Title:

Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields

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26 **SIGNIFICANCE AND IMPACT OF THE STUDY**

27 The exploration of different delivery systems of antimicrobial compounds such as citral
28 in aqueous food products aids in the establishment of successful combined treatments
29 for food preservation. While at room temperature, citral in form of a nanoemulsion
30 shows a higher antimicrobial activity; its combination with heat would imply a partial
31 loss of the outstanding synergistic lethal effect achieved when added in suspension
32 form. Therefore, the most suitable procedure to magnify the synergism between heat
33 and citral when processing juices would merely require an intense homogenization step
34 prior to the combined treatment.

35

36 **ABSTRACT**

37 The application of essential oils in form of nanoemulsions has been proposed as a
38 method to improve their solubility in aqueous solutions, and hence their antimicrobial
39 activity. The objective of this study was to evaluate the antimicrobial activity of citral,
40 applied directly or in combined treatments with heat or pulsed electric fields (PEF), as a
41 function of the inoculation procedure assayed: (i) a simple, vigorous shaking method by
42 vortex agitation (suspension of citral; s-citral) or (ii) the previous preparation of
43 nanoemulsions by the emulsion phase inversion (EPI) method (nanoemulsion of citral;
44 n-citral). N-citral was more effective in either inhibiting or inactivating *Escherichia coli*
45 O157:H7 Sakai than s-citral. However, when combined with heat, a greater synergistic
46 effect was observed with s-citral rather than with n-citral, either in lab media (pH 7.0
47 and 4.0) or apple juice. For instance, while almost 5 $\log_{10}$ cell cycles were inactivated in
48 apple juice after 15 min at 53ºC in the presence of 0.1 μl ml^{-1} of s-citral, the use of n-
49 citral required 30 min. The use of nanoemulsions did not modify the slight synergism
50 observed when citral and mild PEF were combined (150 μs, 30 kV cm^{-1}).

51

52 **INTRODUCTION**

53 The design of successful food preservation processes relies on the establishment of
54 those treatment conditions which guarantee the innocuity of the product with minimal
55 detriment of nutritional and sensory parameters. A way of achieving such effective
56 treatment conditions is through the combination of antimicrobial agents and physical
57 methods, following the hurdle theory proposed by Leistner and Gorris (1995). Over the
58 last few decades, essential oils (EOs) extracted from aromatic and medicinal plants or
59 citrus fruits, as well as their individual constituents, have been tested as antimicrobial
60 agents in combination with heat or pulsed electric field pulses (PEF). In many cases,
61 remarkable synergistic effects in the lethality of these combinations allowed to decrease
62 treatment temperatures and/or antimicrobial doses, or to potentiate the inactivation
63 achieved by PEF (Corbo *et al*. 2009; Espina *et al*. 2010, 2012; de Souza *et al*. 2016).
64 Among EO constituents, citral has displayed a broad-spectrum antimicrobial activity
65 and has been shown to be one of the most effective antimicrobials applied either directly
66 or in combined treatments. For instance, very low doses of citral (0.018-0.2 $\mu l\ ml^{-1}$) in
67 combination with heat were capable of inactivating five log_{10} cell cycles of *Escherichia*
68 *coli* O157:H7, showing promising results for the preservation of apple juice (Espina *et*
69 *al*. 2010).

70 Nevertheless, many authors observe that the hydrophobicity of EOs may hamper their
71 homogenous dispersion in aqueous food products (Maswall and Dar 2013; Piorkowski
72 and McClements 2014). As an effective approach to improve the dispersion of EOs into
73 food products and minimize the phase separation, the formation of food-grade
74 emulsions using low-energy preparation methods is a field of great interest (Komaiko
75 and McClements 2016). Procedures such as the emulsion phase inversion (EPI) method

76 generate metastable oil-in-water nanoemulsions, are simple to implement, and no
77 expensive equipment is required.
78 While the effect of emulsification on the antimicrobial activity of EOs has been studied
79 in depth (Donsi *et al*. 2011; Maswall and Dar 2013; Moghimi *et al*. 2016; Zhang *et al.*
80 2017), few studies have evaluated their influence when applied in combination with
81 other treatments such as heat or PEF. In this regard, to the best of our knowledge, there
82 are no documented studies on the behavior of EPI nanoemulsions of citral under heat or
83 PEF treatments.
84 The first objective of this study was to evaluate the antimicrobial activity of citral
85 against *Escherichia coli* O157:H7 Sakai, applied directly or in combined treatments
86 with heat or PEF, as a function of the citral preparation procedure used: (i) a simple
87 vigorous shaking method by vortex agitation (suspension of-citral; s-citral), and (ii) the
88 previous preparation of nanoemulsions by the EPI method (nanoemulsion of citral; n-
89 citral). As a second objective, the combination of heat with s- and n-citral to inactivate
90 *Escherichia coli* O157:H7 Sakai was assayed in apple juice.
91

