1 **Determination of the combined effect of grape seed extract and cold atmospheric**

2 **plasma on foodborne pathogens and their environmental stress knockout mutants**

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22 **Abstract**

23 The aim of this study was to explore the antimicrobial efficacy of grape seed extract (GSE) and
24 cold atmospheric plasma (CAP) individually or in combination against *L. monocytogenes* and
25 *E. coli* wild type (WT) and their isogenic mutants in environmental stress genes. More
26 specifically, we examined the effects of 1 % (w/v) GSE, 4 min of CAP treatment, and their
27 combined effect on *L. monocytogenes* 10403S WT and its isogenic mutants Δ*sigB*, Δ*gadD1*,
28 Δ*gadD2*, Δ*gadD3,* as well as *E. coli* K12 and its isogenic mutants Δ*rpoS*, Δ*oxyR*, Δ*dnaK*.
29 Additionally, the sequence of the combined treatments was tested. A synergistic effect was
30 achieved for all *L. monocytogenes* strains when exposure to GSE was followed by CAP
31 treatment. However, the same effect was observed against *E. coli* strains, only for the reversed
32 treatment sequence. Additionally, *L. monocytogenes* Δ*sigB* was more sensitive to the
33 individual GSE and the combined GSE/CAP treatment, whereas Δ*gadD2* was more sensitive
34 to CAP*,* as compared to the rest of the mutants under study. Individual GSE exposure was
35 unable to inhibit *E. coli* strains, and individual CAP treatment resulted in higher inactivation
36 of *E. coli* in comparison to *L. monocytogenes* with the strain Δ*rpoS* appearing the most sensitive
37 among all studied strains. Our findings provide a step towards a better understanding of the
38 mechanisms playing a role in tolerance/sensitivity of our model Gram-positive and Gram-
39 negative bacteria towards GSE, CAP and their combination. Therefore, our results contribute
40 to the development of more effective and targeted antimicrobial strategies for sustainable
41 decontamination.

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43 **Importance**

44 Alternative approaches to conventional sterilisation are gaining interest by the food industry,
45 driven by: (i) the consumer demand for minimally processed products and (ii) the need for

46 sustainable, environmentally friendly processing interventions. However, as such alternative
47 approaches are milder than conventional heat sterilisation, bacterial pathogens might not be
48 entirely killed by them, which means that they could survive and grow, causing food
49 contamination and health hazards. In this manuscript, we performed a systematic study of the
50 impact of antimicrobials derived from fruit industry waste (grape seed extract) and cold
51 atmospheric plasma on the inactivation/killing as well as the damage of bacterial pathogens
52 and their genetically modified counterparts, for genes linked to the response to environmental
53 stress. Our work provides insights into genes that could be responsible for the bacterial
54 capability to resist/survive those novel treatments, therefore, contributing to the development
55 of more effective and targeted antimicrobial strategies for sustainable decontamination.

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57 **Keywords**: natural antimicrobials, cold atmospheric plasma (CAP), microbial inactivation, *L.*
58 *monocytogenes*, *E. coli*, environmental stress genes, hurdle technology, food safety.

59

60 ## 1. Introduction

61 Consumers increasingly demand food products that are processed using minimal and
62 environmentally friendly methods (1,2). As a result, researchers and the food industry
63 constantly look for novel sustainable ways to ensure microbiologically safe products via
64 replacing chemical preservatives and antibiotics with natural antimicrobials. Fruit and
65 vegetable by-products are a valuable source of natural antimicrobials that can also help to
66 reduce food waste (3–8).

67 Grape by-products, comprise roughly 20 % of the overall weight of the grape and are a
68 substantial waste stream within the wine and juice industry (9,10). The disposal of these by-
69 products, including the skins, seeds, and stems of the fruit, can be challenging. Grape seed

70 extract (GSE), a natural product derived from grape seeds, is a rich source of antioxidant and
71 antimicrobial compounds, such as polyphenols (11–14). GSE is generally recognized as safe
72 (GRAS) for use in food, but it is not yet commonly utilized as an antimicrobial agent.

73 The inactivation of bacteria by GSE has been linked to multiple modes of action, including
74 the ability of polyphenols to permeate the bacterial cell walls and the potential of tannins to
75 inactivate extracellular enzymes (15–17). Furthermore, the GSE compound shows the ability
76 to form complexes with metal ions, leading to the removal of these ions from the bacterial
77 environment (17,18). The presence of metal ions, such as manganese, iron, cobalt, nickel,
78 copper and zinc, is essential for pathogenic bacteria as it enables the preservation of protein
79 structure and function, hence they are a critical-limiting factor for their successful growth and
80 survival. Consequently, the binding of these metal ions by GSE results in bacterial inhibition
81 (19,20).

82 Previous studies using the agar diffusion method have provided evidence of significant
83 antibacterial efficacy against Gram-positive bacteria including *Listeria monocytogenes,*
84 *Bacillus cereus, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus,*
85 *Staphylococcus epidermidis and Mycobacterium smegmatis* (17,21,22). Additionally,
86 Sivarooban et al. (2007) while studying the microbial dynamics of *L. monocytogenes* (initial
87 load $5x10^6$ CFU/ml) observed an inhibition of 2 log CFU/ml after 24 h of 1 % (w/v) GSE
88 treatment in Tryptone Soy Broth supplemented with Yeast Extract (TSBYE) (23). However,
89 there are contradictory results in literature, on the GSE antimicrobial activity against Gram-
90 negative bacteria. For example, Corrales et al. (2009) reported that in agar diffusion tests, 1 %
91 (w/v) GSE was unable to inactivate *E. coli* and *Salmonella* Typhimurium, whereas Baydar et
92 al. (2006) observed inhibition of both bacteria using the same methodology (17,21). In previous
93 work of our group the microbial dynamics of *L. monocytogenes* and its isogenic mutant Δ*sigB*,
94 *E. coli* and *S.* Typhimurium treated with GSE in TSBYE were explored. We showed that GSE

95 inactivated *L. monocytogenes* by 3 log CFU/ml at 1 % (w/v) GSE. Additionally, a mutant in
96 *sigB,* a gene encoding the central stress gene regulator was more sensitive. On average, there
97 was a 0.6 log CFU/ml difference in the surviving population between the WT and Δ*sigB*. *E.*
98 *coli* and *S*. Typhimurium were more tolerant to GSE in comparison to *L. monocytogenes*. More
99 specifically, for those Gram negative-bacteria a growth inhibition was observed (24). *To the*
100 *best of our knowledge there are no other studies exploring the antimicrobial efficacy of GSE*
101 *using functional genomics (use of isogenic mutants) in environmental stress genes of bacteria*
102 *of importance in food safety.*

103 CAP is a non-thermal emerging technology with multiple applications such as inactivation
104 of microorganisms, wound healing, and cancer treatment (8,25–29). After solid, liquid and gas,
105 plasma has been described as the fourth state of matter. Plasma is achieved by ionizing a
106 gaseous mixture composed of neutral molecules, electrons, positive and negative ions (30–32).
107 To create plasma, energy is applied to the gas to break the bonds between electrons and atoms
108 resulting in the formation of charged particles. Most used ways to supply energy for plasma
109 formation is electricity, heat, or by using lasers. The collision of gas particles in the plasma
110 generates numerous highly reactive species such as high energy UV photons, charged particles
111 including electrons and ions, oxygen reactive species (ROS), nitrogen reactive species (RNS)
112 and hydrogen peroxide (30–34).

113 The exact mode of action of the microbial inactivation of CAP is still elusive. Proposed
114 mechanisms are the destruction of the cell wall, DNA damage, lipid peroxidation and protein
115 dysfunction (32,33,35). Moreover, CAP could potentially reduce the metabolic activity of the
116 cell resulting in growth inhibition, possible loss of pathogenicity and prevention of biofilm
117 formation by destroying the extracellular polymeric substances (26,36).

118 The effectiveness of CAP depends on several parameters. Overall, CAP treatment has
119 shown some promising results for food decontamination. However, the plethora of parameters,
120 affecting the outcome of the CAP treatment, are adding an element of variability when
121 comparing results from different research groups. The magnitude of microbial inactivation
122 ranges from no inhibition to several logs of reduction of the bacterial concentration, depending
123 on the parameters of the treatment, the food properties such as the matrix, the water activity,
124 pH and the sensitivity of the bacterial strain (37–42).

125 The principle of the hurdle technology approach on microbial safety, is the utilization of
126 two or more methods/approaches/processes to enable microbial inactivation and consequently
127 to ensure food safety (8,43–49). Due to their mode of action, mild/alternative technologies can
128 cause less damage and death to bacteria as compared to classic treatments, e.g., heat
129 pasteurisation (37,50,51). This can pose a challenge when employing these approaches, as they
130 might not fully guarantee products that are microbiologically safe. However, the combination
131 of these methods or technologies could potentially exert synergistic or additive effects against
132 bacteria, thereby achieving a substantial microbial inactivation (>5 log CFU/ml), ensuring
133 product safety (52,53). For example, one technology/treatment may be used to damage or
134 increase the permeability of the bacterial cell wall while another could be used to interfere with
135 the intracellular components. The trigger to develop such hurdle approaches has been to protect
136 heat sensitive food products against bacterial growth with minimal processing aiming to
137 maintain their quality (8,44,46,54). Therefore, developing hurdle approaches is more pressing
138 than ever, to meet the rising demand for minimally processed foods and sustainable production.

139 As previously mentioned, novel non-thermal technologies (NTTs) can be sometimes
140 ineffective, depending on external parameters such as the nature of the food, the CAP
141 parameters and the type of microorganism (1,6–8,55–57). Furthermore, natural antimicrobials

142 derived from plants such as essential oils cannot be used in very high concentrations, as they
143 might affect the organoleptic characteristics of the food product (58–61).

144 *Hence, combining NTTs with plant-derived antimicrobials as a hurdle approach could be a*
145 *novel solution to increase the treatment efficacy and achieve food safety.* However, to date,
146 there is a very limited number of studies on such combined treatments and their mechanism of
147 inactivation. In most cases, the current hurdle approaches involve the combination of
148 established methods, such as heat treatment with chemical preservatives, or two NTTs
149 combined together, or an NTT combined with heat treatment (62). Additionally, the limited
150 studies combining NTTs with natural antimicrobials focus on the combination of NTTs with
151 essential oils, rather than other natural antimicrobials derived by plants (63–66). For example,
152 Matan et al. in 2014, studied the synergistic effect of radio frequency plasma with essential oils
153 from clove, sweet basil and lime in concentrations of 0.5 to 2 % v/v. The most effective
154 treatment was that combining plasma with clove oil (1 % v/v), which lead to a total microbial
155 (*E. coli*, *S.* Typhimurium, *S. aureus*) inhibition on eggshells (64). Similarly, Cui et al. (2016b)
156 studied the effect of the combination of cold nitrogen plasma (400 W) and thyme oil (0.05 %
157 w/v) against *S.* Typhimurium and *S.* Enteritidis on eggshells achieving a total microbial
158 inactivation (bacterial counts below detection limit), that lasted for 14 days at 3 different
159 temperatures (4, 12, 25 °C) (67). The same year, Cui et al. (2016a) also showed that cold
160 nitrogen plasma combined with *Helichrysum italicum* essential oil can inhibit *S. aureus* on food
161 packaging. The microbial concentration decreased more than 5 log CFU/cm^2, in contrast with
162 individual treatments that caused only 2 log CFU/cm^2 reduction of the microbial concentration
163 (66).

164 To date, as previously described, GSE has not been extensively studied in terms of its
165 antimicrobial properties nor in combination with other NTTs as a hurdle approach. The only
166 reported combined approach of GSE as a microbial inactivation treatment was with nisin, a

167 natural antimicrobial peptide produced by certain strains of *Lactococcus lactis* (6,7,58,68–70).
168 More specifically, Zhao et al. (2020) reported that the simultaneous treatment of nisin (2000
169 IU/ml) and GSE (1 % w/v) in a liquid broth, was able to reduce the concentration of the
170 bacterial pathogen *L. monocytogenes* by 5 logs after 10 min of treatment, whereas their separate
171 use could not inhibit *L. monocytogenes* more than 2 log CFU/g (58). A similar synergistic
172 effect of the above combination was reported by another study where *L. monocytogenes* was
173 completely inhibited after 12 h in the presence of 6400 IU/ml nisin and 1% w/v GSE (23). The
174 proposed synergistic mechanism of microbial inactivation was common in these studies. Nisin
175 acted on the cell wall surface by forming large pores which allowed the GSE to diffuse in the
176 cytoplasm causing further cell damage (58,68).

