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2 **Mechanism of the synergistic inactivation of *E. coli* by UV-C light at mild temperatures**

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ABSTRACT

UV light only penetrates liquid food surfaces to a very short depth, thereby limiting its industrial application in food pasteurization. One promising alternative is the combination of UV light with mild heat (UV-H), which has been demonstrated to produce a synergistic bactericidal effect. The aim of this paper is to elucidate the mechanism of synergistic cellular inactivation resulting from the simultaneous application of UV light and heat. The lethality of UV-H treatments remained constant below approximately 45 ºC, while lethality increased exponentially as the temperature increased. The percentage of synergism reached a maximum (40.3%) at 55 ºC. Neither the flow regime nor changes in the delivered dose by UV lamps contributed to the observed synergism. UV-H inactivation curves of the parental *E. coli* strain obtained in a caffeic acid-selective recovery medium followed a similar profile to those obtained with *uvrA*$^-$ mutant cells in a non-selective medium. Thermal fluidification of membranes and synergistic lethal effects started around 40-45 ºC. Chemical membrane fluidification with benzyl alcohol decreased the UV resistance of the parental strain but not of the *uvrA*$^-$. These results suggest that the synergistic lethal effect of UV-H treatments is due to the inhibition of DNA excision repair resulting from the membrane fluidification caused by simultaneous heating.

42 **INTRODUCTION**

43 Ultraviolet-C (UV-C) light is an emerging disinfection technology for water and,
44 more recently, for liquid foods due to its multiple advantages (1, 2). UV-C (220-300 nm) has
45 germicidal effect for most types of microorganisms because it produces photochemical
46 modifications of nucleic acids' pyrimidine bases. The major UV-induced DNA lesion is
47 cyclobutane pyrimidine dimers (CPDs), while (6–4) photoproducts (6-4PPs) are also formed
48 on about 25% of CPDs (3). These lesions prevent the proper replication and transcription of
49 DNA, resulting in mutagenesis and, ultimately, in cell death (4). The magnitude of the lethal
50 effect depends on the radiation dose and on the cells' ability to repair damage.

51 Microorganisms have adopted various enzymatic DNA repair pathways to restore
52 DNA molecules from replication errors and the action of both endogenous and exogenous
53 DNA-damaging agents. The DNA repair pathways involved in damage repair prior to
54 replication include photorepair, base excision repair (BER), and nucleotide excision repair
55 (NER) (3, 4). Under extensive DNA damage, repair mechanisms controlled by the SOS
56 regulon, such as RecA mediated excision repair (RAMER), translesion synthesis (TLS), and
57 homologous recombination (HR) repair are induced (4, 5). Overall, the lethality of UV light
58 could be improved by impairing bacterial DNA repair mechanisms.

59 The ability of UV light for liquid food hygienization has been widely demonstrated
60 (2, 6). In fact, UV-based technologies have been approved as alternative treatments to
61 thermal pasteurization of fresh juice products (7). However, the implementation of UV
62 processing in the food industry is still limited due to the large amounts of UV absorbing
63 compounds and suspended particles of foods, which reduce UV light transmittance into
64 liquids preventing thereby to achieve a significant microbial inactivation. To overcome this
65 limitation, new processes have been designed by combining several technologies applied at

66 lower intensities, but with equivalent or even higher degrees of stability and safety. The
67 interaction of UV light applied simultaneously with chemical agents (8, 9, 10) and with
68 different energies, such as ionizing radiation (11) and heat (12, 13, 14), have been reported.
69 Regarding the latter, there is an increased interest in the potential use of UV light and mild
70 heat (UV-H treatments) for pasteurization of high UV absorptivity liquid foods (15), as it has
71 been demonstrated to have a synergistic lethal effect on *E. coli* (12) and *Salmonella enterica*
72 subsp. *enterica* serovar Typhimurium (16) at temperatures around 50-60 ºC. Petin et al. (14)
73 suggested two possible explanations for the synergistic lethal effect of the combined process,
74 which are not contradictory: the reduction of cellular capacity to repair DNA damage by
75 thermal effects and the interaction of sub-lethal lesions induced by each of the agents.
76 Despite its interest, the mechanism of microbial killing improvement by UV light in
77 combination with mild heat is not known.

78 The aim of this paper is to elucidate the mechanism of synergistic cellular inactivation
79 by the simultaneous application of UV light and heat. For this purpose, we evaluated changes
80 in the effective dose either by changes in the flow pattern or UV lamps' efficiency to discard
81 the effect of physical factors, and, in a second step, we studied the biological basis of the
82 synergistic effect. The strain *E. coli* K-12 was selected as model microorganism.

83

84 ## MATERIAL AND METHODS

85 **Bacterial culture.** *Escherichia coli* K-12 substrain BW25113 and its isogenic deletion
86 mutants, listed in Table 1, were obtained from the Keio collection (17). The bacterial cultures
87 were kept frozen at -80 ºC in cryovials. Stationary-phase cultures were prepared by
88 inoculating 10 ml of tryptone soy broth (Biolife, Milan, Italy) supplemented with 0.6% (w/v)
89 yeast extract (Biolife) (TSBYE) with a loopful of growth from tryptone soy agar (Biolife)

90 supplemented with 0.6% (w/v) yeast extract (TSAYE). The cultures were incubated at 35 ºC
91 for 6 h in a shaking incubator. Fifty µl of the cultures were inoculated into 50 ml of fresh
92 TSBYE and incubated for 24 h under the same conditions, which resulted in stationary-phase
93 cultures containing approximately 10^9 CFU ml^{-1}. All media were sterilized at 121 ºC for 20
94 min.