92 **RESULTS AND DISCUSSION**

93

94 **Droplet size and stability of nanoemulsions**

95 Nanoemulsions of citral were prepared by the EPI method and characterized during a
96 period of storage under refrigeration. As shown in Table 1, droplet size remained below
97 200 nm during the 4 months of storage. On the other hand, there were no significant
98 differences (p>0.05) among survival curves of *E. coli* O157:H7 Sakai obtained in the
99 presence of 0.6 µl ml^{-1} at pH 4.0, either from different nanoemulsion preparations and
100 different storage times, which indicates that the EPI method assayed allows the

101 obtention of reproducible and stable nanoemulsions of citral. Figure 1A shows the mean
102 values and the standard deviation of nine survival curves corresponding to different
103 emulsions and storage times. To the best of our knowledge, there are no documented
104 studies on the production and characterization of nanoemulsions of citral using the EPI
105 method. The stability of nanoemulsions of D-limonene obtained with the same
106 methodology has already been shown by Zhang *et al.* (2014) and Mate *et al.* (2016) for
107 6 months.
108
109 **Effect of citral as a suspension or nanoemulsion on antimicrobial activity**
110 The use of citral in the form of a nanoemulsion decreased the MIC from 0.8 (s-citral) to
111 0.7 (n-citral) µl ml^{-1} ($p<0.05$) against *E. coli* O157:H7 Sakai. This result reveals that n-
112 citral can inhibit microbial growth more efficiently than s-citral. Nevertheless, this
113 increase in the antibacterial efficacy of citral after its emulsification was much lower
114 than that observed for other antimicrobials in previous works (Komaiko and
115 McClements 2016). For example, Moghimi *et al*. (2016) demonstrated a 4-fold
116 reduction of the MIC value with a nanoemulsion of sage oil.
117 Moreover, as shown in Figure 1, n-citral was also more effective than s-citral in the
118 inactivation of *E. coli* O157:H7 Sakai in both pH 4.0 and 7.0 treatment media. While
119 the kinetics of inactivation obtained with s-citral showed a prolonged shoulder followed
120 by a rapid decrease, those obtained with n-citral were approximately linear. The greater
121 antimicrobial activity of EOs in the form of nanoemulsions has been associated with
122 their increased polarity, thanks to the coating of the surfactants that reduces surface
123 tension of the oil droplets (Piorkowski and McClements, 2014). Thus, the
124 emulsification of hydrophobic substances might reduce their immiscibility in aqueous
125 solutions, making them readily dispersible in the treatment media. In this regard,

126 Moghimi *et al*. (2016) proved that conversion of sage oil into a nanoemulsion improved
127 its antibacterial activity by enhancing its ability to promote the destruction of bacterial
128 cell membranes.

129 Therefore, the preparation of a nanoemulsion of citral seems to be the best option in
130 treatments applied at room temperature. In addition, the nanoemulsion would also
131 provide the chemical stability required for prolonged inhibitory or bactericidal
132 treatments (Maswall and Dar 2013).

133 On the other hand, the comparison of survival curves shown in Figures 1A and 1B
134 confirms the exceptional higher resistance of *E. coli* O157:H7 Sakai in acid than in
135 neutral pH, already described by Somolinos *et al*. (2010), and shows that this
136 phenomenon also occurs when citral is applied as a nanoemulsion.

