OXIDATIVE STRESS IN *E. COLI* CELLS UPON EXPOSURE TO HEAT TREATMENTS

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Running title: ROS occurrence in *E. coli* heat treated cells

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25 **ABSTRACT**

26 Heat treatments are widely used by the food industry to inactivate microorganisms,
27 however their mode of action on microbial cells is not fully known. In the last years, it
28 has been proposed that the generation of oxidative species could be an important factor
29 contributing to cell death by heat and by other stresses; however, investigations in this
30 field are scarce. The present work studies the generation of reactive oxygen species
31 (ROS) upon heat treatment in *E. coli*, through the use of cell staining with specific
32 fluorochromes. Results obtained demonstrate that ROS are detected in *E. coli* cells
33 when they are subjected to heat exposure, and the amount of fluorescence increases with
34 temperature and time, as does the cellular inactivation. The addition of glutathione or
35 tiron, a potent antioxidant and a superoxide quencher, respectively, to the heating
36 medium protected *E. coli* against heat inactivation and concurrently reduced the
37 detection of ROS, especially in the case of glutathione. Finally, recovery of heated cells
38 under conditions that relief oxidative stress produced an increase in cell survival.

39 Data presented in this work support the view that ROS generation and subsequent
40 control in bacterial cells could be an essential factor determining inactivation and
41 survival upon exposure to heat, and it could be a potential target to increase the efficacy
42 of current treatments.

43

44 KEYWORDS

45 *Escherichia coli*; Reactive Oxygen Species; Heat treatment; Recovery

46 **1.- INTRODUCTION**

47 Food preservation relies on the application of control measures, mainly directed towards
48 pathogenic and spoilage microorganisms. Such measures represent a stress for the
49 microorganisms present in foods, and may be aimed either to control microbial growth,
50 such as high salt or acid concentration, or to inactivate microorganisms, such as heat
51 treatments or high hydrostatic pressure (HHP). In the last years, a great effort has been
52 focused towards gaining knowledge on the cellular events that take place in microbial
53 cells exposed to food preservation related stresses. The final objective of these studies is
54 to obtain a better profit of the existing technologies, through the increase of their lethal
55 effect on microorganisms, or through the design of more effective combinations of
56 different technologies, among other approaches.

57 Thermal treatments are still one of the most widely used methods to inactivate bacteria
58 in foods. With regards to the mode of action of heat on microorganisms, some relevant
59 advances have been reported; however, many aspects still remain unclear. It is generally
60 acknowledged that heat shows a multitarget mode of action in bacteria, causing damage
61 in different cell structures such as the envelopes, DNA, RNA, ribosomes and particular
62 enzymes (Mackey, 2000; Nguyen *et al.,* 2006). Despite much research effort has been
63 dedicated to this field of study in the last decades, the final events leading to cell death
64 in heated bacterial cells are not clear. An aspect that is gaining attention is the
65 involvement of an oxidative component in the inactivation of cells exposed to agents
66 that are not direct oxidant chemical compounds, such as heat, in the so-called secondary
67 oxidative stress (Mols and Abee, 2011).

68 Oxidative stress is defined as an imbalance between prooxidant and antioxidant
69 substances in favor of prooxidant ones. High levels of oxidant species in the microbial
70 cytoplasm, including reactive oxygen species (ROS) and reactive nitrogen species

71 (RNS), may produce damage in different cell components such as proteins, membranes
72 and genetic material (Imlay, 2003). Bacterial cells have certain capacity to eliminate
73 these reactive species and repair injured structures, however if this capacity is exceeded
74 the cell viability is compromised. The cellular defense mechanisms against oxidative
75 stress include detoxifying enzymes, such as superoxide dismutase or catalase, which
76 eliminate superoxide radicals and peroxides, respectively; glutathione and thioredoxin
77 systems which reduce disulfide bonds in oxidized proteins; DNA binding proteins with
78 iron fixing properties and nonspecific reducing activity substances as $FADH_2$ and
79 NADPH (Storz and Zheng, 2000; Gusarov and Nudler, 2005). Besides, there are
80 complex systems which repair damage to macromolecules, such as chaperones and
81 proteases, or DNA-repair enzymes, among others.

82 The occurrence of an oxidative component has been demonstrated for bacterial cells
83 treated by several food preservation-related stresses like HHP, acids and essential oils,
84 by the use of various experimental approaches (Aertsen *et al.*, 2005; Cebrián *et al.*,
85 2009; Mols *et al.*, 2009; Chueca *et al.*, 2014). For instance, it has been reported that
86 some bacterial strains treated by HHP survive better if they are recovered under
87 anaerobic conditions (Aertsen *et al.*, 2005; Cebrián *et al.*, 2009), and that some mutant
88 strains deficient in genes related to redox homeostasis control and DNA repair are more
89 sensitive to this technology (*trxB, gshA, sodA, soxS, polA, recA, xthA*) (Charoenwong *et*
90 *al.*, 2011). With regards to heat treatments, indirect evidences of the involvement of
91 oxidative stress on bacterial inactivation also exist. Some DNA-repair deficient mutants
92 are more sensitive to heat treatments (Mackey and Seymour, 1987), and after heat
93 exposure some strains recover better in conditions of anaerobiosis, in the presence of
94 detoxifying enzymes, in the presence of ROS quenchers, or in minimal medium, which
95 lacks hydrogen peroxide (Mackey and Seymour, 1987; Bromberg *et al.*, 1998; George

96 and Peck, 1998; Cebrián *et al*., 2007; Sagarzazu *et al.,* 2010). More recently, scattered
97 investigations have demonstrated that exposure to heat induces an increase in ROS
98 detection in bacterial cells (Baatout *et al*., 2005; Mols *et al*., 2009; Arku *et al*., 2011;
99 Mols *et al.,* 2011). Mols *et al*. (2011) suggested that this could be a common mechanism
100 involved in cell death by various agents and that ROS formation could be due to
101 malfunctioning of the electron transport chain. However the intensity and relevance of
102 this phenomenon and the possible implication in cell inactivation and survival remains
103 unknown.
104 Despite the importance of oxidative stress and maintenance of the cellular redox
105 homeostasis, this is a factor that has been traditionally underestimated in investigations
106 about microbial inactivation and survival to food preservation technologies. This
107 research studies the presence of ROS in *E. coli* cells subjected to heat treatments, and
108 explores the possible relationship between occurrence of oxidative stress and cell
109 inactivation.