95 **UV treatments.** The UV equipment used in this investigation was previously described by
96 Gayán et al. (12). The entire system was formed by eight annular thin film flow-through
97 reactors connected in series with a sampling valve at the exit of each reactor. Each reactor
98 consisted of a low-pressure UV lamp (TUV 8WT5, Philips, USA) with an input power of 8
99 W (85% of energy emitted at a wavelength of 254 nm) enclosed by a quartz tube. The annular
100 gap between the quartz tube and the outside glass sleeve, where liquid flowed, was 2.5 mm.
101 The reactors were submerged in a tempered 90 l water bath (T ± 1.5 ºC) heated by the
102 circulating water of a peripheral thermostatic bath (Huber, mod. Kattebad K12, Offenburg,
103 Germany). Two thermocouples (Almeco, mod. ZA 020-FS, Bernburg, Germany), located at
104 the input of the first and the outlet of the last reactor, allowed the treatment temperature to be
105 controlled.

106 The treatment medium was added with the bacterial suspension to achieve,
107 approximately, 10^7 CFU ml^{-1} and pumped (8.5 l h^{-1}) through the heat exchanger to the
108 reactors. When the treatment conditions were stabilized, samples were withdrawn through the
109 sampling valves at the outlet of each reactor and 0.1 ml or 1 ml was immediately pour plated
110 in the recovery media. A sterile McIlvaine citrate-phosphate buffer of pH 7.0 (18) with an
111 absorption coefficient of 23.6 cm^{-1}, close to that of clarified apple juice, was used as UV-H
112 treatment medium. To compare the resistance and the inactivation kinetics of *E. coli*
113 derivatives to UV light at room temperature, McIlvaine buffer of pH 7.0 with a lower

114 absorption coefficient (15.3 cm^{-1}) was used. Buffers of different absorption coefficients were
115 prepared by adding after buffer sterilization different quantities of tartrazine (Sigma-Aldrich,
116 St. Louis, USA) and adjusting media absorbance to 254 nm using a Unicam UV500
117 spectrophotometer (Unicam Limited, Cambridge, UK) as described in Gayán et al. (12). Prior
118 to and following each treatment session, the UV equipment was cleaned and sanitized using an
119 ethanol solution (Aldipa, Zaragoza, Spain) (30% v/v) and rinsed with sterile distilled water with UV
120 lamps on. It was checked that plating samples of the rinse water did not show microbial growth.

121

122 **Heat treatments**. Heat treatments were carried out in a specially designed resistometer (19).
123 Briefly, this instrument consists of a 350 ml vessel equipped with an electrical heater for
124 thermostation, an agitation device to ensure inoculum distribution and temperature
125 homogeneity, and ports for injecting the microbial suspension and for sample extraction.
126 Once the preset temperature had attained stability (T ± 0.05 °C), 0.2 ml of an adequately
127 diluted microbial cell suspension were inoculated into the corresponding treatment medium.
128 After inoculation, 0.2 ml samples were collected at different heating times and immediately
129 pour plated. Prior to and following each treatment session, the vessel was sterilized at 121 ºC for
130 20 min.

131

132 **Incubation of treated samples and survival counting.** TSAYE was used as the recovery
133 medium. Where indicated, the maximum non-inhibitory concentration (MNIC) of caffeic acid
134 (TSAYE+CA) (Panreac, Barcelona, Spain) was added to the recovery medium before
135 sterilizing, to estimate the percentage of sub-lethally injured cells. The MNIC of caffeic acid
136 (3.75 mg ml^{-1}) was chosen in previous experiments with non-treated cells (data not shown).
137 The presence of caffeic acid in the recovery medium inhibits nucleotide excision repair by

138 affecting UvrABC excinuclease activity (20, 21). Samples recovered in the non-selective
139 medium were incubated for 24 h at 35 ºC, and those recovered in caffeic acid enriched
140 medium for 48 h at 35 ºC. Previous experiments demonstrated that longer incubation times
141 did not change the profile of survival curves. After incubation, colony-forming units (CFU)
142 were counted with an improved image analyzer automatic colony counter (Protos, Synoptics,
143 Cambridge, UK), as described in previous research (22). For photoreactivation, 20 µl of
144 different dilutions of each sample were spread plated in TSAYE Petri dishes under 13 W
145 daylight fluorescent lamps (T16 13W/827-EVG, G5, Osram, Munich, Germany) that emitted
146 light in the 360 to 700 nm wavelength range. The distance between the surface of the test
147 suspension and the lamp was 10 cm. The mean illuminance was 11.15 Klux, and
148 photoreactivation was performed for 60 min at 20 ºC. Previously, longer incubation times and
149 higher temperatures were demonstrated to not improve survival counts. In each experiment, a
150 sample of UV-irradiated suspension was kept in the dark under the same conditions as a
151 reference. After photoreactivation, the plates were incubated for 24 h at 35 ºC.

152

153 **Curve fitting, resistance parameters, and synergistic effect calculation.** Survival curves to
154 UV treatments were obtained by plotting the logarithm of the survival fraction *versus*
155 treatment doses, expressed in energy consumption unit (J ml^{-1}) (UV treatments at room
156 temperature) or time in min (heat and UV-H treatments). To fit survival curves and calculate
157 resistance parameters, the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT)
158 was used (23). Because our survival curves did not show tails but rather shoulders, the log-
159 linear regression plus shoulder model (24) was used. This model describes the survival curves
160 through two parameters: shoulder length (*Sl*) or time before the exponential inactivation
161 begins and the inactivation rate (*Kmax*), defined as the slope of the exponential portion of the

162 survival curve. For comparison purposes, the GInaFiT also provides the parameter 4*D*,

163 defined as the treatment time necessary to inactivate 99.99% of the microbial population.