137

138 **Effect of citral as a suspension or nanoemulsion on microbial inactivation in**
139 **combined treatments**

140 The synergism observed when combining heat or PEF with EOs has been directly
141 related to the detection of injured cells in the cytoplasmic and outer membranes of
142 Gram-negative bacteria after the application of physical technologies as a single agent
143 (Mackey 2000; Somolinos *et al*. 2009; Arroyo *et al*. 2010; Espina *et al*. 2012). In those
144 studies, heat and PEF treatments were applied, and survivors were recovered in non-
145 selective and selective media (Figure 2). Results obtained in the non-selective medium
146 show that heat and PEF treatments acting as single agents inactivated less than 1 $\log_{10}$
147 cell cycle. Based on the differences in the $\log_{10}$ cycles of inactivation achieved when
148 comparing the non-selective with selective media, heat treatments at pH 4.0 (Figure 2A)
149 injured more survivors in the outer than in the cytoplasmic membrane ($p<0.05$),
150 whereas PEF treatments (Figure 2C) did the opposite. Under these treatment conditions,

151 at least 90% of survivors were injured and susceptible to a citral attack during the
152 combined treatments.
153 Regarding the combination of heat and citral, a remarkable synergism was observed:
154 almost 5 $\log_{10}$ cells cycles of inactivation were achieved at both pH as a function of the
155 citral addition procedure (s- or n-citral) and the antimicrobial concentration. In contrast
156 with the results shown when citral was acting as a single agent (Figure 1), the addition
157 of s-citral was to some extent more effective than n-citral either at pH 7.0 or at pH 4.0,
158 showing the greatest difference in the presence of 0.1 µl ml^{-1} at pH 7.0 ($p<0.05$) (Figure
159 2B). Thus, maintaining the synergism when using n-citral would require higher
160 concentrations of the hydrophobic compound to achieve the desired level of microbial
161 inactivation. It should be noted that the high levels of inactivation achieved with the
162 combined treatment might correspond to the tail of the survival curves. As a
163 consequence, greater differences in the antimicrobial efficacy between s- and n-citral
164 might be expected at lower concentrations or shorter treatment times of the combined
165 processes. Further experiments should be performed to explain this unexpected
166 inversion of the compared activity of s-citral and n-citral when increasing the treatment
167 temperature up to 53°C. Possible hypotheses to consider include the increased solubility
168 of s-citral under mild thermal treatments, providing the optimum dispersion in the
169 treatment medium, and a greater availability of s-citral to interact with microbial cells in
170 the short time span of the treatment in comparison with n-citral, which might be
171 partially retained by the surfactant.
172 Nevertheless, these results differ from those obtained with the EPI method, using
173 propylene glycol instead of ethanol as co-surfactant, to obtain nanoemulsions of D-
174 limonene for the inactivation of *Listeria monocytogenes* (Mate *et al*. 2016). Therefore, it
175 should be considered that the result of using nanoemulsions in combined treatments

176 with heat is likely to vary as a function of the type or complexity of the antimicrobial
177 molecules and/or the microorganism investigated.
178 According to Arroyo *et al*. (2010), the detection of sublethal injury, specifically on the
179 outer membrane of Gram-negative bacteria after PEF, is the key when identifying
180 treatment conditions under which PEF may act synergistically with citral. Thus, the
181 scarce presence of sublethally injured cells on the outer membrane of *E. coli* 0157:H7
182 Sakai after PEF (Figure 2C and D) might justify the limited synergism observed with s-
183 citral. To the best of our knowledge, the bacterial inactivation of PEF treatments with
184 EPI nanoemulsions has never been previously tested. In the present study, no significant
185 differences were found between the efficacy of the combined treatments using PEF and
186 s- or n-citral, and no worsening or improvement of the overall lethality of the combined
187 treatment was observed when emulsifying citral prior to its incorporation. Further
188 experiments combining s-citral or n-citral with PEF applied at higher temperatures
189 could be conducted to further explore the effect of emulsification on each one of these
190 physical preservation treatments.
191

192 **Effect of citral in the form of suspension or nanoemulsion on microbial**
193 **inactivation by combined treatments applied to apple juice**

194 In order to validate in a food model the results obtained with citral in form of
195 suspension or nanoemulsion in lab media, apple juice was contaminated with *E. coli*
196 O157:H7 Sakai and treated with a combined treatment of mild heat and citral (s- and n-
197 citral) (Figure 3). Again, the combined treatment with s- or n-citral was more effective
198 than the use of mild heat as a single agent, showing a remarkable synergistic effect. In
199 addition, the main conclusion obtained in lab media was confirmed in apple juice: s-
200 citral was more effective than n-citral when applied at mild temperature at any treatment