110 **2.- MATERIALS AND METHODS**

111 *2.1.- Bacterial strains and growth conditions*

112 *Escherichia coli* BW25113, W3110, CECT 471 and BJ4 were used in this study. The
113 strains were stored at -80ºC. To prepare precultures, flasks containing 10 ml of sterile
114 TSBYE (Tryptic Soy Broth with 0.6% Yeast Extract, Oxoid, Basingstoke, UK) were
115 inoculated with one single colony from a TSAYE plate (Tryptic Soy Agar with 0.6 %
116 Yeast Extract, Oxoid). The precultures were incubated overnight at 37ºC, under
117 agitation. Subsequently, cultures were obtained by inoculating a flask with 50 ml
118 TSBYE with 100 µl of the preculture, and incubating at 37ºC under agitation, until
119 stationary phase of growth was reached after 18-24 hours incubation (2×10^9 cfu/ml,
120 approximately).

121 *2.2.- Heat treatment*

122 To determine the heat resistance of *E. coli,* a thermoresistometer TR-SC was used
123 (Condón *et al.,* 1993). The instrument consists of a 400 ml vessel provided with an
124 electrical heater for thermostation, an agitation device to ensure inoculum distribution
125 and temperature homogeneity, and ports for injecting the microbial suspension and for
126 the extraction of samples. 350 ml of sterile PBS (Phosphate Buffered Saline, Sigma,
127 San Louis, USA) were placed in the vessel of the TR-SC and heating was switched on.
128 Once the PBS reached the desired temperature (50, 54, 58 or 62 ±0.1ºC), it was
129 inoculated with 0.2 ml of an adequately diluted microbial cell suspension. After
130 inoculation, 0.2 ml samples were collected at different heating times, immediately
131 plated and incubated for survival counting. Occasionally, heat treatments were carried
132 out in test tubes submerged in a thermostated water bath. The objective of these
133 experiments was to obtain treated cells at concentration $\geq 10^7$ cells/ml, to be examined
134 under the microscope, since the concentration normally used in the thermoresistometer
135 TR-SC was insufficient for that purpose.

136 In some experiments, substances with proven redox activity were added to the heating
137 medium. These included L-cysteine hydrochloride (10 mM) (Sigma Aldrich), reduced
138 L-glutathione (10 mM) (Sigma Aldrich), 2,2'-bipyridyl (0.02 mM) (Sigma Aldrich),
139 thiourea (150 mM) (Sigma Aldrich), 4,5-dihydroxy-1,3-benzenedisulfonic acid
140 disodium salt monohydrate (Tiron, 20 mM) (Sigma Aldrich), D-mannitol (50 mM)
141 (Sigma Aldrich) and sodium pyruvate (90 mM) (Panreac, Barcelona, Spain). The
142 concentrations were chosen according to the literature (Kari *et al.,* 1971; Ferguson *et*
143 *al.,* 1998; Yang *et al.,* 2001; Santo *et al.,* 2008; Wang *et al.,* 2010; Johnson e*t al.,* 2014).
144 These concentrations did not affect *E. coli* BW25113 growth either in liquid or in solid
145 media (data not shown).

146 *2.3.- Recovery after heat treatment*

147 After heat treatment, samples were plated in TSAYE, and plates were incubated at 37ºC
148 in aerobic conditions and after 24-72 h colony forming units were counted. Although
149 these were the standard recovery conditions used in most experiments, in some cases
150 cells were also recovered under different conditions. On the one hand, the atmosphere
151 composition was changed. Where indicated in the text, survivors were recovered in a
152 variable atmosphere incubator (MACS VA500, Don Whitley Scientific Limited,
153 Shipley, United Kingdom), with a gas composition of 0% oxygen, 5% hydrogen, 5%
154 carbon dioxide and 90% nitrogen, plus the palladium catalyzer, to create anaerobic
155 atmosphere.

156 On the other hand, the recovery medium was also modified in particular experiments,
157 where cells were also recovered in TSAYE enriched with 10 mM L-glutathione and in
158 minimal M9 glucose-salts agar enriched with 3 mM L-cysteine HCl. These conditions
159 were chosen to create a low-oxidative stress environment in order to improve the
160 recovery of ROS-sensitized cells. M9 agar was prepared as described eslewhere
161 (Gerhardt *et al.*, 1994) and supplemented with $FeSO_4$ (10 mg/L) to improve bacterial
162 growth (Stanier *et al.*, 1992), and with cysteine to create a low redox potential
163 environment (Gerhardt *et al.,* 1994; Suh and Knabel, 2000).

164 To construct survival curves, the fraction of survivors (Log N_t/N_0) was represented *vs*
165 the treatment time (minutes). All the experimental determinations of heat resistance
166 were performed at least 3 times with independent microbial cultures, and data in figures
167 correspond to the average and the mean standard deviation (error bars).

168 *2.4.- ROS determination*

169 The presence of ROS in cells due to heat treatment was studied through staining with
170 specific fluorochromes followed by flow cytometry and/or epifluorescence microscopy.