177 *From the above studies, it can be concluded that, using hurdle approaches to deliver safe*
178 *food products is a very promising strategy which has not been thoroughly studied nor*
179 *understood, especially for fruit by-products, i.e., such as GSE, and other NTTs like CAP.*

180 The aim of this work is to further investigate the antimicrobial effect of (i) GSE (ii) CAP
181 and (iii) GSE combined with CAP in liquid TSBYE broth against two model Gram-positive
182 and Gram-negative bacteria that pose a significant public health concern and their isogenic
183 mutants in environmental stress genes. This study expands upon our prior research, which
184 showed the efficacy of GSE against *L. monocytogenes*. More specifically, the viable
185 populations and sub-lethally damaged cells of *L. monocytogenes* wild type (WT), Δ*sigB,* and
186 GAD system mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD3*, as well as *E. coli* WT, Δ*rpoS*, Δ*oxyR*,
187 Δ*oxyR*, were measured to give insightful information on the mechanisms of microbial
188 resistance to GSE, CAP and their combination. Our study provides insights into the
189 mechanisms of environmental stress response of the above bacteria when exposed to the
190 individual and combined treatments of GSE and CAP, thus contributes to the development of
191 alternative and environmentally friendly methods for microbial inactivation.

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193 **2. Materials and methods**

194 ***2.1. Inoculum preparation***

195 Stock cultures of *L. monocytogenes* 10403S WT, Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δg*adD3*, and
196 *E. coli* K12 WT, Δ*rpoS*, Δ*dnaK*, Δ*oxyR* were stored in Tryptone Soy Broth (TSB, Oxoid Ltd,
197 UK) supplemented with 15% glycerol at -80 °C. **Table 1** provides an overview of the strains
198 and mutants utilised in this study, highlighting their relevance in this study. The inoculum
199 preparation took place as previously described (6–8,24,47–49,57,71–73). More specifically, a
200 loopful of thawed culture was inoculated in 20 ml TSB supplemented with 0.6% w/v of Yeast
201 Extract (Oxoid Ltd, UK) (TSBYE) and cultured for 9.5 h in a shaking incubator at 37 °C and
202 175 rpm. Thereafter, 20 μl were transferred in 20 ml TSBYE and cultured for another 15 h until
203 early stationary phase was reached (approximately 10^9 CFU/ml).

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214 **Table 1**: Strains and isogenic mutants used in this study and function of deleted genes.

<table>
<tr><th>Microorganism</th><th>Deleted gene/ Mutant strain</th><th>Gene function</th><th>Reference</th></tr>
<tr><td rowspan="4">L. monocytogenes 10403S</td><td>ΔsigB</td><td>Central stress (heat, acid, osmotic stress) gene regulator in L. monocytogenes.</td><td>(74–76)</td></tr>
<tr><td>ΔgadD1</td><td rowspan="3">Encode glutamate decarboxylases which are part of the GAD system responsible for the pH homeostasis within the cell.
-gadD1 active in moderately acidic condition
-gadD2 active in severe acidic condition
-gadD3 associated with the intracellular glutamic acid decarboxylase system (GADi).</td><td rowspan="3">(77,78)</td></tr>
<tr><td>ΔgadD2</td></tr>
<tr><td>ΔgadD3</td></tr>
<tr><td rowspan="3">E. coli K12</td><td>ΔrpoS</td><td>Responsible for the general environmental stress response of E. coli and the expression of over 50 genes involved in stress adaptation</td><td>(79–81)</td></tr>
<tr><td>ΔoxyR</td><td>Encodes transcriptional regulators that respond to oxidative stress</td><td>(82–84)</td></tr>
<tr><td>ΔdnaK</td><td>Chaperone that helps in the folding of proteins and prevents protein aggregation under heat stress and/or other environmental stresses (oxidative, osmotic).</td><td>(84–88)</td></tr>
</table>

215

216 ***2.2. Grape seed extracts (GSE)***

217 This study utilised commercially available grape seed extract (GSE) from Bulk, UK. The
218 GSE powder contained a minimum concentration of 95% oligomeric proanthocyanidin.
219 Consequently, the powder is predominantly comprised oligomeric proanthocyanidins. To
220 prepare the GSE solution, the powder was dissolved in Tryptic Soy Broth with 0.6% Yeast
221 Extract (TSBYE) at a concentration of 1% w/v and subsequently autoclaved. The autoclaved
222 TSBYE+GSE was stirred overnight to ensure thorough homogenization. The chosen GSE
223 concentration was selected based on results from our previous study in TSBYE broth. More
224 specifically, we showed that 1% w/v concentration of GSE significantly inactivated *L.*

225 *monocytogenes* WT and its isogenic Δ*sigB* mutant in TSBYE, resulting in a 3 log CFU/ml
226 reduction after 24 h at 37 °C (24).
227

228 ***2.3. CAP experimental set-up***

229 The CAP apparatus utilised in this investigation was developed and supplied by Fourth State
230 Medicine Ltd. The configuration of the device has been previously described in published work
231 of our group (27,72). Briefly, the generator of CAP in this apparatus was a dielectric barrier
232 discharge in a remote and enclosed configuration, whereby the plasma source was contained
233 in an electrically-shielded enclosure and separated from the treatment target by a tube, with no
234 direct line of sight. The gas used for ionization was compressed air (25 °C, 3 bars), and its flow
235 rate (0-5 L/min) was controlled by a needle valve and a flow meter mounted on the enclosure.
236 The chemical composition of the plasma output varies based on the input air flow rate. For
237 example, at flow rate 1 L/min more reactive nitrogen species (RNS– primarily NO_x
238 compounds, NO_2 and NO) are produced in comparison with higher flow rates at which the air
239 flow is enriched with more reactive oxygen species (ROS– primarily O_3). At the used flow rate
240 of the experiment (1 L/min) the concentration of ROS was approximately 320 ppm (72).
241 Additionally, data collected by Fourth State Medicine Ltd showed that the concentration of
242 NOx was approx. 100-200 ppm and NO_z, compounds (mixture of N_2O, HONO, and other
243 compounds, alongside $O_{3)}$ was approximately 200-300 ppm.
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245 ***2.4. Combined treatment: CAP and GSE***

246 To assess the combined treatment of GSE and CAP in liquid, *L. monocytogenes* WT and its
247 isogenic mutants *(*Δ*sigB,* Δ*gadD1,* Δ*gadD2,* Δ*gadD3)* and *E. coli* and its isogenic mutants
248 *(*Δ*oxyR,* Δ*dnaK,* Δ*rpoS)* were inoculated in TSBYE with 1% (w/v) GSE (Figure 1). The initial

249 microbial population was 10^5 CFU/ml. Prior to CAP treatment, the samples were treated with
250 GSE at 37 °C for 2 h. This treatment time in the presence of GSE was selected based on our
251 previously published results for *L. monocytogenes* in liquid nutrient medium (TSBYE), to
252 ensure that a state of stress, i.e., slight reduction but not total inactivation, is caused to the cells
253 (24). The chosen temperature simulates and assesses the impact of the tested treatment on the
254 growth and survival of *L. monocytogenes* under optimal temperature conditions, to exclude the
255 potential effect of a non-optimal temperature (heat stress) on the microbial response.
256 Thereafter, the samples were centrifuged at 5000 rpm for 10 min (Megafuge 16R,
257 ThermoFisher, USA), the supernatant was discarded, and the pellet was resuspended in 20 ml
258 PBS. To enumerate the viable population of the 2 h GSE treatment, the spread-plate method
259 was followed using TSAYE non-selective media. Sub-lethally injured cells exhibit an inability
260 to grow on selective media, while they are capable of normal growth on non-selective media
261 (89). Therefore, to identify the number of cells that were sub-lethally damaged, the samples
262 were also plated into selective media i.e., Polymyxin Acriflavin Lithium-chloride Ceftazidime
263 Esculin Mannitol (PALCAM) agar for *L. monocytogenes* or Violet Red Bile Glucose (VRBG)
264 agar (Oxoid Ltd, UK) for *E. coli*. The number of injured cells was calculated based on the
265 following equation (90) :

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267 $$\%\, Injured\ cells = [1 - \frac{Count\ on\ selective\ agar}{\text{Count on non-selective agar}} (\frac{CFU}{ml})]\ \times\ 100 \qquad (1)$$

268

269 For the experiments involving CAP treatment, 300 μL of PBS containing either GSE pre-
270 treated or untreated cells, were transferred in 12-well plate. The samples were exposed to CAP
271 at 1 L/min flow rate for 4 min. The flow rate of the CAP treatment was determined through
272 initial experiments (results not shown), which demonstrated that lower flow rates in the liquid

273 carrier, enriched with RNS, resulted in more effective inactivation. Additionally, the duration
274 of the CAP treatment was selected in order to induce a slight decrease in the microbial
275 population, therefore allowing the investigation of the potential synergistic effects of the GSE
276 and CAP treatment. The survival of the microbial population and sublethal injury after the
277 treatment was assessed using the spread plate technique as described above (section 2.3).
278 Additionally, for *E. coli*, the treatment sequence was reversed. Initially, the cells were subjected
279 to CAP treatment, and subsequently, they were exposed to GSE (Figure 1). The parameters
280 used for both treatments remained unchanged. This approach was implemented specifically for
281 *E. coli* to explore the potential synergistic or altered effects resulting from the reversed
282 treatment sequence. The decision resulted from the decreased antimicrobial efficacy, in
283 comparison with the efficacy against *L. monocytogenes,* observed when *E. coli* was treated
284 with GSE followed by CAP (see results section).

285

286 ***2.5. Statistical analysis***

287 At least two independent biological experiments with three replicate samples were
288 conducted for all conditions under study. When comparing two mean values, a t-test was used
289 to confirm statistical significance ($p < 0.05$) while for multiple comparisons, a two-way
290 ANOVA followed by Tukey's HSD post hoc was used to confirm statistically significant ($p <$
291 0.05) differences between independent experimental groups. In the plots below, the mean value
292 is presented with error bars representing the standard deviation. In cases where the viable cell
293 count was below the detection limit (<10 CFU/ml) in the general and selective media the
294 number of viable and sub-lethally damaged cells was set to 1 log CFU/ml and/or 100%,
295 respectively. All statistical analysis was performed using GraphPad Prim and Microsoft Excel.

296

297 **3. Results**

298 As previously mentioned, to investigate the combined effect of grape seed extract (GSE)
299 and cold atmospheric plasma (CAP) on *L. monocytogenes, E. coli* and their isogenic mutants
300 (mentioned in section 2.1) in TSBYE, the pathogens were firstly treated with 1 % w/v GSE for
301 2 h. Thereafter, the cells were treated with CAP for 4 min at 1 L/min flow rate. Finally, the
302 viable and sublethal populations of the individual and combined treatments were quantified, to
303 enable a meaningful comparison between the wild types and their isogenic mutants.
304 Furthermore, examining both the individual treatments of GSE and CAP and their combination,
305 allowed for precise evaluation of each treatment's impact on the isogenic mutant strains as well
306 as the evaluation of their combined effect.