164 To determine the magnitude of the synergistic lethal effect of the combined UV-H

165 treatment at each temperature, 4*D* values obtained experimentally (*experimental* $4D_{UV\text{-}H}$)

166 were compared with *theoretical* $4D_{UV\text{-}H}$ values (time to achieve a 99.99% reduction in the

167 microbial inactivation if the two processes act simultaneously but independently) calculated

168 *via* the following equations proposed by Gayán et al. (15):

169 $$Theoretical\ 4D_{UV-H} = \frac{(4D_H\ x\ 4D_{UV})}{(4D_H + 4D_{UV})} \quad (1)$$

170 $$\%Synergism = \frac{Theoretical\ 4D_{UV-H} - Experimental\ 4D_{UV-H}}{Theoretical\ 4D_{UV-H}} x100 \quad (2)$$

171 where $4D_H$, and $4D_{UV}$ values were obtained from the fitting of the inactivation curves to heat

172 and UV light, respectively.

173

174 **Membrane fluidity measurement.** Fluorescence anisotropy was measured using diphenyl-

175 1,3,5-hetratriene (DPH, Sigma-Aldrich, Steinheim, Germany) as a lipophilic marker by a

176 procedure similar to that described by Almeida et al. (25) and calculated according to Chu-

177 Ky et al. (26). Fluorescence measurements were carried out in a spectrofluorometer

178 Cary-Eclipse (Varian Medical Systems, Belrose, Australia) equipped with a manual

179 fluorescence polarizer (Varian Medical Systems). The spectrofluorometer system contains

180 four sample chambers that can be thermostated by an auxiliar thermostatic bath (Digiterm

181 3000613, Selecta S. A., Barcelona, Spain). One ml of cell suspension was centrifuged

182 (10,000 *g* for 5 min) and resuspended at a concentration of 10^8 CFU ml^{-1} in McIlvaine buffer

183 of pH 7.0 with 0.25% (v/v) of formaldehyde (Panreac). The obtained suspension was

184 incubated with a solution of DPH in tetrahydrofuran (Scharlau, Barcelona, Spain) for 45 min
185 at 35 ºC. To determine the fluorescence anisotropy at different temperatures (25-60 ºC),
186 fluorescence measurements were performed after maintaining cells for 3.5 min at the preset
187 temperature. The excitation and emission wavelengths used were 355 ± 5 nm and 425 nm ± 5
188 nm, respectively.

189 To evaluate the effect of cell membrane fluidity on the UV resistance at room
190 temperature, cells were pre-incubated (20 min) and treated in the McIlvaine buffer with
191 benzyl alcohol (BA, Panreac, Barcelona, Spain) added at a concentration of 20 mM.

192

193 **Residence time distribution and delivered dose.** The stimulus-response technique by
194 impulsion was used for experimental determination of residence time distribution (RTD)
195 curves, as previously described by Koutchma and Parisi (27), using saturated sodium chloride
196 solution as a tracer. When the McIlvaine buffer flow achieved a steady-state condition (flow
197 rate 8.5 l h^{-1}), 3 ml of the solution was injected with a syringe into the center of the pipe 3 cm
198 before of the input of the UV reactor. The conductivity (Crison Instruments, Barcelona,
199 Spain) of the output flow of the entire installation was measured as a function of time after
200 injection. The sodium chloride concentration distribution $c(t)$ curve was normalized to obtain
201 the $E(t)$ curve (equation 3), from which the mean residence time ($\bar{t}$, equation 4) was
202 calculated, as well as the variance (σ^2, equation 5) as a measure of the residence time
203 dispersion:

204 $$E(t) = \frac{c(t)}{\int_0^t c(t)dt} \qquad (3)$$

205 $$\bar{t} = \int_0^t tE(t)dt \tag{4}$$

206 $$\sigma^2 = \int_0^t t^2 E(t)dt - \bar{t}^2 \tag{5}$$

207 The chemical quantification of the delivered UV intensity was performed with the
208 iodide-iodate actinometer containing 0.6 M potassium iodide and 0.1 M potassium iodate in
209 0.01 M $Na_2B_4O_7.12H_2O$ at pH 9.25 (Panreac, Barcelona, Spain). Determination of photo-
210 product I_3^- was carried out spectrophotochemically using an extinction coefficient of $\varepsilon_{352} =$
211 27.60 M^{-1} cm^{-1}.

212

213 **Statistical analysis.** Statistical analyses, *t*-test ($p = 0.05$) and ANOVA ($p = 0.05$) tests, were
214 carried out using GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego,
215 USA), and differences were considered significant for $p \leq 0.05$. All microbial resistance
216 determinations and analytical assays were performed at least three times on different working
217 days. The error bars in the figures correspond to the mean standard deviation.

218

219 **RESULTS**

220 **Synergistic lethal effect of UV-H treatments on *E. coli* wild type inactivation.** Figure 1
221 shows the survival curves of *E. coli* wild type (WT) treated by UV light at different
222 temperatures (25.0-60.0 ºC) in a McIlvaine buffer with an absorption coefficient of 23.6 cm^{-1}
223 and recovered in a non-selective medium. As observed in the figure, survival curves showed
224 an initial lag phase followed by an exponential inactivation rate. Concave downward survival
225 curves were fitted with the log linear regression plus shoulder model described by Geeraerd

226 et al. (24). Estimated parameters of the model (*Sl* and *Kmax*) and 4*D* values are compiled in
227 Table 2. The table includes the coefficient of determination (R^2) and the root mean square
228 error (*RMSE*) values obtained from the fit. The simultaneous combination of UV light with
229 mild heating significantly improved microbial inactivation. This improvement was explained
230 by the reduction of the *Sl* and the increase of the *Kmax* with increasing treatment
231 temperatures (Table 2).

232 To quantify the contribution of thermal effects to the lethality of the UV-H combined
233 treatment, the heat resistance of *E. coli* WT was ascertained (Table 3). Figure 2 shows the
234 relationship between treatment temperature and the Log_{10} 4*D* values to heat (thermal death
235 time curve-TDT) and to UV-H treatments (UV-TDT). As expected, the TDT curve obtained
236 for heat treatments showed an exponential course, from which log linear regression equation
237 a *z* value of 5.56 ºC ($R^2 = 0.999$) was deduced (degrees of temperature increase necessary to
238 reduce $4D_H$ value 10 times). In contrast, the UV-TDT curve showed a concave downward
239 profile. The lethality of UV-H treatments remained constant below approximately 45 ºC, and
240 above this temperature, the lethality increased exponentially but with a lower thermo-
241 dependence than heat treatments: The $4D_{UV\text{-}H}$ value would decrease ten times by increasing
242 the temperature by 12.02 ºC.