201 time. For instance, while almost 5 $\log_{10}$ cell cycles were inactivated in apple juice after 202 15 min at 53ºC in the presence of 0.1 μl ml^{-1} of s-citral, the use of n-citral required 203 doubling the treatment time. Thus, the vigorous agitation in vortex method for 204 suspending citral, which might simulate the action of the actual industrial homogenizers 205 employed as a previous stage in the pasteurization process of liquid foods, seems to be 206 sufficient to disperse the oil correctly and favor its antimicrobial action in apple juice, 207 providing enough stability at least during the short duration of the combined treatment. 208 In this regard, the treatment time required to comply with FDA regulation (FDA 2001), 209 which recommended that juices should be hygienized reaching 5-$\log_{10}$ reductions 210 (99.999%) of pathogens of concern such as *E. coli* O157:H7, would be approx. 2 times 211 shorter when using s- than n-citral. Nevertheless, it should be highlighted that citral in 212 the form of nanoemulsion also attained the 5-$\log_{10}$ reductions of the pathogen. Thus, if 213 its use represented any advantage – for instance, for the purpose of limiting the 214 modification of the flavor in food due to high EO concentrations in comparison with 215 non-encapsulated ones – then it would be interesting to reconsider its use in the 216 development of combined treatments for food preservation. Further studies are required 217 in order to evaluate the influence of this and other encapsulation methods on the 218 efficacy of combined processes with heat, PEF, or other successful emerging 219 technologies, such as high hydrostatic pressure, and other EOs and EO constituents.

220

221 MATERIALS AND METHODS

222 Bacterial strain and cultures

223 *E. coli* O157:H7 Sakai *stx* $1A^{-}$/*stx* $2A^{-}$ was kindly provided by Kyu-Tae Chang 224 (The National Primate Research Center, KRIBB, Ochang, South Korea). This strain was 225 isolated from an outbreak involving white radish sprout (Michino *et al.* 1999). During

226 this investigation, the cultures were maintained and kept frozen at −80 °C in cryovials.
227 Broth subcultures were prepared by inoculating one single colony from a plate into a
228 test tube containing 5 mL of sterile tryptic soy broth (Biolife, Milan, Italy) with 0.6%
229 yeast extract added (Biolife) (TSBYE). After inoculation, the tubes were incubated
230 overnight at 37 °C. Along with these subcultures, 250-ml Erlenmeyer flasks containing
231 50 mL of TSBYE were inoculated to a final concentration of 10^4 cells ml^{-1}. These flasks
232 were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at
233 the appropriate temperature until the stationary growth phase was reached (24 ± 2 h).
234

235 **Antimicrobial and direct addition procedure**

236 Citral was obtained from Sigma Aldrich Chemie (Steinheim, Germany). Following the
237 procedure described by Friedman *et al.* (2002), a vigorous shaking method was used to
238 prepare citral suspensions (s-citral) in the treatment media: citrate-phosphate buffer
239 (McIlvaine's buffer) at pH 7.0 and pH 4.0 (Dawson *et al.* 1974) and apple juice.
240

241 **Preparation of nanoemulsions**

242 The preparation of nanoemulsions of citral (n-citral) was based on the catastrophic
243 phase inversion method (Zhang *et al.* 2014, 2017), also known as the emulsion phase
244 inversion (EPI) method. The aqueous phase was prepared by mixing 1.5 ml of ethanol
245 (Sigma) with 40.5 ml of sterile distilled water. The oily phase was prepared by mixing 3
246 ml of Tween 80 (Panreac, Barcelona, Spain) with 5 ml of citral. Nanoemulsions were
247 prepared from a mixture of oily phase by slowly adding aqueous phase with gentle
248 magnetic agitation. The addition rate of aqueous phase was kept constant at
249 approximately 1.0 ml min^{-1}. A water-in-oil (W/O) emulsion with a high oil-to-water
250 ratio was formed, and then increasing amounts of water were added to the system with

continuous stirring. The amount of water added to a W/O emulsion was progressively increased, until a phase inversion occurred and an oil-in-water (O/W) emulsion was formed. Final concentration of citral in the nanoemulsion was 587 mM.

Droplet size and stability of nanoemulsions

The emulsion droplet size and size distribution (polydispersity index-PDI) was determined using a particle size analyzer (Brookhaven, 90 Plus, New York, USA). Droplet size was analyzed using dynamic light scattering (DLS) technique. Prior to all the experiments, the nanoemulsion formulations were diluted with water to eliminate the multiple scattering effects. Emulsion droplet size was estimated by an average of three measurements and is presented as the mean diameter of volume distribution. Droplet size was evaluated after fresh preparation, and then after 1 month of storage under refrigeration. The reproducibility of the protocol for preparing nanoemulsions and their stability during 30 days was also evaluated by comparing the survival curves of *E. coli* O157:H7 Sakai in the presence of 0.6 μl ml^{-1} of n-citral at pH 4.0, as described below.