307 *To the authors' best knowledge this is the first study investigating the impact of the combined*
308 *antimicrobial effect of GSE with a novel non-thermal technology such as CAP in a liquid*
309 *carrier on L. monocytogenes and E. coli and their isogenic mutants in environmental stress*
310 *genes. Therefore, this study provides valuable insights into the microbial mechanisms of stress*
311 *response to this combined treatment.*

312

313 ***3.1 The effect of GSE and CAP against L. monocytogenes WT and its isogenic mutants.***

314 Figures 2 and 3 show the level of microbial inactivation caused by individual and combined
315 GSE and CAP treatments, for all tested strains of *L. monocytogenes* (WT, Δ*sigB*, Δ*gadD1,*
316 Δ*gadD2,* Δ*gadD3*). More specifically, Figure 2 presents the data arranged by treatment type
317 while in Figure 3 the results are organised by the strain of *L. monocytogenes*, to enable clearer
318 multiple comparisons. Overall, the combined treatment of 1 % (w/v) GSE for 2 h followed by
319 4 min of CAP treatment at flow rate 1 L/min had a good synergistic effect against all strains of
320 *L. monocytogenes.* After the individual GSE treatment, i.e., a 2 h exposure to 1 % (w/v) GSE,

321 there was no significant decrease in the population of *L. monocytogenes* WT (Figure 3a). For
322 most mutant strains of *L. monocytogenes,* the cell concentration was reduced by an average of
323 0.4 log CFU/ml following a 2 h exposure to GSE ($p > 0.05$) (Figure 2 & 3b-e). Additionally,
324 the sublethal injury assessment showed that the GSE treatment led to a greater percentage of
325 sub-lethally injured cells among the mutant strains (Figure 4b & 5). The cells of Δ*sigB* and
326 Δ*gadD1* were the most sensitive, as all the microbial population was sub-lethally injured (100
327 %) after 2 h in the presence of GSE. The WT strain exhibited the lowest percentage of sub-
328 lethal injury following the individual GSE treatment, which was approximately 60 % (Figure
329 4 & 5). The high yield of sub-lethally injured cells for all *L. monocytogenes* strains emphasises
330 the great potential of GSE as a sustainable solution for decontamination.

331 After the individual CAP treatment (4 min, 1 L/min), all strains of *L. monocytogenes* except
332 the mutant strain Δ*gadD2* were inhibited by an average of 0.5 log CFU/ml. The inactivation of
333 *L. monocytogenes* Δ*gadD2* mutant was higher as compared to all other mutant strains, with an
334 approximate reduction of 1.7 log CFU/ml ($p < 0.05$) (Figure 2a & 3c). Additionally, when
335 measuring the sublethal population it was observed that all mutants had higher percentage of
336 sub-lethally injured cells, in comparison to the WT, with the highest percentage of 24%
337 belonging to Δ*gadD2* (Figure 4a & 5). These results indicate that the *gadD2* might have a
338 significant role in the tolerance of *L. monocytogenes* to CAP treatment.

339 As previously mentioned, the combined treatment of GSE (1 % w/v, 2 h) and CAP (1 L/min,
340 4 min) had a great synergistic effect against all strains of *L. monocytogenes.* As can be seen in
341 Figures 2 and 3, the viable population of *L. monocytogenes* WT after the combined treatment
342 was equal to 3.2 log CFU/ml i.e., the combined treatment led to a 2.5 log CFU/ml reduction
343 when compared to untreated controls (Figure 2c & 3a). Additionally, the microbial inactivation
344 of most mutant strains (Δ*gadD1*, Δ*gadD2*, Δ*gadD3*) was comparable to the WT strain with an
345 average reduction of 2.3 log CFU/ml. *L. monocytogenes* Δ*sigB* was the only mutant strain for

346 which a higher level of microbial inactivation (3.2 log CFU/ml) was observed, in comparison
347 to all other *L. monocytogenes* strains. ($p < 0.05$) (Figure 2c). However, when assessing the
348 extend of sub-lethal injury (Figure 4c & 5), it was noted that cells of all strains could not grow
349 on selective medium (PALCAM) i.e., most cells of all strains were in the state of sub-lethal
350 injury indicating the great antimicrobial efficacy of this hurdle approach.

351

352 ***3.2. Combined treatment of GSE and CAP against E. coli WT and its isogenic mutants.***

353 For the inactivation of *E. coli,* the same treatments as *L. monocytogenes* were performed. In
354 addition, the combined treatment in reverse sequence, i.e., CAP treatment followed by GSE,
355 was examined due to the observed inefficient microbial inactivation of *E. coli* by the initial
356 sequence of the combined treatment.

357 The individual GSE treatment was unable to reduce the population of *E. coli* WT, Δ*oxyR*,
358 Δ*rpoS*, Δ*dnaK* (Figure 6b & 7). The inability of GSE to inactivate all strains of *E. coli* was also
359 observed in the sub-lethally damaged microbial population, were the percentage of sub-lethally
360 damaged cells after the GSE treatment was similar to that of the control ($p > 0.05$) (Figure 8b
361 & 9).

362 The individual CAP treatment was more effective against *E. coli* (Figure 6) in comparison
363 to *L. monocytogenes* (Figure 2). As can be seen in Figures 6 and 7, the microbial inactivation
364 of *E. coli* WT and Δ*oxyR* were similar and on average 1.4 log CFU/ml ($p > 0.05$). When
365 subjected to CAP treatment, the mutant strain *E. coli* Δ*dnaK* showed increased inactivation in
366 comparison to *E. coli* WT and Δ*oxyR* resulting in a reduction of 2.3 log CFU/ml (Figure 6a &
367 7c). However, the count of sub-lethally injured cells of *E. coli* Δ*oxyR* showed a higher
368 percentage of sub-lethally damaged cells (67 %) as compared to *E. coli* WT (average of 31.7
369 %) (Figure 8). Overall, our results show that both mutant strains *E. coli* Δ*oxyR* and Δ*dnaK,* are

370 more sensitive to CAP treatment than the WT. The most significant reduction in microbial
371 concentration following CAP treatment was observed in *E. coli* Δ*rpoS* with a population
372 decrease of 3.8 log CFU/ml (Figure 6a & 7b) with the surviving population being 100% sub-
373 lethally injured (Figure 8a & 9b).

374 For the combined treatment of GSE and CAP, when treating the cells with GSE followed
375 by CAP, no synergistic or additive effects were observed against any of the strains of *E. coli*
376 under study, as shown in Figure 6c & 7. The results indicated that there was an increase in the
377 tolerance to CAP treatment after a 2 h exposure to 1% (w/v) GSE, as seen by the viable counts
378 (Figure 7). However, it was noted that nearly all cells of the *E. coli* Δ*oxyR* and Δ*rpoS* strains
379 were in a sub-lethal injury physiological state, indicating that the combined treatment affected
380 those mutants, inducing injury, but did not affect the overall cell viability (Figure 8c, 9b, 9c).
381 Moreover, while *E. coli* Δ*dnaK* showed increased tolerance to CAP treatment after GSE
382 treatment, it did not demonstrate an equivalent level of tolerance as compared to the WT strain
383 (Figure 6c, 7a, 7d). As previously mentioned, due to the inability of the combined treatment of
384 CAP and GSE to inactivate *E. coli*, the reversed combined treatment was investigated.
385 Interestingly, in a combined CAP/GSE treatment where the samples were first treated with
386 CAP followed by a 2 h exposure to GSE, all strains of *E. coli* were completely inactivated (~5
387 log CFU/ml reduction as compared to the controls; Figure 6d). This suggests that the sequence
388 of the CAP/GSE treatments can have a detrimental effect on the microbial inactivation *E. coli*.
389 As the combined treatment of GSE and CAP achieved total inactivation of all strains *E. coli*,
390 there was no scope to enumerate the sub-lethally damaged population.

391

392 **4. Discussion**

393 In this study the antimicrobial effect of grape seed extract (GSE, 1 % w/v, 2 h), cold
394 atmospheric plasma (CAP, 1 L/min, 4 min) and their combination against *L. monocytogenes,*
395 *E. coli* and their isogenic mutants in environmental stress genes was systematically explored.
396 *To the best of our knowledge this is the first study combining natural antimicrobials with non-*
397 *thermal technologies like CAP in a controlled liquid system against L. monocytogenes, E. coli*
398 *along with functional genomics work (usage of targeted knockout mutants) to identify*
399 *mechanisms of resistance and modes of action*. Overall, our results show that most mutant
400 strains were more susceptible to the individual and combined treatments than the wild type
401 (WT) strains, but the level of susceptibility was strain dependent. Additionally, the sequence
402 of the combined treatment played a significant role on the efficacy of the combined treatment
403 against *E. coli.*

404

405 *4.1 Individual GSE and CAP treatments*

406 For the individual GSE treatment against *L. monocytogenes* and its isogenic mutants, after
407 2 h of exposure to GSE, the only mutant strain having significant difference in the microbial
408 inactivation, in comparison to the WT strain, was *L. monocytogenes* Δ*sigB* ($p > 0.05$) (Figure
409 2b). However, all mutant strains of *L. monocytogenes* (Δ*sigB,* Δ*gadD1*, Δ*gadD2*, Δ*gadD3*)
410 demonstrated higher percentage of sublethal injury as compared to the WT (Figure 4b & 5),
411 indicating that GSE causes significant damage to those mutants, and it is a promising agent for
412 the design of antimicrobial strategies. Additionally, *L. monocytogenes* Δ*sigB* showed a slightly
413 increased sensitivity to CAP treatment when compared to the WT, as demonstrated in the
414 evaluation of sublethal injury (Figure 2a). The higher antimicrobial effect of GSE against Δ*sigB*
415 can be explained by the fact that SigB (σ^B) regulates the general stress response of Gram-
416 positive bacteria like *L. monocytogenes* (91,92). More specifically, the gene regulator SigB

417 plays a crucial role in controlling the expression of more than 100 genes involved in various
418 stress responses (see also Table 1). Therefore, it plays a major role in the resistance of *L.*
419 *monocytogenes* to various treatments (75,92–94). Results showing the effect of SigB in a
420 treatment are important as they suggest that at least one of the genes controlled by SigB plays
421 a role in the resistance to this stress. This narrows our investigation regarding the specific
422 mechanisms that contribute to the resistance under a certain stress. SigB has been reported to
423 exhibit increased expression in *L. monocytogenes* as a response to stress, significantly
424 contributing to the adaptability of the bacterium to various types of stress including heat, acid,
425 and osmotic stress (74–76,95). However, for oxidative stress, the results existing in the
426 literature are contradicting (74,96). For example, Patange et al. (2019) showed that the mutant
427 in *sigB* was more susceptible as compared to the WT, when exposed to CAP treatment (directly
428 applied dielectric barrier discharge, sealed container, 1-5 min). However, in a study by Boura
429 et al. (2016), Δ*sigB* was more tolerant to oxidative stress (H_2O_2 treatment) than the WT. The
430 latter authors demonstrated that the discrepancies were due to different oxygen levels during
431 growth, with presence of SigB resulting in high sensitivity to oxidative stress under aerobic
432 conditions and the opposite effect under anaerobic conditions. In our results, the percentage of
433 sub-lethally damaged cells of Δ*sigB* (grown in aerobic condition) was higher in comparison to
434 the WT, but the viable count was not significantly different in comparison to the WT (Figure
435 2a, 3b, 4a). Overall, to date, the studies on the contribution of SigB in the tolerance to natural
436 antimicrobial treatments are very limited, and there is absence of studies examining its impact
437 to GSE treatment (97,98). According to the limited studies on natural antimicrobials (other than
438 GSE), SigB impacts the antimicrobial resistance to certain bacteriocins like nisin and lacticin
439 3147 (75,98) which is in accordance to our results on the sensitivity of Δ*sigB* to GSE (24).