Heated cells were collected after different exposure times and stained separately with H_2DCFDA (2',7'– dichloro-dihydro-fluorescein diacetate) or DHE (dihydroethidium). Treated cells, at approximately 10^5 cells per ml in PBS, were incubated with the fluorescent dye, then centrifuged and resuspended in filtered PBS (0.22 μm). A positive and a negative control of staining were always included. The negative control corresponded to untreated cells, whereas the positive control consisted of cells exposed to hydrogen peroxide (10 mM/30 min) (Sigma Aldrich) in the case of H_2DCFDA, and of cells treated with the superoxide-generating agent plumbagin (Sigma Aldrich) (5.3 mM/10 min) in the case of DHE (Hassan *et al.,* 1979; Farr *et al.,* 1985).

Fluorescent signals obtained with H_2DCFDA and DHE stained cells were quantified through flow cytometry. The cytometer (Guava EasyCyte, Merck Millipore, Darmstadt, Germany) was calibrated with fluorescent beads (Guava easyCheck Bead Reagent, Merck Millipore), according to the manufacturer´s instructions. The samples were introduced into the cytometer at 0.59 μl/s and a 488 nm laser was used for excitation. The threshold was set with the forward scatter (FSC) to 80 mV, to distinguish the cells from the debris and electronic noise, and the values for the photomultiplier tube were: 53.8 (FSC), 79.5 (SSC) and 29.3 (GRN and RED). A band-pass filter of 695 nm was used to collect the red fluorescence and a band-pass filter of 525nm was used to collect the green fluorescence. Data were obtained (5,000 events) and analyzed using the Software GuavaSoft 2.7 (Millipore, Darmstadt, Germany). Results were expressed using histograms, which represent the distribution of fluorescence level among the cells in the population (number of events *vs* level of fluorescence). The area under the curve in the histogram was used as a total fluorescence measurement, and was calculated with the Excel software (Microsoft Office 2007).

195 To choose adequate staining conditions, different concentrations and different contact
196 times of each dye were tested. In the case of H_2DCFDA three concentrations (5, 50 and
197 500 µM) and different contact times (5, 30, 60, 90 and 120 minutes) were tested. After
198 applying these conditions, the histograms (number of cells *vs* green fluorescence level)
199 corresponding to untreated and hydrogen-peroxide treated cells stained with H_2DCFDA
200 were observed and final staining conditions were chosen. In general, total fluorescence
201 increased with the dye concentration and contact time up to a maximum, thus the
202 conditions chosen were those which allowed a better separation of peaks corresponding
203 to the positive and the negative controls. A similar procedure was followed for DHE.
204 Final staining conditions chosen were 50 µM/90 minutes in both cases.
205 Occasionally, the results obtained with flow cytometry were completed using
206 fluorescence microscopy, in order to obtain a more direct estimation of the percentage
207 of cells stained with DHE, since flow cytometry did not always allow peaks to be
208 completely resolved. In this case cells were stained following the same procedure, but
209 using a higher cell concentration (10^7-10^8 cfu/ml). A Nikon Eclipse E400 phase contrast
210 and fluorescence microscope (Nikon Corporation, Japan) was used, images were
211 obtained with a high resolution camera (AxioCam MRc, Zeiss, Germany) and processed
212 with the software ZEN 2012 (Zeiss, Germany). Total and fluorescent cells were counted
213 from photographs taken from each sample.
214 All the staining experiments, either by cytometry or microscopy, were carried out at
215 least in duplicate with independent bacterial cultures. Data in figures correspond to the
216 average and the mean standard deviation (error bars).
217 *2.5.- Propidium iodide staining*
218 Additionally, the permeabilization of the membrane due to the exposure of cells to heat
219 treatment was also studied. For this purpose, after heat treatments, cells were stained

220 with propidium iodide (PI, 3 µM/30 min), following a similar protocol to that described
221 for H_2DCFDA and DHE, and observed by fluorescence microscopy. Cells with
222 permeabilized membranes allow the entrance of the dye inside the cell, rendering cells
223 with intense red fluorescence.

224 *2.6.- Statistical analysis*

225 Student´s *t* tests were carried out using the GraphPad PRISM 5 software (GraphPad
226 Software, Inc., San Diego, CA, USA), and differences were considered significant for p
227 ≤ 0.05.

228 **3.- RESULTS**

229 **3.1.- Exposure of *E. coli* cells to heat causes an increase of ROS**

230 The heat resistance of *E. coli* BW25113 was evaluated in PBS at 50, 54, 58 and 62ºC.
231 Figure 1 shows the survival curves of *E. coli* at the four temperatures studied. The
232 inactivation level was similar to that obtained by other authors with other strains of *E.*
233 *coli* (Benito *et al.*, 1999; Cebrián *et al.*, 2007).

234 Subsequently, the presence of ROS in heat treated cells was assessed. For this purpose,
235 cells were stained with H_2DCFDA and DHE. H_2DCFDA (2',7'– dichloro-dihydro-
236 fluorescein diacetate) is a membrane-permeant dye that is enzymatically hydrolyzed by
237 intracellular esterases to DCFH, which is oxidized to render a green fluorescent
238 compound (DCF, 2, 7-dichlorofluorescein). H_2DCFDA is considered as a general
239 oxidative stress indicator, being able to react with H_2O_2, hydroxyl (OH•) and peroxyl
240 radicals (ROO•) and also with some reactive nitrogen species (RNS) (Gomes *et al.*,
241 2005). On the other hand, DHE has been widely used as a permeant probe for the
242 detection of superoxide ($O_2^{\bullet -}$) due to its specificity for this radical. DHE is oxidized by
243 superoxide, originating oxyethidium, which is a compound that bounds to DNA
244 rendering fluorescence in red (Gomes *et al.*, 2005). Despite it has been shown that