243 The magnitude of the synergistic lethal effect was calculated by comparing
244 experimental $4D_{UV\text{-}H}$ values with those estimated mathematically (theoretical $4D_{UV\text{-}H}$ values),
245 assuming that the effect of the combined treatment was additive (equation 1). The theoretical
246 UV-TDT curve is also included in Figure 2 (dotted line), so that the area between the
247 experimental and theoretical UV-TDT curves illustrates the magnitude of the synergistic
248 lethal effect.

249

250 **Residence time distribution and delivered dose at different temperatures**. The RTD
251 curves of the entire installation at 25.0, 50.0, 55.0, and 60.0 ºC were obtained by the stimulus-
252 response method in a McIlvaine buffer flowing at 8.5 l h^{-1}. RTD curves were normalized and
253 the mean residence time ($\bar{t}$) and the variance (σ^2) from the point of tracer injection were
254 obtained. No statistically significant differences ($p > 0.05$) were found among $\bar{t}$ and σ^2 values
255 obtained at 25.0 ($\bar{t}$ = 4.36 ± 0.14 min, σ^2 = 34.66 ± 1.69 min^2), 50.0 ($\bar{t}$ = 4.41 ± 0.09, σ^2 =
256 35.10 ± 5.78 min^2), 55.0 ($\bar{t}$ = 4.31 ± 0.11 min, σ^2 = 31.04 ± 0.77 min^2), and 60.0 ºC ($\bar{t}$ = 4.21
257 ± 0.12 min, σ^2 = 30.23 ± 2.75 min^2).

258 The actinometry iodide-iodate technique was used to study the effect of temperature
259 on the delivered dose. Figure 3 illustrates the absorbance of I_3^- photo-products induced by
260 UV light in the McIlvaine buffer with an absorption coefficient of 23.6 cm^{-1}. As observed in
261 the figure, the delivered UV dose decreased at temperatures between 50.0 and 60.0 ºC, the
262 range of treatment temperatures at which the UV susceptibility of *E. coli* WT increases.

263

264 **UV resistance of *E. coli* derivatives deficient in DNA repair mechanisms.** To study the
265 UV inactivation kinetics of the wild type strain and its isogenic mutants (Table 1) they were
266 treated in McIlvaine buffer of low absorption coefficient (15.4 cm^{-1}), and recovered in the
267 non-selective medium (TSAYE) (Figure 4). As observed in the figure, the Log_{10} of survivors
268 of *uvrA*$^-$ and *recA*$^-$ mutants decreased linearly with the delivered dose. Meanwhile,
269 inactivation curves of *phr*$^-$, *nth*$^-$, and *umuD*$^-$ mutants showed an initial shoulder followed by
270 an exponential inactivation phase similar to that of the parental strain. Survival curves were
271 fitted to Geeraerd et al.'s model and calculated parameters are presented in Table 4. No
272 statistically significant differences ($p > 0.05$) were found between the 4*D* values, as well as *Sl*

273 and *Kmax* parameters, of the parental strain and the *nth*⁻ mutant. The UV sensitivity of the
274 *phr*⁻ and *umuD*⁻ mutants (4*D* = 26.30 ± 1.15 and 26.77 ± 1.13 J ml^{-1}, respectively) increased
275 slightly as compared to the parental strain (4*D* = 29.93 ± 1.54 J ml^{-1}), decreasing the shoulder
276 length of the survival curve (Table 4). However, the UV resistance of the *uvrA* and *recA*
277 deficient strains diminished drastically (4*D* = 12.21 ± 0.93 and 11.07 ± 0.43 J ml^{-1},
278 respectively). The concave downward profile of the survival curve of the parental strain
279 became linear in *uvrA*⁻ and *recA*⁻ mutants (Figure 4) as the shoulder disappeared, and, in
280 addition, the inactivation rate almost duplicated. Table 4 also includes the resistance
281 parameters of the wild type strain treated by UV light at room temperature and recovered in
282 the caffeic acid-selective medium (TSAYE+CA) and in the non-selective medium after a
283 photoreactivation step before incubation (TSAYE+visible light -11.15 Klux for 60 min). The
284 photoreactivation process improved the UV survival of *E. coli* WT, increasing the 4*D* value
285 by 17.6%. The parental strain inactivation curve obtained in the TSAYE+CA medium
286 followed a similar profile to those obtained with *uvrA*⁻ and *recA*⁻ mutant cells in TSAYE
287 (Figure 4): The shoulder disappeared and the inactivation rate increased (Table 4). In fact,
288 there were no statistically significant differences ($p > 0.05$) among the mean 4*D* values of the
289 parental strain recovered in TSAYE+CA (12.37 ± 0.32 J ml^{-1}) and that of its *uvrA*⁻ and *recA*⁻
290 derivatives recovered in TSAYE. Similar results were obtained with buffer of high absorption
291 coefficient (data not shown).

292 **Heat resistance characterization of *uvrA*⁻ and *recA*⁻ mutants.** The heat resistance
293 characterization of the two most UV sensitive *E. coli* derivatives (*uvrA*⁻ and *recA*⁻ mutants)
294 was performed (Table 3). The data of Table 3 show that heat survival curves of the *uvrA*⁻
295 mutant had long shoulders, like the parental strain, whereas shoulders were not present in the
296 *recA*⁻ mutant. Moreover, 4*D* values obtained from the *recA*⁻ mutant were about half of those
297 obtained for the parental strain and for the *uvrA*⁻ mutant at all temperatures tested. As the

298 *uvrA*$^{-}$ mutant showed the same heat resistance as the wild type strain, it was chosen for further
299 studies.