440 As previously mentioned, there was no significant difference in the microbial inactivation
441 (viable count) between the Δ*gadD1, D2* and WT strains after the GSE treatment (Figure 2b)

442 which can be explained by the fact the GAD system has been primary linked to acid stress
443 responses (Table 1). However, the percentage of sublethal injury of Δ*gadD1, D2 and D3*
444 mutants was higher following the GSE treatment in comparison to the WT (Figure 4b, & 5).
445 After CAP treatment, Δ*gadD2* exhibited the highest level of inactivation in terms of viable
446 count among all strains (Figure 2a & 3d). Additionally, the absence of *gadD1* and *gadD3* did
447 not result in a higher level of inactivation as compared to *L. monocytogenes* WT after CAP
448 treatment (Figure 2a, 3a, 3c, 3e). The GAD system is crucial for the viability of *L.*
449 *monocytogenes* under acid stress as is responsible for maintaining the cellular pH in certain
450 optimal range for survival and growth. It comprises of 5 or 3 proteins, depending on the strain.
451 Although all strains possess both *gadT2D2* and *gadD3*, the *gadD1T1* operon is missing from
452 serotype 4 *L. monocytogenes* strains (99). Two of the proteins namely GadT1 and GadT2 are
453 glutamate/GABA antiporters while GadD1, GadD2, GadD3 are glutamate decarboxylases
454 (77,78,95,100). The five corresponding proteins are encoded in three transcriptional units,
455 namely *gadD1T1*, *gadT2D2*, and *gadD3*. Previous studies have shown that the *gadT2D2* locus
456 has a significant impact on the survival of *L. monocytogenes* in highly acidic environments,
457 whereas the *gadD1T1* locus has been observed to promote growth in moderately acidic
458 conditions (77,78,101). Additionally, it has been shown that GadD2 might be the dominant
459 gene within the GAD system of *L. monocytogenes* 10403S (77,78). The full functionality of
460 the GAD system in stress adaptation has not been yet completely elucidated and the studies
461 exploring its role to other treatments like natural antimicrobial or oxidative stress are extremely
462 limited. For instance, Begley et al. (2010) observed that Δ*gadD1* in *L. monocytogenes* LO28
463 exhibited increased susceptibility to nisin treatment in BHI broth at a concentration of 300
464 μg/ml, when compared to the WT strain (102). Nisin's mechanism of inactivation is based on
465 its ability to bind to Lipid II, a precursor involved in the synthesis of peptidoglycan of the cell
466 wall. This binding process hinders the cell wall synthesis resulting to pore formation in the cell

467 membrane and ultimately causes release of the cell's intracellular content and ATP (6,102,103).
468 Begley et al. (2010), proposed that under specific circumstances, the presence of *gadD1* may
469 play a role in increasing the intracellular ATP pools, therefore increasing the resistance to nisin.
470 It is possible that, similarly, there is a GAD system-mediated mechanism protecting cells from
471 GSE and its absence results in increased percentage of sub-lethally damaged cells, as indicated
472 by our results (Figure 4b, 5c, 5d, 5e).

473 As previously mentioned, for the individual CAP treatment, *L. monocytogenes* Δ*gadD2* was
474 the most sensitive strain with the highest microbial inactivation and the highest percentage of
475 sub-lethally damaged cells (Figure 2a & 3c). Boura et al. (2020) investigated the role of GAD
476 system in oxidative stress (H_2O_2) in 3 strains of *L. monocytogenes* namely EGD-e, LO28 and
477 10403S. The study demonstrated that *gadD3 and gadD2* play a role in oxidative stress
478 resistance of EGD-e, *gadD1* in LO28 while no role of the GAD system was found in 10403S
479 (104). Therefore, overall, several components of the GAD system play a role in oxidative stress
480 and this can depend on the strain, the stage of growth and other environmental conditions such
481 as the type of growth medium. The CAP treatment utilised in this study, generates reactive
482 oxygen species (ROS) leading to oxidative stress and nitrogen reactive species (RNS), that
483 might result in microbial inactivation (24). Hence, the increased microbial inactivation of
484 Δ*gadD2*, observed in this study, may be attributed to the RNS or at the different ROS present
485 in the CAP output species, in comparison to H_2O_2 treatment (Figure 2d). In addition, the lack
486 of response of the GAD system to the CAP treatment might also be related to the stage of
487 growth or the medium used (78). RNS are very reactive and have the ability to modify DNA,
488 lipids, and proteins (105) while they can also reduce the intracellular pH. The conversion of
489 glutamate to γ-aminobutyric acid (GABA) carried out by the GAD system, might have an
490 indirect role as a cellular defence mechanism against the RNS (77,78,101). Additionally,
491 similar to nisin, CAP treatment can cause pore formation leading to the release of ATP.

492 Therefore, the GadD2, which has been shown to be the dominant gene in the GAD system of
493 *L. monocytogenes* 10403S, might help in sustaining the intracellular ATP levels (77,102,106).
494 As a result, the absence of this gene might increase the sensitivity of *L. monocytogenes* 10403S
495 to CAP treatment, as shown by our results (Figure 2a & Figure 3d).

496 In the evaluation of the efficacy of the individual treatments on WT *E. coli* K12 and its
497 mutants *(ΔrpoS, ΔoxyR, ΔdnaK*), it was observed that the individual GSE treatment was
498 inefficient in reducing the microbial population, for all strains under study (Figure 6b & 7).
499 This trend was expected as it is known in literature that Gram-negative bacteria have a higher
500 level of resistance to natural antimicrobials, as compared to Gram-positive bacteria (24,107–
501 109). This difference/resistance, arises from the presence of an outer lipid membrane, which
502 acts as a protective barrier, limiting the penetration of antimicrobial compounds (17).
503 Additionally, during the mild GSE treatment stress adaptation mechanisms could be activated,
504 which help *E. coli* to overcome the imposed stressor (110–112).

505 The efficacy of the individual CAP treatment was found to be higher against all strains of
506 *E. coli* when compared to its effectiveness against most strains of *L. monocytogenes* (Figure 2a
507 and 6a). This is in accordance to literature (for the WT), as it has been generally observed that
508 Gram-negative bacteria are more sensitive to plasma treatment in comparison to Gram-positive
509 bacteria (106,113,114). For example, Smet et al., (2018) examined the inactivation of Gram-
510 positive *L. monocytogenes* and Gram-negative *S.* Typhimurium by CAP (directly applied
511 dielectric barrier discharge, mixture of 4 L/min helium and 40 ml/min oxygen) and observed
512 that *L. monocytogenes* was more tolerant to the CAP treatment. The microbial inactivation of
513 *S.* Typhimurium after 10 min of CAP treatment was approx. 2 log CFU/ml. However, the
514 population of *L. monocytogenes* was reduced by less than 0.5 log CFU/ml (Cindy Smet et al.,
515 2018). One of the contributing factors to this difference is the structural characteristics of their
516 cell walls. The thinner peptidoglycan layer in Gram-negative bacteria allows reactive species,

517 such as ROS and RNS generated by CAP, to penetrate more easily into the bacterial cell and
518 cause damage to essential cellular components i.e., proteins and nucleic acids (35,115–117).
519 However, the sensitivity to CAP can still vary among different bacterial species of the same
520 cell structure or strains of the same species. Other factors affecting the sensitivity are the
521 physiological state of the cells and the initial microbial population existing in the sample
522 (27,33).

523 For the individual CAP treatment, when comparing the different strains of *E. coli*, the most
524 sensitive mutant strain was *E. coli* Δ*rpoS* followed by Δ*dnaK* (Figure 6a, 7b, 7d). In addition,
525 despite having a similar number of viable cells after CAP treatment (Figure 6a & 7c), the *E.*
526 *coli* Δ*oxyR* strain had a much higher percentage of sub-lethally injured cells as compared to the
527 WT strain (Figure 8a & 9c). The high sensitivity of *E. coli* Δ*rpoS* can be explained by the fact
528 that in Gram-negative bacteria like *E. coli,* the general stress response is regulated by the RpoS
529 (σ^s; see also Table 1). Similarly to SigB for Gram-positive bacteria, RpoS is an alternative
530 sigma factor responsible for the expression of >50 genes involved in stress adaptation of Gram-
531 negative bacteria (79–81). However, the genes affected by the central stress gene sigma factor
532 are not the same in Gram-positive and Gram-negative bacteria and there are differences
533 between species and strains of the same species (118,119).

534 According to our results, DnaK could have an impact on the sensitivity of *E. coli* to CAP
535 treatment (Figure 6a & 7d). This is due to the existence of another sigma factor, namely RpoH,
536 which regulates the expression of genes that are involved in the heat shock response, such as
537 chaperones and heat shock proteins like DnaK. DnaK is a chaperone that helps in the folding
538 of proteins and prevents protein aggregation under heat stress and/or other stresses. Therefore,
539 it is crucial for the maintenance of the cellular protein homeostasis and in its absence the cells
540 could become more sensitive to CAP treatment, as it causes protein denaturation (120,121).
541 Other important gene regulators worth mentioning are OxyR and SoxR (82,83) that respond to

542 oxidative stress and subsequently activate *soxS* and *sod* that are associated with reactive oxygen
543 species (ROS) defence mechanisms (122). Therefore, when cells are under oxidative stress,
544 they produce proteins that contribute to DNA repair or the free radicals elimination. The results
545 of the current study indicate that the transcriptional regulator OxyR plays an important role in
546 the CAP treatment tolerance of *E. coli*, as evidenced by the increase sublethal injury (Figure
547 5). However, in the absence of *oxyR*, the presence of *soxS* is possibly sufficient for the cells to
548 cope with the oxidative stress caused by CAP treatment or cover for the absence of the former.
549 The sensitivity of the isogenic mutants of *E. coli (*Δ*rpoS*, Δ*oxyR*, Δ*dnaK)* to CAP treatment has
550 been reported in previous studies (123–126). The results of these studies are in accordance with
551 the results of the current study, suggesting that RpoS, OxyR and DnaK might play a role in the
552 tolerance of *E. coli* to CAP treatment. However, it is challenging to compare the level of
553 inactivation due to various factors that influence the efficiency of cold atmospheric plasma
554 (CAP). These factors, include the plasma source, the duration of treatment, the system on which
555 it is implemented and the treated level of microbial population (8,37–40,113). For example,
556 Connolly et al. (2013) explored the inactivation of *E. coli* K12 and its isogenic mutants Δ*soxR*,
557 Δ*soxS*, Δ*oxyR*, Δ*rpoS* and Δ*dnaK* by treating cells of *E. coli* on agar with CAP (dielectric
558 barrier discharge, fixed volume of helium and air mixture) for 5 min. After the treatment, the
559 microbial inactivation of all strains was 1.5 log CFU/cm^2. However, it was noted that Δ*oxyR*,
560 Δ*rpoS* and Δ*dnaK* had a much slower recovery compared to the WT strain indicating that these
561 gene regulators impact the cell's repair mechanisms (123). Additionally, Han et al. (2016)
562 investigated the effects of CAP (dielectric barrier discharge, fixed volume of atmospheric air)
563 on *E. coli* K12 using the same mutant strains as our study i.e., *E. coli* Δ*rpoS*, Δ*oxyR*, and Δ*dnaK*
564 genes. The cells were treated in a sealed container for 1, 3, and 5 min and their inactivation
565 levels were assessed after being stored for 0, 1, and 24 h at room temperature. The results
566 demonstrated increased sensitivity of Δ*rpoS* to CAP treatment whereas Δ*oxyR* did not show a

567 sensitive phenotype until after 5 min of treatment. In this study, the importance of *dnaK* was
568 more apparent after analysing the viable population after storage time, suggesting that its role
569 is in contributing to the repair mechanism rather than the immediate reaction right after CAP
570 treatment (124).