245 oxidation of DHE is not completely specific to superoxide anion (Patsoukis *et al.,*
246 2005), DHE and its derivatives continue to be a commonly used probe for superoxide
247 detection.
248 The adequacy of the two dyes here used was checked through the use of the controls.
249 Untreated cells did not exhibit significant fluorescence upon staining with any of the
250 dyes. Cells exposed to H_2O_2 were strongly stained with H_2DCFDA, whereas
251 plumbagin-exposed cells showed red fluorescence when they were stained with DHE.
252 These results were tested by cytometry and also by fluorescence microscopy (data not
253 shown). In order to check the specificity of the dyes, cells exposed to H_2O_2 were stained
254 with DHE and cells exposed to plumbagin were stained with H_2DCFDA. Whereas in
255 this latter case no staining was detected, cells exposed to H_2O_2 showed some low-
256 intensity fluorescence when stained with DHE (data not shown). In fact, Patsoukis *et al.*
257 (2005) showed that DHE can be also oxidized by H_2O_2 via non-specific peroxidase
258 catalysis. However we cannot discard that exposure to H_2O_2 provoked the generation of
259 superoxide due to extensive damage to the cytoplasmic membrane and thus, to the
260 electron transfer chain. In summary, although the two dyes showed clear affinity for
261 particular ROS, H_2DCFDA for peroxide and DHE for superoxide, in the case of DHE it
262 cannot be discarded that other oxidative species may somehow contribute to the
263 fluorescence detected.
264 As a first approach, the presence of ROS in cells treated at 58ºC was evaluated. Figure 2
265 includes the phase contrast and epifluorescence microphotographs of *E. coli* BW25113
266 cells stained with H_2DCFDA (2A) and DHE (2B). It can be observed that heat-treated
267 cells (58ºC/3 min) presented staining with the two dyes. These results confirmed that
268 ROS formation occurred in *E. coli* BW25113 upon heat treatment.

269 It is also remarkable that, under the treatment conditions here used (58ºC/3 min), most
270 of the cells were stained with DHE, whereas in the case of H_2DCFDA, only a
271 percentage of the cells showed fluorescence. Fig. 3A and 3B include the total
272 fluorescence calculated from the histograms after different treatment times at 58ºC.
273 Results are expressed as the total area under the curve in the histogram (A_t) minus the
274 area corresponding to non-treated cells (A_0). As it can be observed in the graphs,
275 whereas for cells stained with H_2DCFDA a maximum peak of fluorescence was
276 observed after approximately 4 minutes at 58ºC and further exposure to heat caused a
277 decrease in fluorescence, for cells stained with DHE a gradual increase in fluorescence
278 was observed up to 4-6 minutes of treatment.
279 In order to know if the generation of ROS was particular to this *E. coli* strain,
280 experiments were carried out with other three strains (W3110, CECT 471 and BJ4).
281 Results obtained confirmed that ROS formation occurred in other strains and it could be
282 a common effect during thermal treatment. DHE staining increased with treatment time
283 in all the strains (Fig 4). In addition, in all the strains, the detection of fluorescence with
284 the dye H_2DCFDA was similar to that previously observed with strain BW25113, and
285 fluorescence decreased after a maximum peak (data not shown).

286 **3.2.- Influence of treatment temperature and time on ROS generation**

287 In order to study the kinetics of ROS production, cells were treated at different
288 temperatures and times, stained with DHE and analyzed by flow cytometry. Figure 5A
289 represents the evolution of total fluorescence along treatments at 50, 54, 58 and 62 ºC.
290 Figure 5B includes data obtained after a constant treatment time (2 min) at the four
291 temperatures tested, for comparison purposes.
292 As it can be observed in the graphs, the amount of fluorescence increased with
293 treatment time for all the treatment temperatures tested, and it appeared that tended to

294 reach a plateau (Fig 5A). The increase in fluorescence was faster the higher the
295 temperature, and for a fixed exposure time (Fig 5B), the amount of fluorescence
296 increased with temperature.

297 **3.3.- Influence of the presence of different substances with redox activity in the**
298 **treatment medium on *E. coli* survival and ROS formation.**

299 The influence of the addition of a variety of substances to the heating medium on cell
300 survival was studied. These substances were selected because they possess either
301 antioxidant activity or they have been used as quenchers of several ROS or ROS-related
302 compounds. Glutathione was added due to its proven antioxidant activity at different
303 cellular levels (Smirnova and Oktyabrsky, 2005). Cysteine was added due to its
304 capacity of eliminating oxygen from the medium and decreasing its redox potential
305 (Suh and Knabel, 2000). Bipyridyl was added because it is a permeant iron-binding
306 substance, thus reducing the intensity of the formation of hydroxyl radicals *via* Fenton
307 reaction (Imlay *et al*., 1988; Gusarov and Nudler, 2005). Thiourea and mannitol have
308 been used as hydroxyl radical quenchers (Whiteman and Halliwell, 1997; Wisselink *et*
309 *al*., 2002); pyruvate was added to culture media as H_2O_2 quencher (Mackey, 2000), and
310 tiron has been described as superoxide quencher (Krishna *et al*., 1992). Results obtained
311 are shown in Table 1, where the Log cycles of inactivation after a 3 minutes exposure to
312 58ºC in PBS with and without these compounds are included. From the variety of
313 substances used, glutathione and tiron were selected since they exerted a significant
314 thermo-protective effect on *E. coli* cells ($p \leq 0.05$), which was especially relevant in the
315 case of glutathione.

316 Figure 6A includes the inactivation attained after a heat treatment at 58ºC of *E. coli*
317 BW25113 in PBS alone and with tiron (20 mM) and glutathione (10 mM) added. Cells
318 were stained with DHE and the total fluorescence determined (Fig 6B). Cells were

319 stained in the same medium that had been used before for heating, thus, the effect of
320 glutathione and tiron has to be ascribed to heating plus the following post-treatment
321 staining period. As it can be observed in the graphs, the presence of either tiron or
322 glutathione protected *E. coli* against heat inactivation, and also reduced the intensity of
323 ROS occurrence in the cells.

