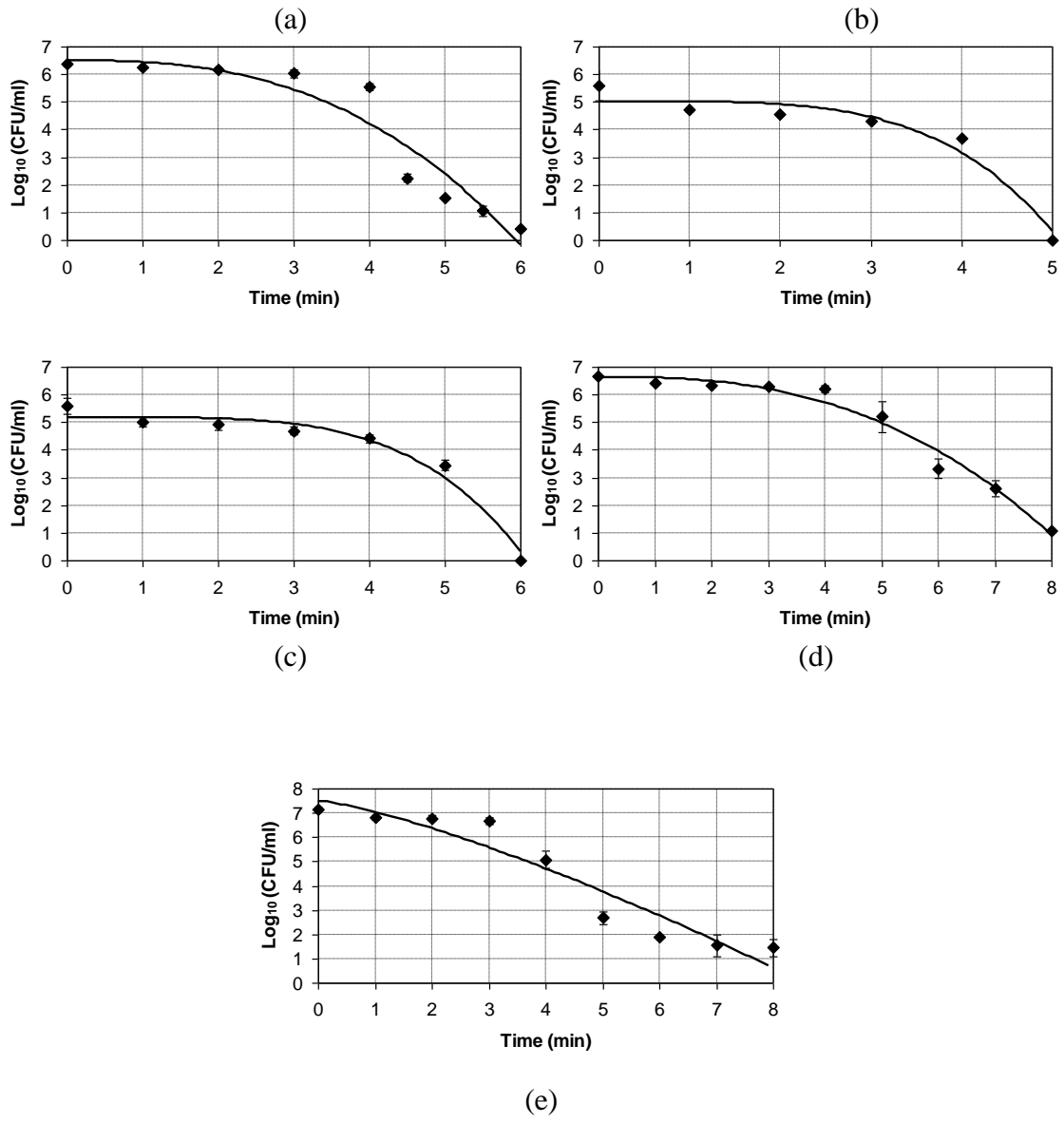


Fig. 4