300 **UV-H resistance of the *uvrA*$^{-}$ mutant and the wild type strain recovered in non-
301 selective and caffeic acid-selective medium.** Resistance parameters of the *uvrA* deficient
302 microorganism recovered in the non-selective medium and its isogenic wild type strain, also
303 recovered in the caffeic acid-medium, are shown in Table 2. As observed in the table, the
304 survival curves of the *uvrA*$^{-}$ mutant recovered in TSAYE and those of the parental strain
305 recovered in TSAYE+CA did not show shoulders, and no statistically significant differences
306 ($p > 0.05$) were found between their *Kmax* at all temperatures tested. A comparison of the 4*D*
307 values of the *uvrA*$^{-}$ mutant with those of the parental strain recovered in the non-selective
308 medium shows that the former was significantly less UV-H resistant. However, this
309 difference in resistance was reduced by increasing treatment temperature. This phenomenon
310 is also illustrated in Figure 5, where the experimental UV-TDT curves of the *uvrA*$^{-}$ mutant
311 and of the parental strain are plotted. For the mutant strain, the lethal contribution of heat was
312 not observed at temperatures below 55 ºC, approximately, and above this temperature, the
313 lethality increased with a similar thermo-dependence to heat treatments. In addition, the
314 experimental and theoretical UV-TDT of the *uvrA*$^{-}$ mutant overlapped (Figure 5), which
315 means that no synergistic lethal effect occurred, contrary to that observed for the parental
316 strain (Figure 2).

317

318 **Role of membrane fluidity on the UV-H resistance of *E. coli*.** Figure 6 shows the
319 percentage decrease of DPH fluorescence anisotropy in the *E. coli* WT and *uvrA*$^{-}$ mutant
320 membrane after maintaining cells for 3.5 minutes at temperatures between 25.0 and 58.0 ºC.
321 The percentage of synergism previously calculated for UV-H treatments of *E. coli* WT

322 (equation 2) has also been included (dotted line) for comparison. The decrease of DPH
323 fluorescence anisotropy with temperature in the *E. coli* WT and *uvrA*$^{-}$ mutant membranes
324 showed the same tendency. The fluidity of the membrane of *E. coli* remained constant as the
325 temperature increased to 40-45 ºC, approximately. Further increases in temperature caused a
326 decrease in DPH fluorescence anisotropy, which indicates an increase in membrane fluidity.
327 This profile reflected the curve corresponding to the percentage of synergism (Figure 6),
328 which started to be relevant at temperatures of 40-45 ºC, approximately, and increased
329 steadily up to 55 ºC. Further increases in temperature did not induce higher UV-H synergistic
330 lethal effects due to the predominance of heat lethality over the lethal effects of the UV light.
331 These results suggested that the fluidification of the membrane could contribute to the
332 synergistic lethal effect of UV-H treatments.

333 To test this hypothesis, *E. coli* WT and the *uvrA*$^{-}$ mutant were treated by UV light at
334 room temperature after inducing its membrane fluidification with benzyl alcohol (BA). BA
335 has been widely used to investigate the correlation between membrane fluidity and membrane
336 function because it is able to induce physical membrane disorders without impairing cell
337 viability (28). Before treatments, cells were incubated in the McIlvaine buffer with 20 mM
338 BA added for 20 min. This treatment triggered a decrease of 30-40% of DPH fluorescence
339 anisotropy in the parental and in the *uvrA*$^{-}$ mutant strains, indicating a fluidification of the
340 membrane (data not shown). The survival curves of the BA-exposed cells of the parental and
341 the *uvrA*$^{-}$ mutant strains against UV treatments (23.6 cm^{-1}) are represented in Figure 7. As
342 can be observed in the figure, the UV resistance of the parental strain was significantly
343 reduced (4*D* value from 64.62 ± 2.86 to 29.52 ± 1.52 J ml^{-1}) when cells had been previously
344 exposed to BA. However, membrane fluidification in the *uvrA*$^{-}$ mutant did not change its UV
345 resistance ($p > 0.05$).

346

DISCUSSION

348 The present results show that the UV susceptibility of *E. coli* WT was augmented with
349 increasing treatment temperatures (Figure 1 and Table 2). This sensitization was due to a
350 decrease of the shoulder length and an increase of the inactivation rate. Shoulder length has
351 been related with damage repair capacity (12, 16), therefore shorter shoulders at higher
352 temperatures could be due to a reduction in cellular capacity to repair damages or to the
353 appearance of additional lethal damages that arises from the interaction of lesions induced by
354 both agents.

355 The relationship between treatment temperature and the Log_{10} 4*D* values of the UV-H
356 treatments (UV-TDT curve) showed a concave downward profile (Figure 2). Accordingly,
357 the UV lethality of *E. coli* WT did not change by increasing the temperature to 45 ºC,
358 approximately, and above this temperature, it exponentially augmented at a rate 10 fold for
359 each temperature increase of 12.02 ºC. The theoretical 4*D* values of UV-H treatments were
360 calculated assuming that the lethal effect of the combined treatment was additive (equation 1)
361 and plotted in Figure 2 (theoretical UV-TDT curve). There was a range of temperatures
362 where theoretical 4*D* values were higher than experimental values, from which was deduced
363 that there was a synergistic lethal effect. The area between experimental and theoretical UV-
364 TDT curves illustrates the evolution of the magnitude of the synergism. The maximum
365 synergistic lethal effect of all tested temperatures was found at 55.0 ºC (40.3% of synergism),
366 a temperature value that was similar to that reported for *E. coli* STCC 4201 (12) and
367 *Salmonella Typhimurium* (16). Above 55 ºC the synergistic lethal effect decreased. This was
368 probably due to the higher heat dependence of the thermal inactivation (z = 5.56 ºC) as
369 compared to UV-H inactivation (z = 12.02 ºC). The faster increase with temperature of the

370 thermal lethality would finally make the lethality of UV negligible and therefore 4*D* values
371 for both technologies become the same. It would be also possible that above 55 ºC changes
372 may occur in some cellular components that would lead to a reduction in the synergistic
373 effect.