324 **3.4.- Relationship between ROS formation, membrane permeabilization and cell**
325 **inactivation**

326 In order to gain a deeper knowledge about the cellular mechanisms involved in cell
327 inactivation and survival, cells were stained with DHE and with PI, with the aim of
328 studying the possible link between ROS formation, membrane permeabilization and cell
329 inactivation. The percentage of cells stained with each of the dyes used was determined
330 microscopically by epifluorescence and phase contrast. Results are shown in Figure 7,
331 where the percentage of inactivated cells, evaluated as cells unable to outgrow in
332 TSAYE and aerobiosis, and the percentage of fluorescent cells upon staining either with
333 DHE or with PI after different heating times are included.

334 As it can be observed in the graph, the percentage of inactivated cells was closer to the
335 percentage of cells stained with DHE than to PI-permeabilized cells. For instance, after
336 2 minutes of exposure to 58ºC, 93% of the cells had lost viability, 95% of cells were
337 stained by DHE, and only 19% were permeable to PI.

338 **3.5.- Cell recovery after treatment under conditions that relief oxidative stress**

339 Cells were subjected to a treatment at 58ºC, and they were subsequently recovered
340 under different experimental conditions. Conditions included the standard recovery
341 conditions (TSAYE, aerobiosis) and two conditions to reduce the level of oxidative
342 stress: TSAYE plus 10 mM glutathione (aerobiosis), and minimal medium with 3 mM
343 cysteine added plus anaerobic incubation. Figure 8 shows the survival curves obtained.

344 As it can be observed in the graph, cellular recovery improved in both conditions, as
345 compared to TSAYE/aerobiosis, and the protective effect was more notable for the
346 recovery in cysteine-supplemented minimal medium under anaerobic atmosphere.

347 **4.- DISCUSSION**

348 In this investigation, we have demonstrated the occurrence of ROS in various strains of
349 *E. coli* heat treated cells, supporting the results previously reported with other bacterial
350 species (Baatout et al., 2005; Mols et al., 2009; Arku et al., 2011; Mols et al., 2011).
351 Mols et al. (2011) affirmed that superoxide radicals were formed in *B. cereus*
352 exponentially growing cells due to the treatment applied, and this coincides with the
353 results obtained in this investigation with stationary phase *E. coli* cells. ROS detection
354 increased with the intensity of the heat treatment, i.e. with longer exposure time and/or
355 higher temperature, suggesting that the generation of ROS and/or the unbalance in the
356 subsequent control within the cell could be a critical factor influencing cell death and
357 survival upon exposure to heat.

358 We have observed that after particular treatment conditions, generally after the most
359 intense treatments, a majority of the cells were stained with DHE, whereas only a
360 percentage of the cells showed fluorescence with H_2DCFDA. These results may indicate
361 that superoxide radical is formed more intensely, since superoxide is a precursor of
362 other ROS including hydrogen peroxide and hydroxyl radical (Lushchak, 2011; Imlay,
363 2013). Also, perhaps hydrogen peroxide and other radicals able to react with H_2DCFDA
364 would be more efficiently removed by the cellular detoxifying machinery, i.e. by the
365 action of catalases. Another possibility is that the fluorescent dye H_2DCFDA could be
366 lost across a permeabilized membrane, after longer treatments. Finally, given the fact
367 that the development of fluorescence upon staining with H_2DCFDA requires esterase
368 enzymatic activity (Gomes *et al.,* 2005), the heat treatment applied might have partly

369 affected cellular esterases; thus the fluorescence observed would be the result of the
370 balance between ROS generation and enzymatic activity drop. We would like to point
371 that we also tested the use of HPF (hydroxyphenyl fluorescein), which is an indicator of
372 the presence of hydroxyl radical whose signal does not depend on enzymatic activity.
373 However, we did not detect strong enough fluorescence with this dye by the use of the
374 flow cytometer, under our experimental conditions, in any of the samples of *E. coli*,
375 including the positive controls and with a variety of staining conditions, so we discarded
376 its use.
377 We also studied the effect of different substances which possess either antioxidant
378 activity or they have been used as quenchers of several ROS or ROS-related
379 compounds, and it was found that the presence of either tiron or glutathione protected *E.*
380 *coli* against heat inactivation. It has to be noted that all the substances were used at a
381 single concentration, with the only objective of detecting particular conditions that
382 protected cells against heat inactivation through a redox-related mechanism. We cannot
383 discard that a wider study, including different concentrations and experimental
384 conditions, could reveal also protective effects for the rest of quenchers and substances
385 here used. Tiron and glutathione protected cells against heat inactivation and also
386 reduced the intensity of ROS formation in the cells. The protection exerted by tiron
387 would be attributed to its superoxide quenching activity; supporting the view that
388 superoxide was formed and was involved in the development of DHE fluorescence. The
389 magnitude of the protective effect of glutathione was particularly important, both in cell
390 inactivation and preventing ROS formation in cells. Glutathione is present in some
391 Gram-negative prokariotes at relatively high concentrations (Smirnova and Oktyabrsky,
392 2005), and it exerts its antioxidant action through several mechanisms, which include
393 direct reaction with several free radicals, and thiol-disulfide interchange that help to