Evaluation of the antimicrobial activity of citral

Citral (s-citral and n-citral) was evaluated to determine the minimum inhibitory concentration (MIC) and to obtain survival curves against *E. coli* O157:H7 Sakai.

Regarding the MIC, tubes containing 5 mL of TSBYE and different concentrations of citral (0.5–1 μl ml^{-1}) were inoculated to a final concentration of 10^5 cells ml^{-1}. Negative control (without microorganisms), positive control (without citral), and diluent control (the amount of ethanol corresponding to the maximum n-citral concentration assayed -1 μl ml^{-1}) were also prepared. After 24 h of incubation at the appropriate temperature in a

276 shaking thermostatic incubator (Bunsen, mod. BTG, Madrid, Spain), survivors were
277 enumerated, as described below. The MIC was the lowest concentration of citral at
278 which bacteria failed to grow, showing counts equal to the initial concentration.
279 Moreover, the antimicrobial properties of s-citral and n-citral were evaluated by
280 determining survival curves in treatment media of different pH. Cells from stationary-
281 phase cultures were added at final concentrations of 10^7 cells ml^{-1} to buffers with citral
282 (0.6 μl ml^{-1} at pH 4.0 and 0.4 μl ml^{-1} at pH 7.0). Buffer pH was not modified as a
283 consequence of adding antimicrobial compounds. Antimicrobial compound treatments
284 were carried out at room temperature (23 ± 2 ºC). Samples were taken at preset intervals
285 and survivors were enumerated, as described below. Previous experiments showed that
286 untreated cells of *E. coli* O157:H7 Sakai at concentrations of 10^7 cells ml^{-1} were
287 insensitive to incubation at pH 7.0 or 4.0 for 1 h at room temperature (data not shown).
288

289 **Evaluation of microbial inactivation by heat and heat combined with citral**
290 For the preparation of heat-treated samples, microorganisms were resuspended at a
291 concentration of 10^7 cells ml^{-1} in treatment media thermostated at 53 ± 0.2°C (FX
292 Incubator, A.F. Ingeniería S. L., Valencia, Spain). Buffer of pH 7.0 and 4.0, as well as
293 the same treatment media with s-citral or n-citral to a final concentration of 0.1 and 0.2
294 μl ml^{-1} were used. Antimicrobials were added once the treatment media were
295 thermostated, and prior to microbial inoculation. The actual temperature was controlled
296 with a thermocouple wire introduced in a 0.9 mL buffer test tube inside the incubator.
297 After 15 min at 53 ºC, samples were taken, immediately placed on ice, and survivors
298 and sublethally-injured cells were evaluated, as explained below.
299 Following the same methodology, heat treatments were also carried out in apple juice
300 (Don Simón, Murcia, España), as well as in the presence of s-citral and n-citral (0.1 μl

301 ml^{-1}). Samples were collected at pre-set intervals and survivors were evaluated to obtain
302 survival curves.
303

304 **Evaluation of microbial inactivation by PEF and PEF combined with citral**

305 PEF treatments were carried out using ScandiNova equipment (Modulator PG,
306 ScandiNova, Uppsala, Sweden), described by Saldaña *et al*. (2010).
307 Before treatments, micro-organisms were centrifuged at 6000 x *g* for 5 min and
308 resuspended at a concentration of 10^7 cells ml^{-1} in citrate–phosphate buffer of pH 7.0
309 and 4.0 (electrical conductivity was adjusted to 1 mS/cm), as well as in the same
310 treatment media with s-citral or n-citral to a final concentration of 0.2 µl ml^{-1}.Then, 0.5
311 ml of the microbial suspensions was placed into the treatment chamber with a sterile
312 syringe. Exponential waveform pulses at an electrical field strength of 30 kV cm^{-1} and a
313 pulse repetition rate of 1 Hz were used in this study. The specific energy input of each
314 pulse 2.7 kJ kg^{-1}. Cell suspensions were treated for 50 pulses (pulse width 3 µs).
315 Experiments started at room temperature (23 ± 2 ºC). In all experiments, the
316 temperature of the samples after the application of 50 pulses was lower than 35 ºC.
317 After treatment, samples were taken, and survivors and sublethally-injured cells were
318 evaluated, as explained below.
319