571

572 *4.2 Combined GSE and CAP treatments*

573 The combined treatment of GSE and CAP achieved a synergistic effect against all strains of
574 *L. monocytogenes* with the mutant strain Δ*sigB* to be the most sensitive to the combined
575 treatment (Figure 2c & 3b). However, for *E. coli* a synergistic effect was only achieved when
576 CAP preceded the GSE treatment (Figure 6c, 6d, 7). The combined effect of GSE and CAP has
577 been investigated in previous work from our group in which similar results were observed when
578 1 % (w/v) GSE was incorporated in various 3D *in vitro* models with varying rheological
579 properties. *L. monocytogenes* was treated with GSE on the surface of the 3D models for either
580 2 h and/or 8 h and treated with CAP for 2 min at flow 5 L/min (higher concentration of ROS
581 species). To the best of our knowledge, no other studies to date have investigated the
582 combination of GSE and CAP against *L. monocytogenes* and *E. coli* and their isogenic mutants.
583 However, prior research has investigated the combined use of CAP with different natural
584 antimicrobials. During these studies it was shown that employing a combined approach led to
585 more effective microbial inactivation compared to applying the treatments individually
586 (8,64,65,96,127). For example, De la Ossa et al. (2021) evaluated the synergistic effect of olive
587 leaf extract (with a total phenolic content of 100 mg/ml) and CAP treatment (using the same
588 apparatus as utilised in this study, with a flow rate of 5 L/min for 1 min) in a liquid nutrient
589 broth against exponential and stationary phase cells of *Listeria innocua, E. coli*, and *S. aureus*.
590 The combination of CAP and olive leaf extract resulted in total inactivation of exponential cells

591 of all tested strains, while no inhibitory effects were observed with either treatment applied
592 individually. Additionally, cells in stationary phase appeared to be more resistant to the
593 combined treatment therefore the same synergistic effect was not observed. In another study,
594 Costello et al. (2021a) investigated the hurdle strategy of nisin in sublethal concentration (35
595 IU/ml, 30 min) and CAP (directly applied dielectric barrier discharge, 4 L/min helium and 40
596 ml/min oxygen, 30 min) against *L. innocua* in/on liquid and solid like 3D *in vitro* models (1.5%
597 w/v XG). Again, a combined effect was reported when the hurdle approach of CAP and nisin
598 was tested, in comparison to the individual treatments (8). Furthermore, the combination of
599 CAP with other natural antimicrobials has been explored using real food products (65,67,128).
600 For example, Matan et al. (2015), reported a synergistic effect of plasma (radio frequency 40W)
601 and green tea extract (5% w/v) against *L. monocytogenes, E. coli,* and *S.* Typhimurium, on the
602 surface of dragon fruit (10^6 CFU/g initial microbial concentration). More specifically, when
603 the combined treatment of CAP and green tea extract was applied, complete inactivation was
604 achieved for all bacterial strains. The individual plasma treatment caused a reduction in
605 bacterial population by 1-1.5 log CFU/g depending on the strain. The individual treatment with
606 green tea extract did not exhibit a significant antimicrobial effect against the tested Gram-
607 negative bacteria, however *L. monocytogenes* was reduced by 1 log CFU/g (65).

608 There is only one study examining the combination of CAP (directly applied dielectric barrier
609 discharge, atmospheric air, 1-5 min) with other treatments (4 °C and/or acetic acid at pH 4.0
610 for 1 h) against *L. monocytogenes* and its mutants (Δ*sigB,* Δ*gadD1,* Δ*gadD2,* Δ*gadD3,*
611 Δ*gadD2D3,* Δ*prfA,* Δ*rsbR,* Δ*lmo0799,* Δ*lmo0799-C56A*). In this study, it was shown that the
612 susceptibility of various strains of *L. monocytogenes* bacteria to CAP treatment was enhanced
613 by exposing them to cold stress. However, the efficacy of CAP treatment was shown to be
614 comparable among the various strains, with the exception of the Δ*rsbR* mutant, which showed
615 an increased inactivation after the combined cold stress and CAP treatment (96). After the

616 combined acid stress and CAP treatment, all strains of *L. monocytogenes* were completely
617 inactivated indicating a synergistic effect of the tested treatments. *To the author's best*
618 *knowledge there are no studies exploring the combined effect of CAP or natural antimicrobials*
619 *with other treatments against E. coli and its mutants.*

620 As previously stated, the total inactivation of *E. coli* was achieved through a sequenced
621 treatment approach, starting with the application of CAP followed by GSE treatment (Figure
622 6d & 7). Previous studies have demonstrated that the order of antimicrobial treatments can
623 influence their efficacy and the microbial response, depending on the cellular component they
624 targeted (5,129–131). For instance, Chaplot et al. (2019) investigated the hurdle approach of
625 CAP (dielectric barrier discharge, 6 min) and peracetic acid (100 ppm, 6min) against *S.*
626 Typhimurium in raw poultry meat. The CAP treatment followed by peracetic acid resulted in
627 a 3.8 log CFU/cm^2 reduction, however when the reversed order was applied, *S.* Typhimurium
628 was inhibited by 2.5 log CFU/cm^2. In this study it was proposed that the release of active
629 oxygen by peracetic acid, disrupted the sulfhydryl and sulphur bonds present in the cellular
630 membrane resulting in the effective penetration of RONS in the cell and further inactivation
631 caused by RONS interacting with the intracellular components (131). The proposed
632 inactivation mechanism for the combined treatment against *L. monocytogenes* involves firstly
633 the penetration of GSE in the bacterial cells and the interaction with their intracellular
634 components (17–19). According to our results GSE treatment causes a moderate stress to the
635 cells resulting in sublethal injury (>60 %), making them more susceptible to CAP treatment,
636 which targets other cellular structures (Figures 2b, 3, 4b, 5). Therefore, the increased sensitivity
637 of the Δ*sigB* strain to the combined treatment can be attributed to its higher susceptibility to
638 GSE treatment (24). The same combined effect could not be observed when GSE followed by
639 CAP treatment was tested against *E. coli* as the GSE treatment, according to our viable and
640 sublethal count, imposed a mild stress from which the cells could easily adapt (Figures 6c and

641 8b). This suggests that the exposure of *E. coli* to GSE could lead to a higher tolerance to the
642 CAP treatment via cross-protection mechanisms. The proposed mechanism for the total
643 inactivation of *E. coli* by CAP followed by GSE treatment, is the ability of CAP to cause cell
644 wall disruption hence allowing increased penetration of the GSE components in the cell
645 (32,33,35).

646

647 **Conclusion**

648 In this work we investigated the antimicrobial activity of grape seed extracts (GSE), cold
649 atmospheric plasma (CAP, a remote air plasma with an ozone-dominated RONS output) and
650 their combination against *L. monocytogenes, E. coli* and their environmental stress isogenic
651 mutants in liquid nutrient medium (TSBYE). More specifically, all bacteria under study were
652 treated with 1% (w/v) GSE for 2h, CAP at flow rate 1 l/min for 4 min and/or their combination.
653 The combined treatment was applied sequentially by exposing the cells first to GSE followed
654 by CAP. For *E. coli*, the treatment sequence was also reversed i.e., treating the cells with CAP
655 prior to GSE. The hypothesis of testing the reverse treatment sequence against *E. coli* was that
656 CAP would be able to increase the permeability of the bacterial cell wall, allowing GSE to
657 easily penetrate through the outer lipopolysaccharide membrane and target the intracellular
658 components.

659 A synergistic effect was achieved when GSE and CAP treatments were combined to
660 inactivate *L. monocytogenes* (WT, Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δ*gadD3*) *and E. coli* (WT,
661 Δ*rpoS*, Δ*oxyR*, Δ*dnaK*). Specifically, GSE followed by CAP treatment effectively inactivated
662 all strains of *L. monocytogenes* with Δ*sigB* having the highest microbial inactivation. However,
663 this combined treatment sequence did not exhibit the same efficacy against *E. coli*.
664 Interestingly, when the reverse sequence was explored i.e., first applying CAP and then GSE,

665 a total inactivation of all strains of *E. coli* was observed. For the individual treatments, *L.*
666 *monocytogenes* Δ*sigB* was more sensitive to GSE treatment, *while L. monocytogenes* Δ*gadD2*
667 was more susceptible to CAP treatment, as compared to all other *L. monocytogenes* strains
668 under study. The individual GSE treatment did not inhibit *E. coli* (WT, Δ*rpoS*, Δ*oxyR*, Δ*dnaK*)
669 after 2 h and the individual CAP treatment was more effective against *E. coli* Δ*rpoS* as
670 compared to all other *E. coli* strains under study.

671 Our research suggests that GSE, CAP, and their combination could be used as sustainable
672 antimicrobial strategies in the food industry. However, the sequence of the combined
673 treatments can have an effect on the microbial inactivation depending on the bacterial species.
674 Additionally, our work sheds light on the genes responsible for sensitivity/tolerance of the
675 tested bacteria to the individual treatment of GSE and CAP, therefore contributing to the
676 development of more effective and targeted antimicrobial strategies for sustainable
677 decontamination.

678

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687

688

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1093 **Figure legends**

1094 **Figure 1**: Experimental procedure of the individual and combined treatment of GSE and CAP
1095 (created with BioRender.com).

1096 ***Figure 2*****:** Reduction (log CFU/ml) of the viable population of *L. monocytogenes* 10403S (WT
1097 and mutants) following (a) CAP (4 min), (b) GSE (2h), (c) GSE (2h) + CAP (4 min) treatment
1098 in TSBYE. Data are normalised for each strain/condition with respect to untreated controls. In
1099 all plots, (■) WT, (■) Δ*sigB*, (■) Δ*gadD1*, (■) Δ*gadD2*, (■) Δ*gadD3*. Each bar represents the
1100 average of two independent experiments with three technical replicates per experiments while
1101 error bars represent standard deviation. Connecting lines with asterisks indicate significant
1102 differences between samples (* if $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq$
1103 0.001, *** if $p \leq 0.0001$)

1104 ***Figure 3*****:** Viable counts of *L. monocytogenes* 10403S (a) WT, (b) Δ*sigB*, (c) Δ*gadD1*, (d)
1105 Δ*gadD2*, (e) Δ*gadD3* in TSBYE for all treatments under study. In all plots, (■) control
1106 (untreated sample), (■) CAP treatment for 4 min at flow rate of 1 L/min, (■) 1% (w/v) GSE
1107 treatment for 2h, (■) Combination of 1% (w/v) GSE (2h) and CAP treatment (4 min). Each bar
1108 represents the average of two independent experiments with three technical replicates per
1109 experiment while error bars represent standard deviation. Connecting lines with asterisks
1110 indicate significant differences between samples (* if $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, ***
1111 if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

1112 ***Figure 4*****:** Sublethal injury (%) of *L. monocytogenes* 10403S (WT and mutants) induced by (a)
1113 CAP (4 min), (b) GSE (2h), (c) GSE (2h) + CAP (4 min) treatment in TSBYE. In all plots, (■)
1114 WT, (■) Δ*sigB*, (■) Δ*gadD1*, (■) Δ*gadD2*, (■) Δ*gadD3*. Data are normalised with respect to
1115 untreated samples for each condition under study. Each bar represents the average of two
1116 independent experiments with three technical replicates per experiment. In cases where the

1117 viable cell count in the selective media was below detection limit (<10 CFU/ml) the number of
1118 sublethal damaged cells was set to 100 % (bar with stripes) while error bars represent standard
1119 deviation. Connecting lines with asterisks indicate significant differences between samples (*
1120 if $0.01 < p \leq 0.05$, ** if $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1121 ***Figure 5***: Quantification of sub-lethally injured cells (%) of *L. monocytogenes* 10403S (a) WT,
1122 (b) Δ*sigB*, (c) Δ*gadD1*, (d) Δ*gadD2* (e) Δ*gadD3* in TSBYE for all treatments under study. In
1123 all plots, (■) control (untreated sample), (■) CAP treatment for 4 min at flow rate of 1 L/min,
1124 (■) 1 % (w/v) GSE treatment for 2 h, (■) Combination of 1 % (w/v) GSE (2 h) and CAP
1125 treatment (4 min). In cases where the viable cell count in the selective media was below
1126 detection limit (<10 CFU/ml) the number of sublethal damaged cells was set to 100 % (bar
1127 with stripes). Each bar represents the average of two independent experiments with three
1128 technical replicates per experiment while error bars represent standard deviation. Connecting
1129 lines with asterisks indicate significant differences between samples (* if $0.01 < p \leq 0.05$, ** if
1130 $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1131 ***Figure 6***: Reduction (log CFU/ml) of the viable population *E. coli* K12 (WT and mutants)
1132 followed by (a) CAP (4 min), (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP
1133 treatment (4 min) (d) CAP (4 min) following with 1% (w/v) GSE treatment in TSBYE. In all
1134 plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*, (■) Δ*dnaK*. Data are normalised with respect to untreated
1135 controls for all conditions under study. In cases where the viable cell count in the selective
1136 media was below detection limit (<10 CFU/ml) the reduction is portrayed as total inactivation
1137 (bar with stripes). Each bar represents the average of two independent experiments with three
1138 technical replicates per experiment while error bars represent standard deviation. Connecting
1139 lines with asterisks indicate significant differences between samples (* if $0.01 < p \leq 0.05$, ** if
1140 $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1141 ***Figure 7*:** Viable counts of *E. coli* K12 (a) WT, (b) Δ*rpoS* (c) Δ*oxyR*, (d) Δ*dnaK* in TSBYE for
1142 all treatments under study. In all plots, (■) control (untreated sample), (■) CAP treatment for 4
1143 min at flow rate of 1 L/min, (■) 1 % (w/v) GSE treatment for 2 h, (■) Treatment with 1 % (w/v)
1144 GSE (2 h) following with CAP treatment (4 min), (■) Treatment with CAP (4 min) following
1145 with 1% (w/v) GSE treatment (2 h). In cases where the viable cell count was below the
1146 detection limit (<10 CFU/ml) the number was set to 1 log CFU/ml. Each bar represents the
1147 average of two independent experiments with three technical replicates per experiments while
1148 error bars represent the standard deviation. Connecting lines with asterisks indicate significant
1149 differences between control and treated samples (* if $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, ***
1150 if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