374 Temperature affects the rheological properties of liquids and consequently modifies
375 the flow behavior and the velocity profile of flowing liquids (29); therefore, it can influence
376 the residence time distribution. As a first step, we observed that increasing the temperature
377 between 25.0 and 60.0 ºC did not change the flow pattern in our experimental conditions ($\bar{t}$ =
378 4.36 ± 0.14 min, σ^2 = 34.66 ± 1.69 min^2 and $\bar{t}$ = 4.21 ± 0.12 min, σ^2 = 30.23 ± 2.75 min^2,
379 respectively). Therefore, the synergistic lethal effect was not due to rheological changes. On
380 the other hand, the delivered dose by UV lamps could change with temperature and could
381 perhaps explain the increase of UV-H lethality at higher temperatures. However, results
382 demonstrated that the delivered dose that reached the treatment medium was reduced between
383 50.0 and 60.0 ºC (Figure 3), coinciding with the temperature range in which UV-H lethality
384 increased. These results are consistent with the fact that the production of 254 nm UV
385 irradiation by low pressure lamps increases with respect to ambient temperature up to a
386 maximum level at 40 ºC, and then drops with increasing temperature (6). Thus, the change of
387 the delivered dose emitted by lamps does not explain the synergistic lethal effect. Overall, we
388 conclude that no photo-physical effect contributed to the observed synergism of the combined
389 UV-H process and it should be therefore explained through its biological effects.

390 The synergistic lethality of UV-H treatments could be due to an improvement of the
391 UV effects on the DNA molecule, either through an interference with DNA repair
392 mechanisms or through an increase of the damage to the DNA. To evaluate the effect of the
393 combined UV-H process on *E. coli*'s DNA repair capacity, we used derivative strains

394 deficient in different repair mechanisms. According to our results (Figure 4 and Table 4), the

395 microorganism carrying the *nth*$^{-}$ mutation exhibited the same degree of UV sensitivity as the

396 wild type strain, which indicates that base excision repair mediated by the TG-DNA

397 glycosylase would not intervene in the UV-C resistance of *E. coli*. This finding was not

398 surprising since TG-DNA glycosylase is involved in the repair of DNA oxidative damage (5-

399 hydroxycytosine, urea, and thymine glycol lesions) (3), induced primarily at higher UV-

400 wavelengths (UV-A and UV-B) (30) than those used in this study. The deletion of the *umuD*

401 gene, essential to construct polymerase V, which is involved in the translesion synthesis

402 pathway (5), scarcely increased UV cell sensitivity. On the other hand, the deletion of the *phr*

403 gene also resulted in a slight increase in *E. coli* UV sensitivity (17.6% after a

404 photoreactivation step). Sancar et al. (31) demonstrated that DNA photolyase may interact

405 with CPD lesions, facilitating the incision of the UvrABC exinuclease. Strains harboring

406 mutations in one of the *uvr* loci (*uvrA*, *uvrB*, and *uvrC*) are unable to remove thymine dimers

407 by nucleotide excision repair, failing to survive even a small UV dose (32). Accordingly, the

408 UV-survival curve obtained with the parental strain recovered in caffeic acid added medium

409 produced the same pattern as the *uvrA*$^{-}$ mutant (Table 4). RecA nucleofilament protein is

410 directly involved in the implementation of SOS response repair mechanisms (5). The *recA*$^{-}$

411 mutant showed the same degree of UV sensitivity as the *uvrA*$^{-}$ strain. All of these results

412 taken together indicate that the most relevant to UV-C survival in the *E. coli* WT strain are

413 UvrABC-mediated nucleotide excision repair and RecA-dependent repair systems. Given the

414 coincidence among survival curves of the *recA*$^{-}$ and the *uvrA*$^{-}$ mutants, it is also feasible that

415 the loss of UV resistance in both strains is due to a defect in the same repair pathway, which

416 would be nucleotide excision repair mediated by RecA protein (RAMER). This hypothesis

417 agrees with results of Bichara et al. (33).

418 The heat resistance characterization of the most UV sensitive mutants (*uvrA*$^-$ and
419 *recA*$^-$) showed that the *recA*$^-$ mutant was more sensitive to heat treatments since 4*D* values
420 were approximately half of those corresponding to the parental strain at all tested
421 temperatures (Table 3), indicating that the RecA protein is also involved in cellular protection
422 to heat. The *E. coli uvrA*$^-$ mutant was considered the most appropriate derivative strain to
423 continue our investigation due to its defect in DNA damage repair and its similarity in heat
424 tolerance to the parental strain. Subsequently, the UV-H resistance at different temperatures
425 of the strain carrying the *uvrA*$^-$ mutation was performed. The UV-TDT curve of the *uvrA*$^-$
426 mutant showed, as the parental strain, a downward concave profile (Figure 5). However, its
427 experimental UV-TDT curve overlapped with the theoretical curve, confirming that the UV-
428 H treatments of the *uvrA*$^-$ mutant did not exhibit a synergistic lethal effect. Moreover, the
429 resistance parameters of the *uvrA*$^-$ mutant recovered in the non-selective medium and the wild
430 type strain recovered in the caffeic acid-medium were similar (Table 2). Therefore, it can be
431 concluded that the synergistic lethal effect observed in UV-H treatments was due to the
432 inhibition of DNA excision repair mechanisms by the action of heat, and not to a higher
433 degree of direct DNA damage as previously suggested.