320 **Counts of viable cells**

321 After treatments, samples were adequately diluted in 0.1% w/v peptone water (Biolife).
322 Subsequently, 0.1 ml samples were pour-plated onto Tryptic Soy Agar (Biolife) with
323 0.6% Yeast Extract added (Biolife) (TSAYE). Plates were incubated for 24 h at 37ºC.
324 Previous experiments showed that longer incubation times did not influence the
325 surviving cell counts. After incubation, colonies were counted with an improved image

326 analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United
327 Kingdom), as previously described (Condón *et al*. 1996).

328

329 **Detection of sublethal injury**

330 In order to determine bacterial cell injury, treated samples were also plated onto TSAYE
331 with 4% sodium chloride (Panreac) added (TSAYE-SC) and onto TSAYE with 0.25%
332 bile salts (Oxoid, Hampshire,United Kingdom) added (TSAYE-BS) in order to evaluate
333 cytoplasmic membrane damage and outer membrane damage, respectively (Mackey,
334 2000). These levels of sodium chloride and bile salts were previously determined as the
335 maximum non-inhibitory concentrations for native cells (data not shown). Samples
336 recovered in selective media were incubated for 48 h. Previous experiments showed that
337 longer incubation times did not influence survival counts.
338 The proportion of sublethally injured cells was estimated by the difference in the
339 number of $\log_{10}$ cycles of colony forming units (CFU) obtained after plating treated
340 cells in the nonselective (TSAYE) and selective (TSAYE-SC) media.

341

342 **Data analyses**

343 The error bars in the figures indicate the mean ± standard deviations from the data
344 obtained from at least three independent experiments. All analyses were performed with
345 GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA). Unpaired t-
346 Student and one-way ANOVA tests were performed to test statistically significant
347 differences among two or more groups, respectively ($p = 0.05$).

348

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354

355 **CONFLICT OF INTEREST**

356 No conflict of interest declared.

357

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426

427

428

429 **Table 1.** Droplet size and polydispersity index (PDI) of nanoemulsions of

430 citral stored under refrigeration. Data represent the mean ± standard error of

431 the mean of at least three independent experiments.

Storage time (months)	Droplet size (nm)	PDI
0	161 ± 5	0.096 ± 0.012
1	160 ± 2	0.159 ± 0.011
4	191 ± 0	0.291 ± 0.005

432

433

434 **FIGURE LEGENDS**

435

436 **Figure 1.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x
437 10^7 CFU/mL) after exposure to s-citral (●) or n-citral (○) in buffer of pH 4.0 (A) (0.6 µl
438 ml^{-1}) and 7.0 (B) (0.4 µl ml^{-1}) at room temperature and recovered in TSAYE. Data
439 represent the mean ± standard error of the mean (error bars) of at least three
440 independent experiments. The dotted line represents the detection limit.

441

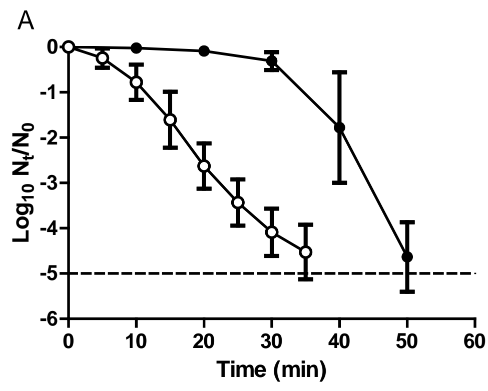
442 **Figure 2.** Cycles of inactivation of *Escherichia coli* O157:H7 Sakai (initial
443 concentration: 3 x 10^7 CFU/mL) after a heat treatment at 53 ºC for 15 min (A,B) and
444 after a PEAV treatment at 30 kV cm^{-1} for 150 µs (50 pulses of 3 µs) (C, D) in buffers of
445 pH 4.0 (A, C) and 7.0 (B, D), and recovered in TSAYE (grey bars; NS), TSAYE-SC
446 (grey bars; SC), TSAYE-BS (grey bars; BS), or recovered in TSAYE after combined
447 treatment with heat and s-citral (black bar) or n-citral (white bar) (0.1 and 0.2 µl ml^{-1}).
448 Data represent the mean ± standard error of the mean (error bars) of at least three
449 independent experiments. Statistical differences (Student's t-test, $p<0.05$) among
450 combined treatments is represented by different superscript letters. The dotted line
451 represents the detection limit.