1151 ***Figure 8*:** Sublethal injury (%) of *E. coli* K12 (WT and mutants) induced by (a) CAP (4 min),
1152 (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP treatment (4 min) (d) CAP (4 min)
1153 following with 1% (w/v) GSE treatment in TSBYE. Data are normalised with respect to
1154 untreated controls for all conditions under study. In all plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*,
1155 (■) Δ*dnaK*. In cases where the viable cell count in the selective media was below detection
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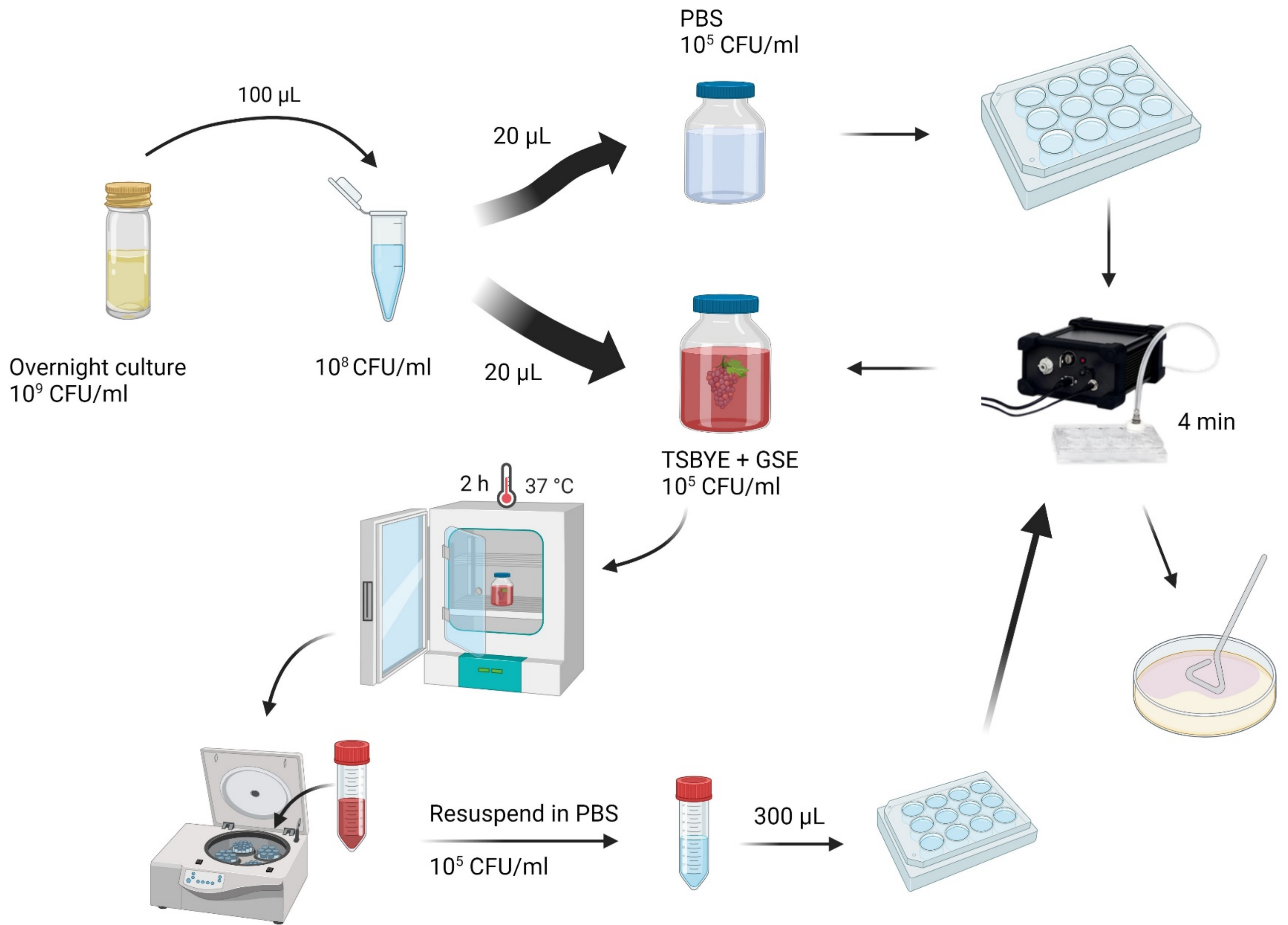
1161 ***Figure 9*:** Quantification of sub-lethally injured cells (%) of *E. coli* K12 (a) WT, (b) Δ*rpoS*, (c)
1162 Δ*oxyR*, (d) Δ*dnaK* in TSBYE for all treatments under study. In all plots, (■) control (untreated
1163 sample), (■) CAP treatment for 4 min at flow rate of 1 L/min, (■) 1% (w/v) GSE treatment for
1164 2h, (■) treatment with 1% (w/v) GSE (2h) following with CAP treatment (4 min), (■) treatment
1165 with CAP (4 min) following with 1% (w/v) GSE treatment (2h). In cases where the viable cell

count in the specific media was below detection limit (<10 CFU/ml) the number of sublethal damaged cells was set to 100% (indicated with stripes). Each bar represents the average of two independent experiments with three technical replicates per experiments while error bars represent the standard deviation. Connecting lines with asterisks indicate significant differences between samples (* if $0.01 < p \le 0.05$, ** if $0.001 < p \le 0.01$, *** if $0.0001 < p \le 0.001$, *** if $p \le 0.0001$)

1187 ## List of Figures

1188 ## Figure 1



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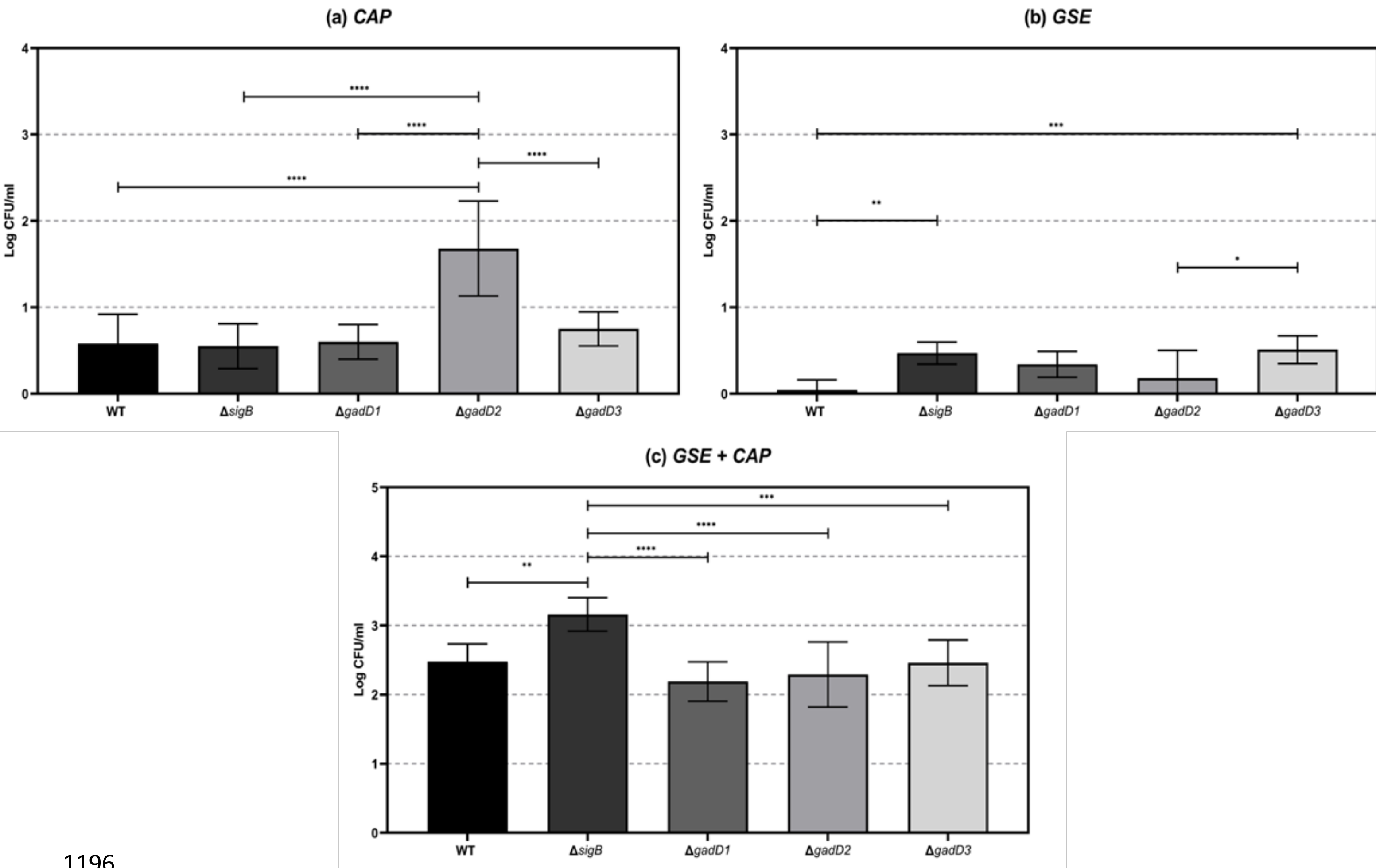

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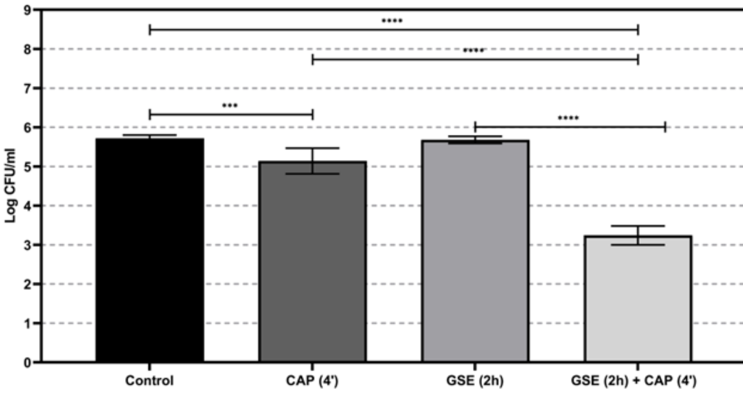