394 recover the structure in altered proteins (Smirnova and Oktyabrsky, 2005). Further work
395 is being carried out in our laboratory to gain insight into the actual mode of action of
396 this compound.
397 Furthermore, this study tried to stablish a relationship between the permeabilization of
398 the membrane to PI and the formation of ROS. The results indicate that the
399 permeabilization of the membrane to PI and the occurrence of ROS due to the heat
400 treatment were independent phenomena, since most DHE-positive cells were not
401 permeable to PI. This would suggest that the formation of ROS upon heating would not
402 be a consequence of membrane damage and subsequent electron transport chain
403 disturbance, as suggested by other authors (Mols *et al.,* 2011). Other mechanisms may
404 be playing a role, such as for instance the destabilization of other cellular structures or
405 the loss of activity of cellular defensive systems, although it cannot be discarded that
406 more subtle membrane alterations may occur in heated cells, before permanent
407 permeabilization to PI takes place.
408 All the results described so far in this investigation suggested that the occurrence of
409 ROS would be a cellular event that could be related somehow to cell inactivation.
410 However, it is well known that inactivation depends on the recovery conditions used.
411 Bacterial cells subjected to a variety of stresses may suffer sublethal alterations in one
412 or several structures and/or functions. This fact is particularly important in those agents
413 that have a multitarget mode of action, such as heat (Mackey, 2000; Wuytack *et al*.,
414 2003). If the environmental conditions are appropriate, sublethally injured cells may
415 recover their damages, maintain cell internal homeostasis and resume growth after
416 treatment. On the contrary, under adverse conditions, damaged cells may not be able to
417 repair damages and may be inactivated. Standard recovery conditions used in this
418 investigation were a nutritionally complex agar (TSAYE), and aerobic atmosphere. It

419 has been reported that traces of peroxides and other ROS are formed in complex media
420 upon autoclaving and exposure to UV light (Mackey, 2000; Stephens *et al.,* 2000), and
421 that factors like the tryptone that is added to culture media may influence the oxidative
422 stress level of the cells (Stephens *et al.,* 2000; De Spiegeleer *et al*., 2004). Thus, if
423 heated cells contained a higher amount of ROS, either due to the direct action of heat on
424 several components of the bacterial cell, or due to subtle alterations in the membrane, it
425 is reasonable to think that these cells could be more sensitive after treatment to adverse
426 conditions that involve an oxidative burden (Stephens *et al.,* 2000). In fact, our results
427 showed that cellular recovery was improved by the addition of glutathione, and also
428 when recovery was carried out in minimal media enriched with cysteine and anaerobic
429 atmosphere. It was checked that anaerobic recovery did not improve cell survival
430 neither in TSAYE, nor in TSAYE plus glutathione (data not shown), fact that illustrates
431 the complex relationship between media composition, atmosphere composition and cell
432 physiological state. The results also suggest that the presence of superoxide radicals,
433 despite being a good indicator of the level of cell alteration upon heating, could be
434 partly counteracted in *E. coli* cells under some favorable environmental conditions. This
435 fact is particularly important for food preservation strategies, where conditions which
436 permit and favor cell recovery should be strongly avoided.

437 **5.- CONCLUDING REMARKS**

438 In summary, our results show that heat treatment causes a progressive increase of ROS
439 in *E. coli* cells. While many authors have suggested that heat treatment produces
440 oxidative stress in cells, only scattered investigations have described the direct
441 formation of ROS in cells subjected to heating. Moreover we have also demonstrated
442 for the first time that the detection of ROS and the amount of cells with ROS increase
443 with treatment time and treatment temperature, and that this increase is always

444 coincident with increases in cell inactivation degree upon exposure to heat. The addition
445 of glutathione or tiron, a potent antioxidant and a superoxide quencher, respectively, to
446 the heating medium protected *E. coli* against heat inactivation and also reduced the
447 detection of superoxide. Our results also show that ROS presence seems to be
448 independent from permanent membrane permeabilization to PI. Whether the formation
449 of ROS derives from imbalances in the electron transfer chain due to more subtle
450 membrane alterations, or from other cellular mechanisms, is something that remains to
451 be elucidated.

452 Data presented in this work support the view that ROS generation and ulterior balance
453 maintenance in bacterial cells is an essential factor determining inactivation and
454 survival upon exposure to stressing agents, and it could be a potential target for the
455 optimization of current food preservation procedures.

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589

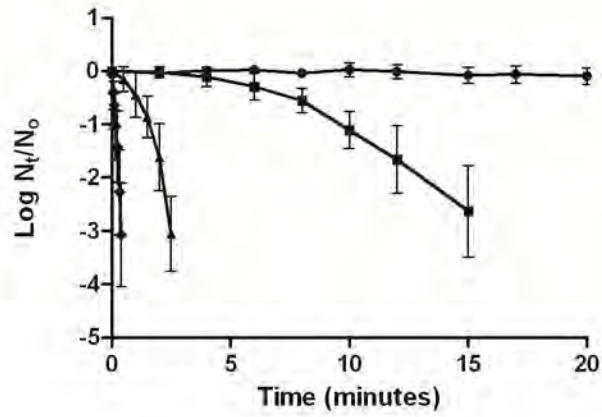
590 **TABLE 1.** Influence of the addition of substances with selected quenching and/or redox
591 activity to the heating medium (58ºC/3 min) on the inactivation of *E. coli* BW25113.

	Log cycles of inactivation
PBS (control)	2.83±0.15
Glutathione	0.95±0.06*
Cysteine	3.46±0.31
Bipyridyl	2.82±0.64
Thiourea	3.87±0.10*
Mannitol	3.07±0.42
Pyruvate	2.13±0.60
Tiron	1.75±0.24*

592 *Significantly different from control ($p<0.05$)