452

453 **Figure 3.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 10^7
454 CFU/mL) to a heat treatment at 53 ºC in apple juice (▲), and a combined treatment of
455 heat and s-citral (●) or n-citral (○) (0.1 µl ml^{-1}) and recovered in TSAYE. Data represent
456 the mean ± standard error of the mean (error bars) of at least three independent
457 experiments. The dotted line represents the detection limit.

458

Figure 1



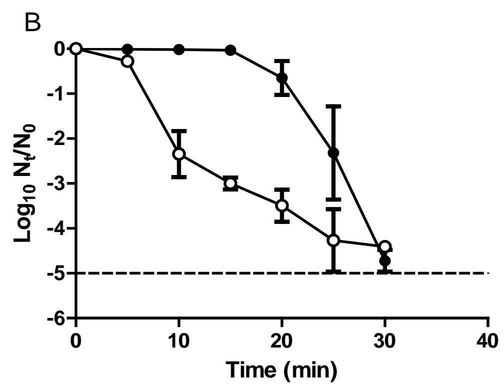


Figure 2

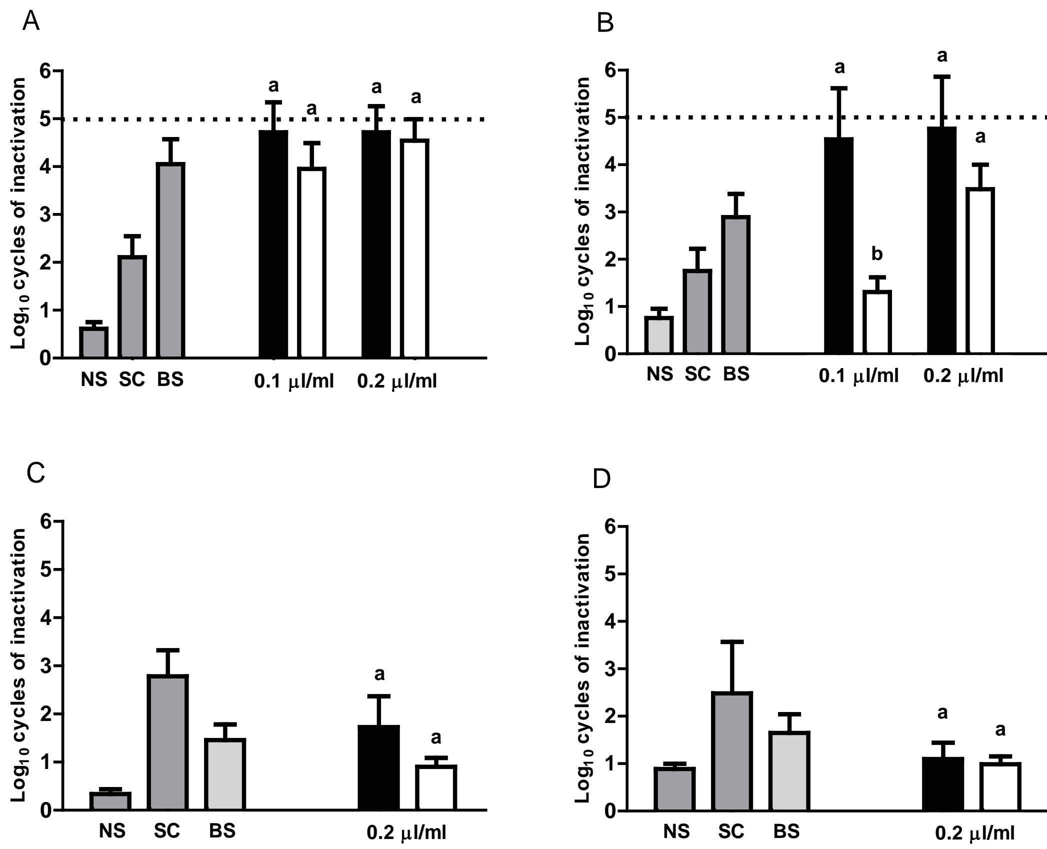


Figure 3