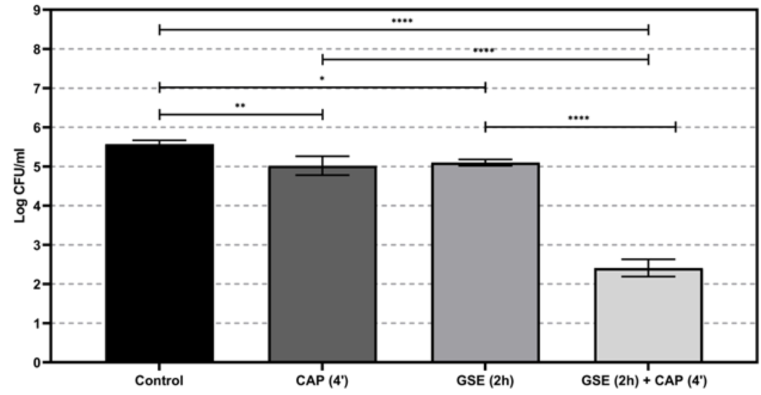

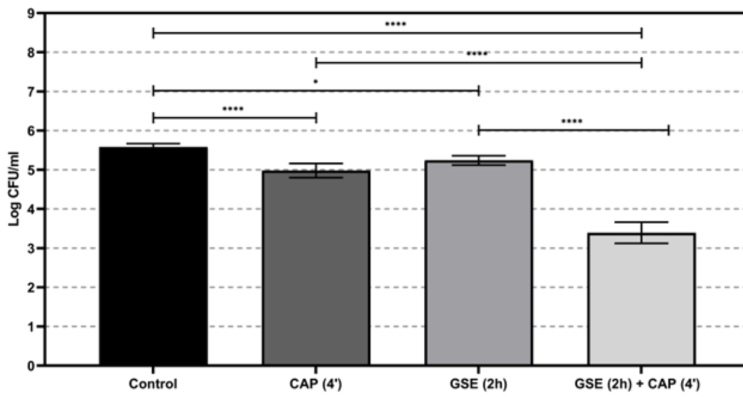

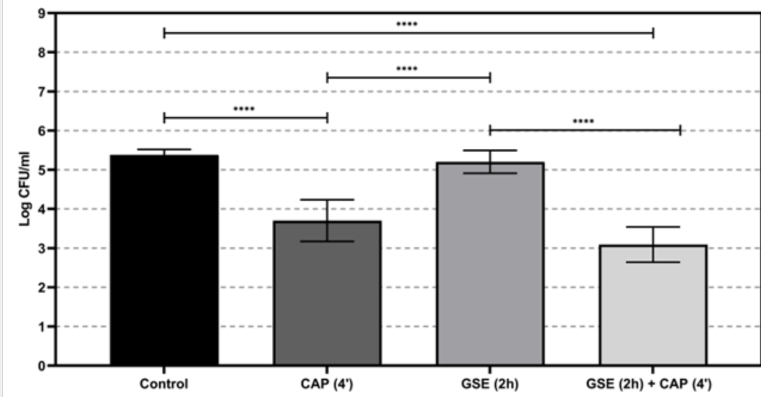

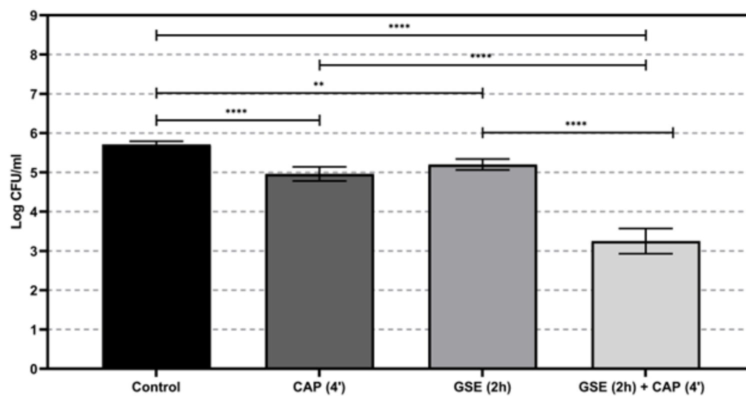

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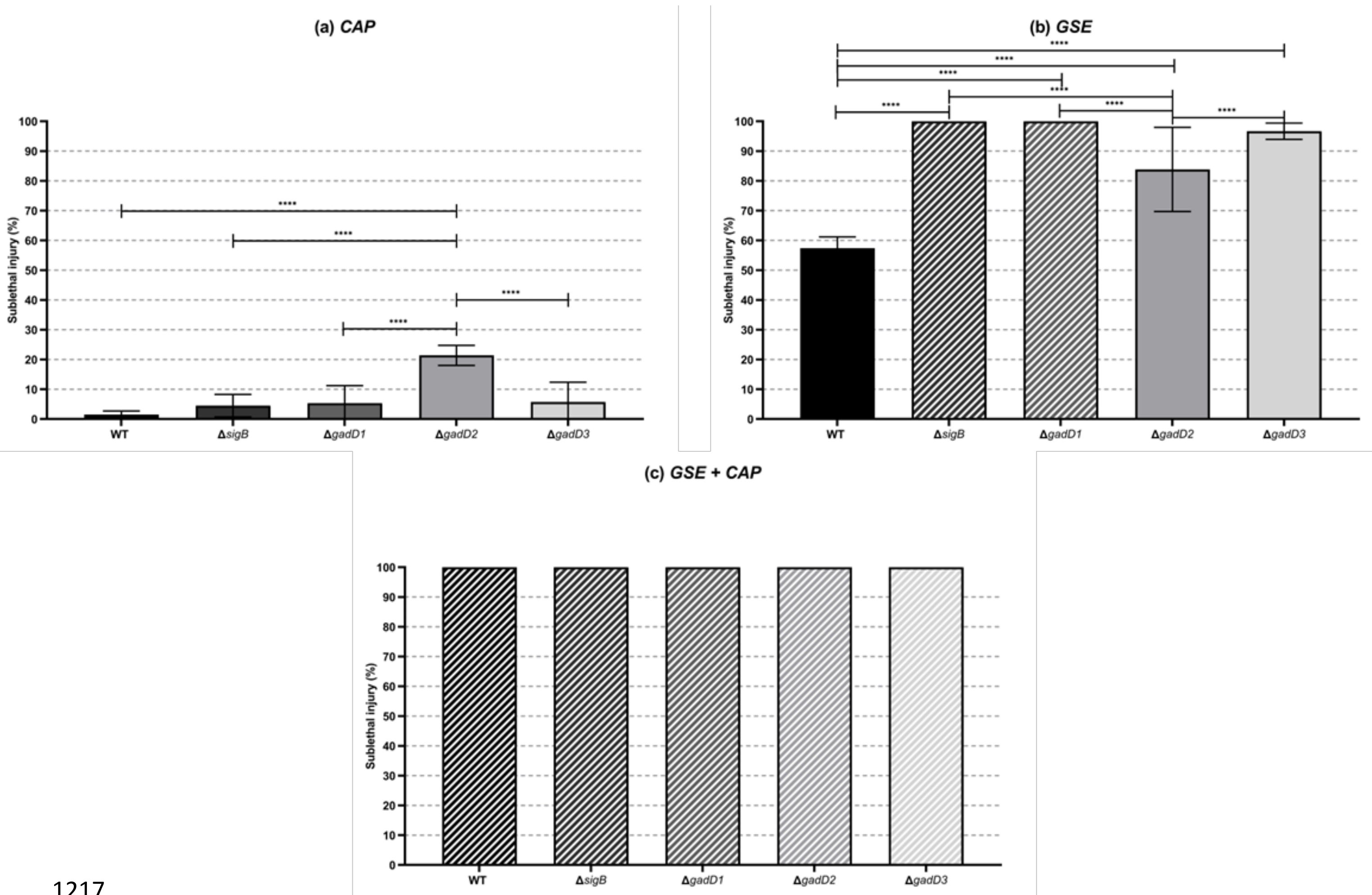
1216 **Figure 4**



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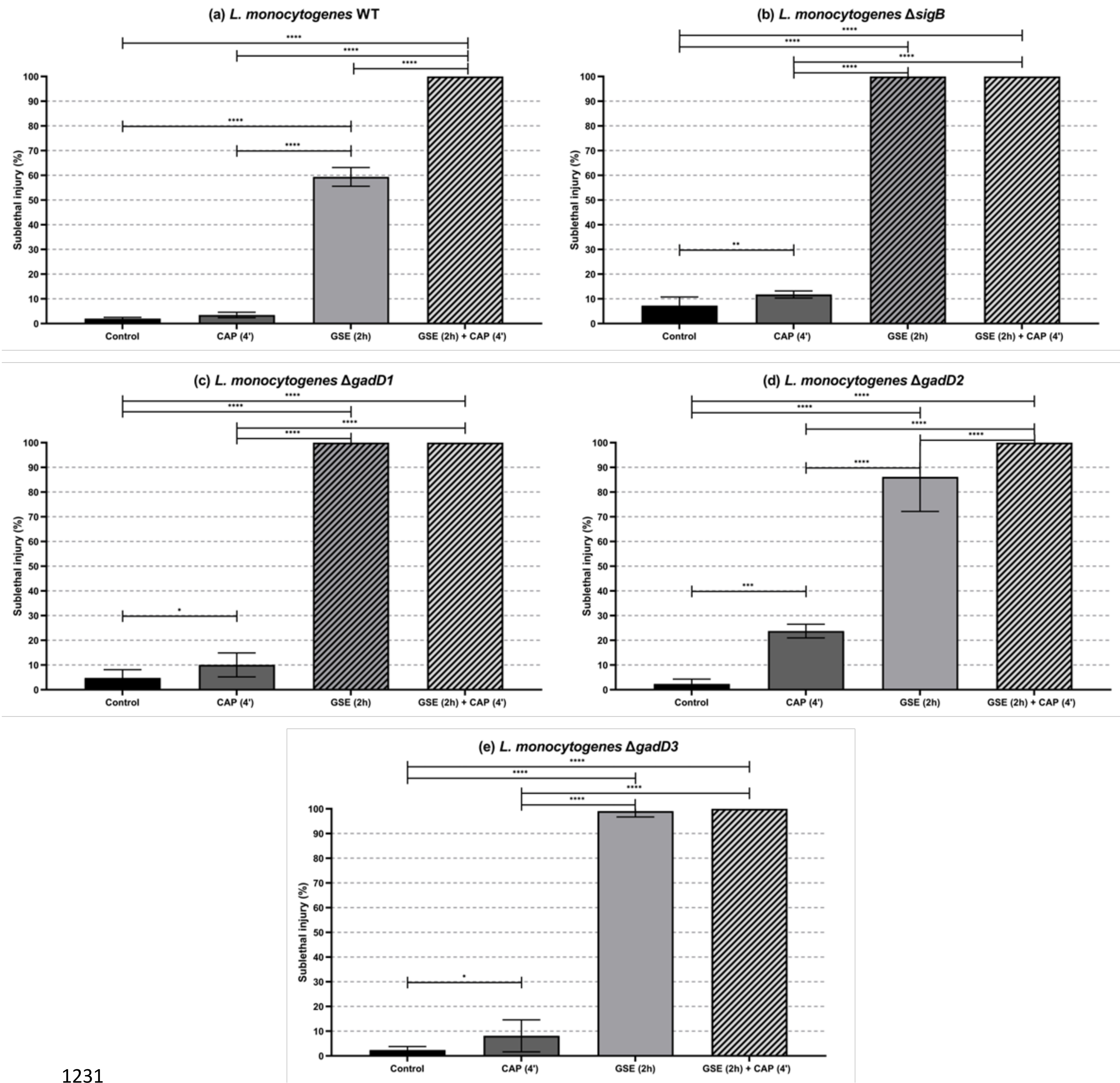
1230 **Figure 5**