593

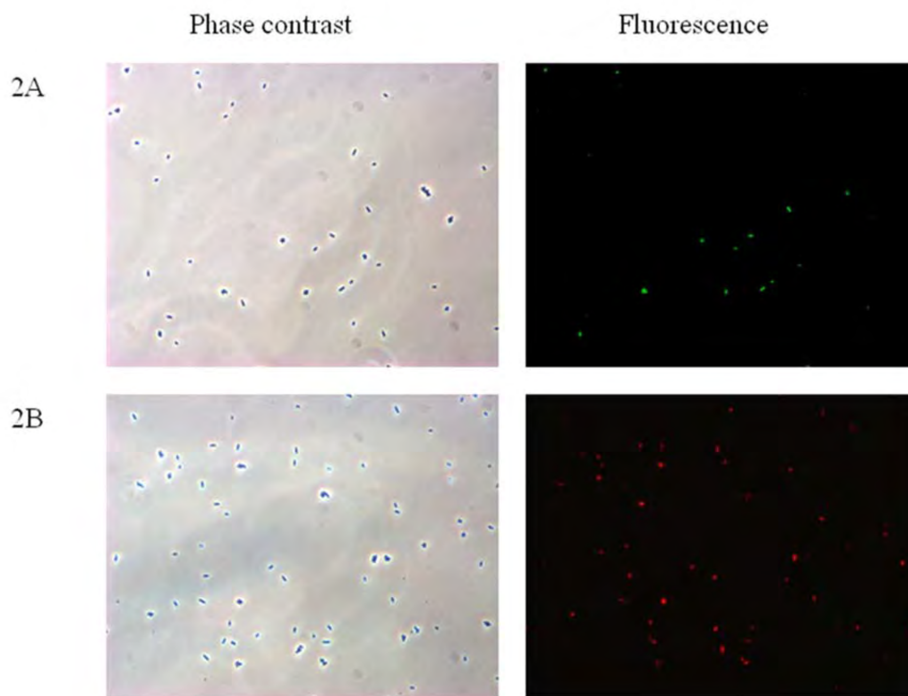
594



595 **Figure 1.** Survival curves of *E. coli* at 50ºC (●), 54ºC (■), 58ºC (▲) and 62ºC (♦). Data

596 points in figures correspond to the average ± standard deviation ($n \geq 3$).

597



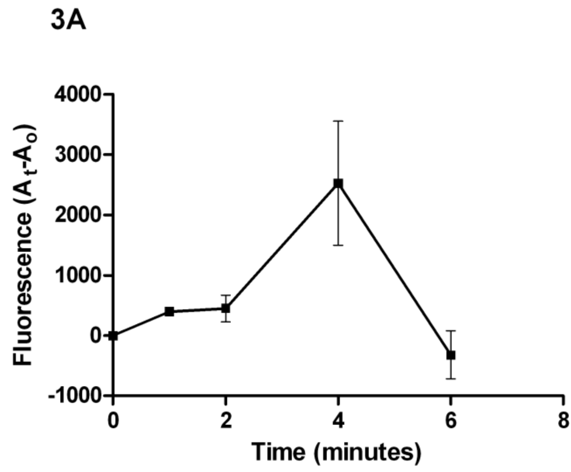
598

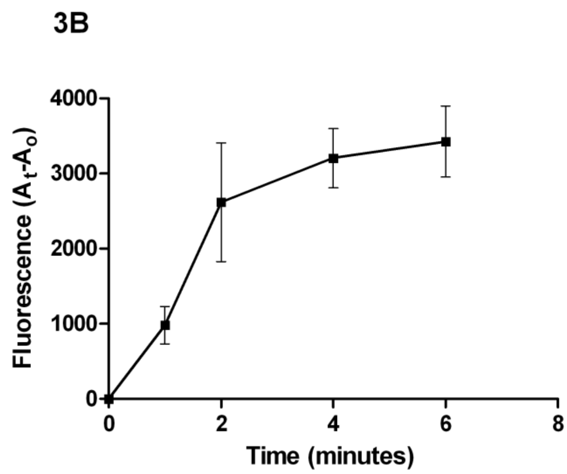
599 **Figure 2.** Presence of ROS in cells treated at 58ºC: representative phase contrast and

600 epifluorescence microphotographs of *E. coli* cells heated for 3 min and stained with

601 H_2DCFDA (2A) and DHE (2B).

602





603

604 **Figure 3.** Total fluorescence of *E. coli* cells heated at 58ºC up to 6 min and stained with

605 H_2DCFDA (A) and DHE (B) (n≥3).

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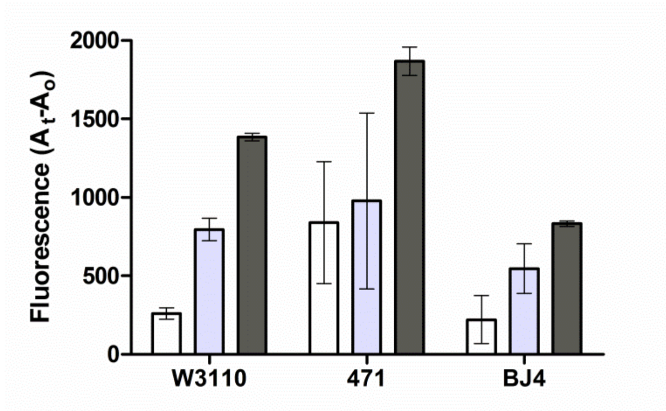

608 **Figure 4.** ROS generation during heat of different strains of *E. coli* (W3110, CECT
609 471, BJ4): total fluorescence of cells treated at 58ºC for 1 (white), 2 (light grey) and 5
610 minutes (dark grey) and stained with DHE (*n*=2).