434 This phenomenon may explain the inactivation of UV-H kinetics. The UV-H survival
435 curves of the wild type strain (Figure 2) showed initial shoulder phases, which decreased with
436 increasing treatment temperature, followed by an exponential order of death. Shoulder phases
437 obtained in our experimental conditions can be attributed to the action of DNA repair
438 mechanisms, given the lack of the lag phase in the UV survival curves of the *uvrA*$^-$ and *recA*$^-$
439 derivatives, and in the survival curves of the parental strain in the presence of caffeic acid,
440 which prevents UvrABC action.

441 Finally, the motivation behind inhibiting the excision repair pathway requires
442 clarification. The idea that heat could induce conformational and structural changes in
443 UvrABC proteins inhibiting its enzymatic activity was immediately discarded since, as we
444 have reported before (16), the application of a heat treatment before the UV treatment did not
445 increase the UV susceptibility of *Salmonella* Typhimurium, merely showing an additive
446 lethal effect. Geveke (13) suggested that the UV-H synergistic lethal effect observed in *E.*
447 *coli* treated in egg whites (30-50 ºC) could be related to changes in the physical state of the
448 cell membrane, when induced by heat. This hypothesis may be consistent with our results
449 since the incision step of UvrABC and RecA activity during SOS are reportedly associated
450 with the inner membrane of *E. coli* (34). Moreover, Todo et al. (35) demonstrated, using an
451 unsaturated fatty acid auxotroph of *E. coli*, that the fluidification state of cell membranes
452 influences the recovery of UV irradiated cells. Hence, we proceeded to determine the
453 membrane fluidity changes induced by heating. The relationship between the DPH
454 fluorescence anisotropy decreased in *E. coli* WT and *uvrA*$^-$ mutant and the treatment
455 temperature showed a similar profile to the experimental UV-TDT curves of both strains
456 (Figure 6). Both the thermal fluidification of membranes and the synergistic lethal effects of
457 *E. coli* WT started around 40-45 ºC. In addition, the incubation of *E. coli* cells in the
458 treatment medium with the membrane fluidizing agent BA reduced the UV resistance of the
459 parental strain, whereas it did not change the UV resistance of the *uvrA*$^-$ mutant strain (Figure
460 7). These results indicate that the physical state of the membrane during treatment in the
461 parental strain of *E. coli* (i.e., its fluidity) influenced its UV resistance. Cells with a more
462 fluid membrane were more sensitive to the action of UV, and this increase in fluidity upon
463 heating may explain the synergistic lethal effect observed in UV-H treatments applied to the
464 parental strain. Since this effect did not occur in the *uvrA*$^-$ mutant strain, we have to assume

465 that the decrease in survival caused by membrane fluidization is linked to the poor
466 functioning of the nucleotide excision repair mechanisms in the parental strain.

467 In conclusion, results obtained in this investigation demonstrate that the synergistic
468 lethal effect of UV-H treatments in *E. coli* resulted from the inhibition of DNA excision
469 repair due to membrane fluidification caused by the simultaneous heating. Thus, UV-induced
470 DNA lesions remained unrepaired, decreasing *E. coli*'s UV survival. This raises the
471 possibility of combining fluidizing membrane agents with UV light treatments to improve its
472 lethality in, for example, food processing applications.

473

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TABLE 1. *E. coli* BW25113 derivatives used in this investigation with different DNA repair pathways deleted.

Derivative	Relevant genotype	Description	DNA repair mechanisms affected
JW0698	Δ*phr-758::kan*	Deoxyribodipyrimidine photolyase	Photoreactivation
JW1625	Δ*nth-782::kan*	Thymine Glycol-DNA glycosylase (TG-DNA glycosylase) or endonuclease III	Base excision repair
JW4019	Δ*uvrA753::kan*	ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC	Nucleotide excision repair
JW1172	Δ*umuD772::kan*	Subunit D of DNA polymerase V	Translesion synthesis
JW2669	Δ*recA774::kan*	DNA strand exchange and recombination protein with protease and nuclease activity	SOS response: RecA mediated excision repair, translesion synthesis, and homologous recombination repair

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TABLE 2. Resistance parameters (*Sl*, *Kmax,* and time for 4*D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *E. coli* WT and its isogenic mutant *uvrA*[-] treated by the UV-H combination in McIlvaine buffer with an absorption coefficient of 23.6 cm^{-1} at different temperatures and recovered in non-selective (TSAYE) and selective media with caffeic acid added (TSAYE+CA).

	Wild type strain (TSAYE)					**Wild type strain (TSAYE+CA)**					***uvrA*[-] strain (TSAYE)**				
Temperature (ºC)	***Sl* (min)**	***Kmax* (min^{-1})**	***4D* (min)**	***R^2***	***RMSE***	***Sl* (min)**	***Kmax* (min^{-1})**	***4D* (min)**	***R^2***	***RMSE***	***Sl* (min)**	***Kmax* (min^{-1})**	***4D* (min)**	***R^2***	***RMSE***
25.0	0.96 (0.07)[a]	1.13 (0.23)[a]	9.07 (0.43)[a]	0.958	0.116	0[b]	3.63 (0.25)[b]	2.56 (0.13)[b]	0.960	0.501	0[b]	3.79 (0.56)[b]	2.37 (0.12)[b]	0.985	0.260
40.0	0.91 (0.03)[a]	1.38 (0.31)[a]	7.57 (1.05)*[a]	0.967	0.119	0[b]	4.11 (0.32)[b]	2.24 (0.18)[b]	0.992	0.241	0[b]	3.72 (0.23)[b]	2.47 (0.11)[b]	0.989	0.208
50.0	0.85 (0.05)[a]	1.77 (0.05)*[a]	6.06 (0.19)*[a]	0.986	0.104	0[b]	4.48 (0.34)[b]	2.15 (0.06)[b]	0.982	0.183	0[b]	4.53 (0.09)[b]	2.03 (0.22)[b]	0.991	0.256
52.5	0.81 (0.12)*[a]	2.24 (0.08)*[a]	4.92 (0.13)*[a]	0.994	0.084	0[b]	4.49 (0.14)[b]	2.05 (0.31)[b]	0.970	0.473	0[b]	4.58 (0.39)[b]	2.01 (0.26)[b]	0.978	0.377
55.0	0.75 (0.03)*[a]	3.41 (0.28)*[a]	3.45 (0.22)*[a]	0.988	0.212	0[b]	4.63 (0.07)*[b]	1.99 (0.28)*[b]	0.986	0.350	0[b]	4.90 (0.29)*[b]	1.88 (0.32)*[b]	0.988	0.320
57.5	0.45 (0.01)*[a]	5.12 (0.28)*	2.25 (0.06)*[a]	0.990	0.287	0[b]	5.66 (0.29)*	1.63 (0.15)*[b]	0.994	0.241	0[b]	5.82 (0.37)*	1.58 (0.21)*[b]	0.993	0.276
60.0	0*	8.01 (0.34)*	1.15 (0.07)*	0.990	0.339	0	9.04 (1.16)*	1.02 (0.08)*	0.993	0.321	0	8.91 (0.44)*	1.04 (0.11)*	0.996	0.185