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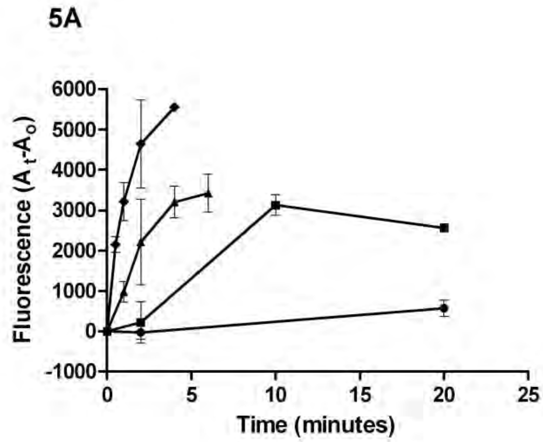
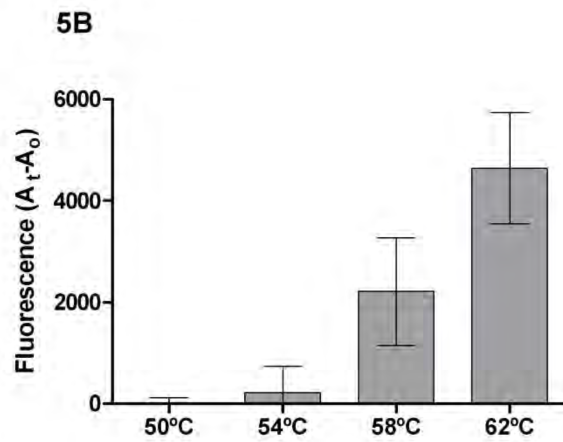
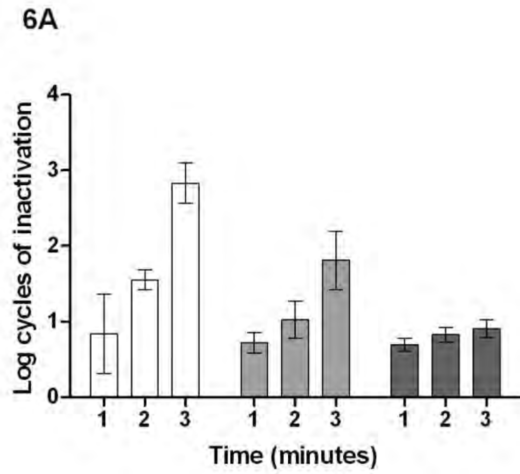


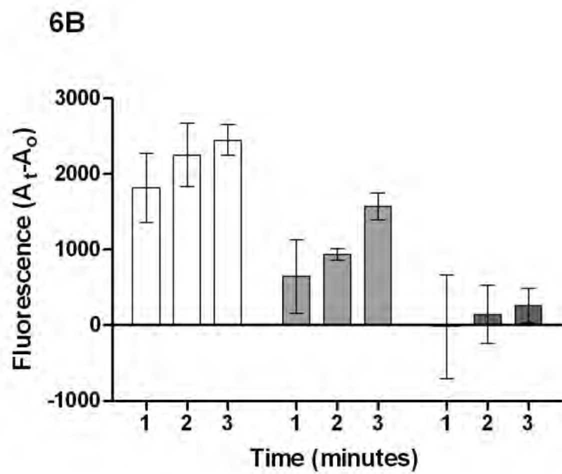


612

613 **Figure 5.** Influence of treatment temperature and time on ROS formation (DHE
614 staining) in heat treated *E. coli* BW25113 cells: (A) total fluorescence along treatments
615 at 50ºC (●), 54ºC (■), 58ºC (▲) and 62ºC (♦); and (B) total fluorescence after 2 minutes
616 of treatment (*n*=2-4).

617

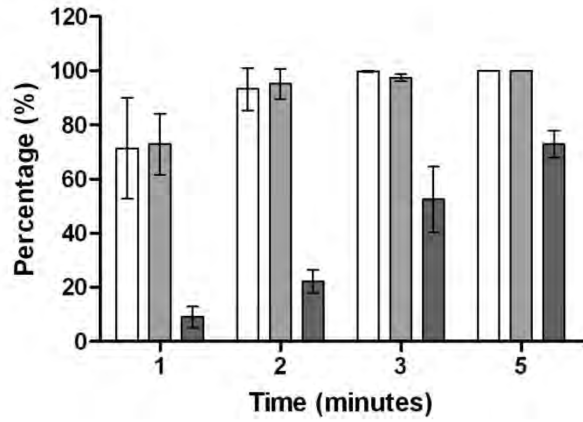




618

619 **Figure 6.** Inactivation of *E. coli* BW25113 after a heat treatment (58ºC; 1, 2 and 3 min)
620 in PBS (white), PBS with 20 mM tiron (light grey) and PBS with 10 mM glutathione
621 (dark grey) (6A), and fluorescence corresponding to DHE staining after heating (6B)
622 (*n*=3).

623



624

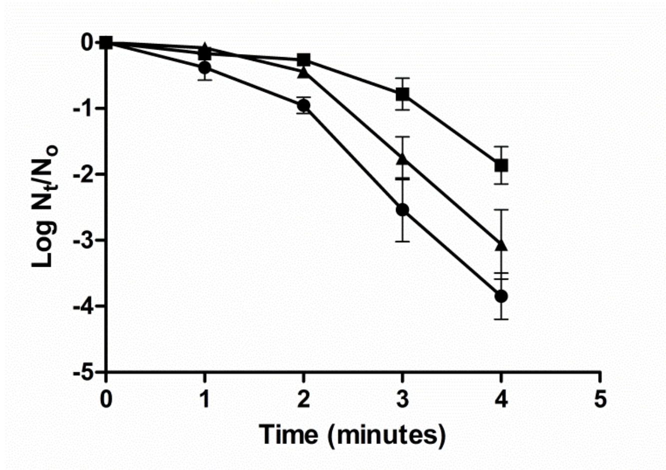
625 **Figure 7.** Percentage of *E. coli* BW25113 inactivated cells (white), cells fluorescent to

626 DHE (light grey) and to PI (dark grey) after different exposure times to heating at 58ºC

627 ($n \geq 3$).

628

629



630 **Figure 8.** Survival curves of *E. coli* BW25113 obtained at 58ºC when cells were

631 recovered in TSAYE/aerobiosis (●), TSAYE with 10 mM of glutathione/aerobiosis (▲)

632 and minimal medium plus 3mM of cysteine/anaerobiosis (■) (*n*=3).

633