Values in parentheses represent the standard deviations of the means.

* Significant differences ($p \leq 0.05$) between mean values at treatment temperature between 40.0 to 60.0 ºC and those at room temperature in each strain and recovery condition.

[a b] Significant differences ($p \leq 0.05$) among mean values of strains recovered in TSAYE and the wild type strain counts obtained in TSAYE+CA medium.

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TABLE 3. Heat resistance parameters (*Sl*, *Kmax,* and time for 4*D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *E. coli* WT and its isogenic mutants *uvrA*[-] and *recA*[-] treated in McIlvaine buffer at different temperatures and recovered in the non-selective medium.

	Wild type strain					***uvrA*[-] *strain***					***recA*[-] *strain***				
Temperature (ºC)	***Sl* (min)**	***Kmax* (min^{-1})**	**4*D* (min)**	R^2	***RMSE***	***Sl* (min)**	***Kmax* (min^{-1})**	**4*D* (min)**	R^2	***RMSE***	***Sl* (min)**	***Kmax* (min^{-1})**	**4*D* (min)**	R^2	***RMSE***
55.6	1.13 (0.51)[a]	0.73 (0.13)[a]	13.67 (0.95)[a]	0.988	0.125	2.29 (0.49)[a]	0.98 (0.07)[a]	11.76 (0.24)[a]	0.994	0.075	0[b]	1.39 (0.28)[b]	6.84 (1.52)[b]	0.976	0.213
58.1	0.61 (0.04)[a]	2.16 (0.62)[a]	4.87 (0.15)[a]	0.991	0.110	0.72 (0.28)[a]	2.38 (0.73)[a]	4.58 (1.06)[a]	0.968	0.177	0[b]	4.24 (0.21)[b]	2.45 (0.36)[b]	0.976	0.214
60.6	0.27 (0.03)[a]	7.59 (1.09)[a]	1.48 (0.02)[a]	0.982	0.164	0.54 (0.03)[a]	8.74 (0.07)[a]	1.60 (0.03)[a]	0.991	0.129	0[b]	10.34 (1.05)[b]	0.87 (0.13)[b]	0.982	0.264
63.1	0.11 (0.02)[a]	20.58 (1.31)[a]	0.55 (0.03)[a]	0.996	0.185	0.19 (0.04)[a]	22.32 (1.46)[a]	0.60 (0.02)[a]	0.987	0.163	0[b]	23.21 (2.19)[a]	0.39 (0.05)[b]	0.985	0.157

Values in parentheses represent the standard deviations of the means.

[a b] Significant differences ($p \leq 0.05$) among mean values of strains at each treatment temperature.

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TABLE 4. UV resistance parameters (*Sl*, *Kmax,* and dose for 4*D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *E. coli* WT and its isogenic mutants *nth*$^-$, *phr*$^-$, *umuD*$^-$, *uvrA*$^-$, and *recA*$^-$ in McIlvaine buffer with an absorption coefficient of 15.3 cm^{-1} at room temperature. Table includes resistant parameters of the wild type strain recovered in nonselective (TSAYE) and selective media with caffeic acid added (TSAYE+CA), and with a previous photoreactivation step before incubation (TSAYE+visible light -11.15 Klux for 60 min).

Derivative	**Recovery medium**	***Sl* (J ml^{-1})**	***Kmax* (ml J^{-1})**	**4*D* (J ml^{-1})**	**R^2**	***RMSE***
WT	TSAYE	8.78 (0.20)[a]	0.44 (0.03)[a]	29.93 (1.54)[a]	0.989	0.145
WT	TSAYE+visible light	10.05 (1.50)[a]	0.36 (0.08)[a]	35.19 (1.28)[b]	0.938	0.254
WT	TSAYE+CA	0[b]	0.80 (0.05)[b]	12.37 (0.32)[c]	0.986	0.368
nth$^-$	TSAYE	6.95 (0.44)[a]	0.42 (0.02)[a]	28.54 (1.50)[a]	0.969	0.196
phr$^-$	TSAYE	6.84 (1.11)[c]	0.47 (0.05)[a]	26.30 (1.15)[d]	0.977	0.269
umuD$^-$	TSAYE	5.95 (1.56)[c]	0.46 (0.08)[a]	26.77 (1.13)[d]	0.984	0.208
uvrA$^-$	TSAYE	0[b]	0.77 (0.05)[b]	12.21 (0.93)[c]	0.992	0.271
recA$^-$	TSAYE	0[b]	0.79 (0.01)[b]	11.07 (0.43)[c]	0.986	0.372

Values in parentheses represent the standard deviations of the means.

[a b c d] Significant differences ($p \leq 0.05$) between values of the wild type strain recovered in different conditions and its derivatives recovered in TSAYE.

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