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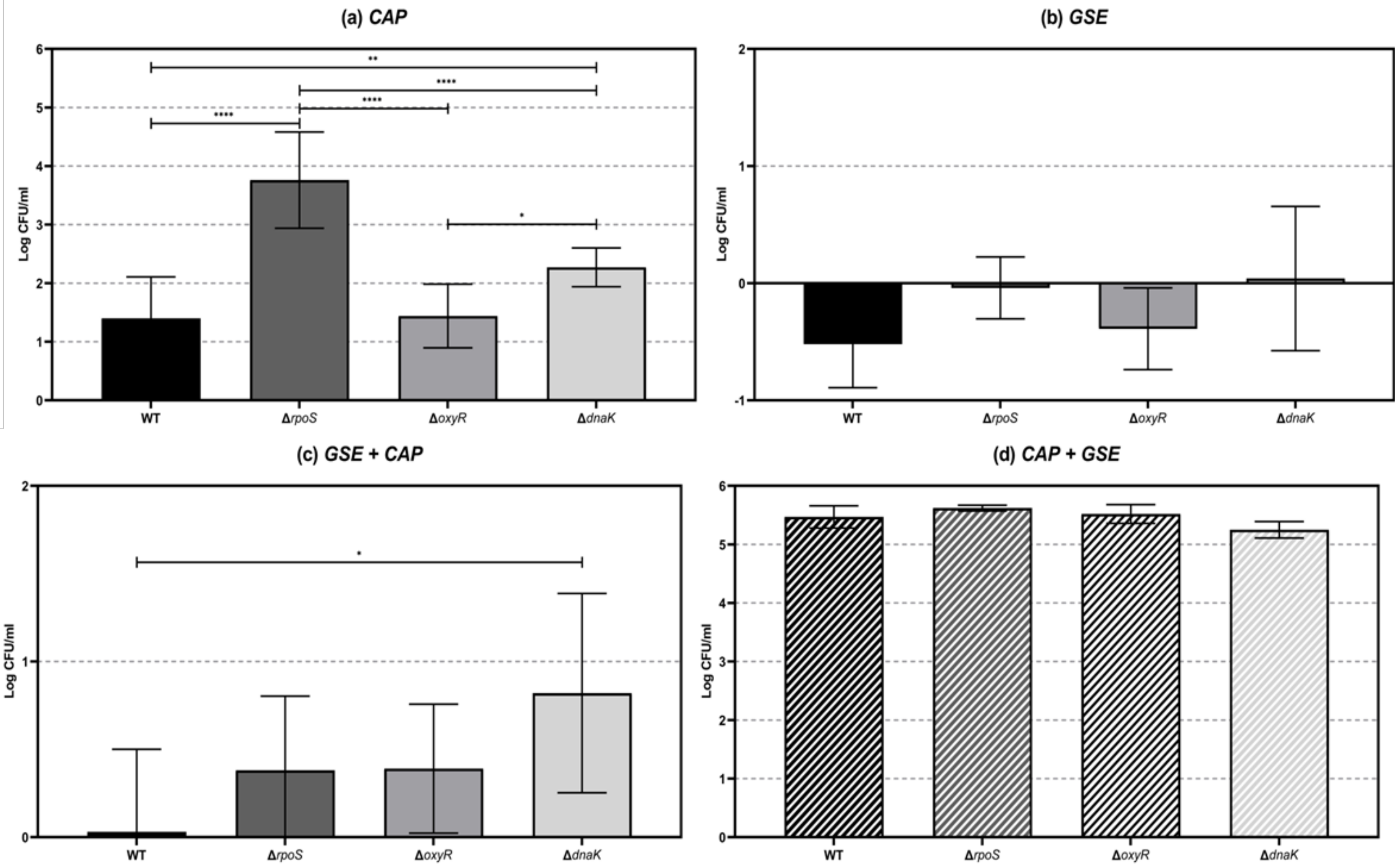

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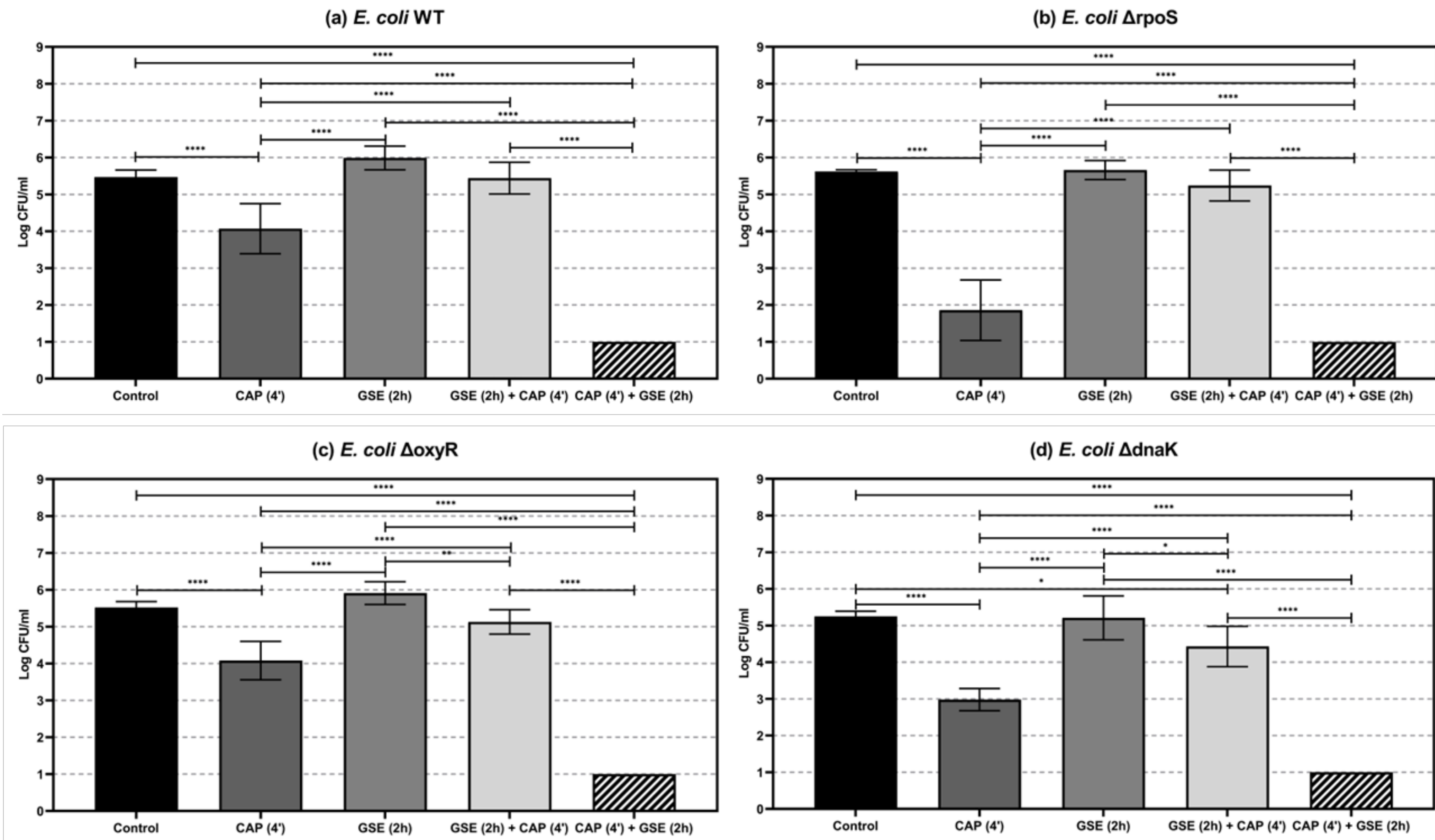
1248 **Figure 7**



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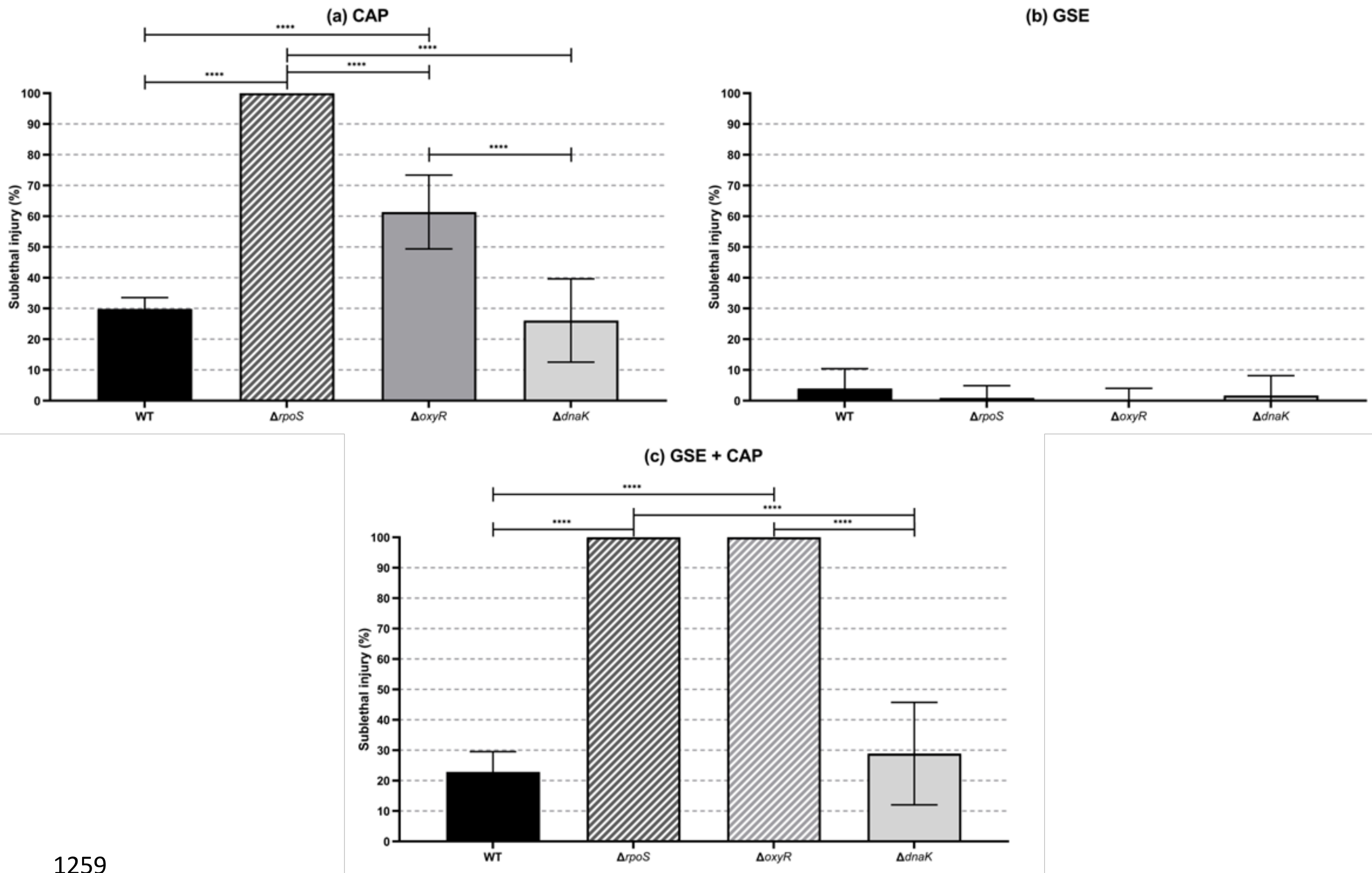
1258 **Figure 8**



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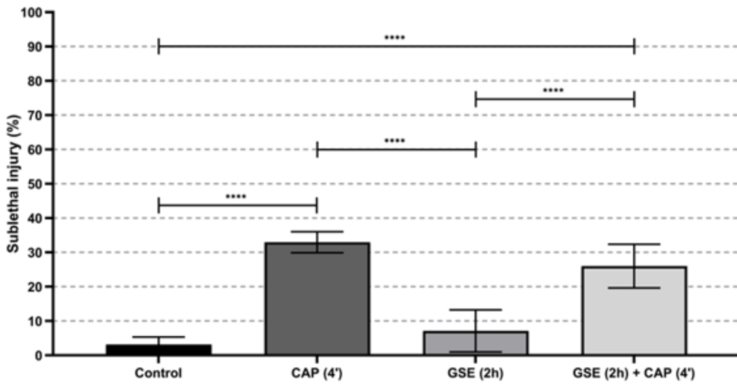
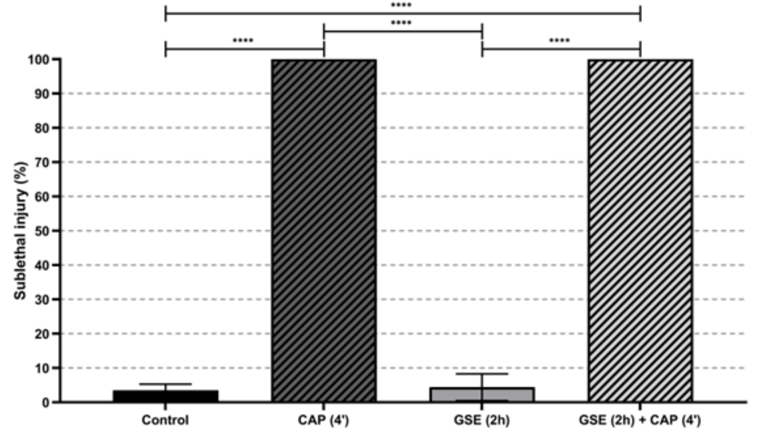
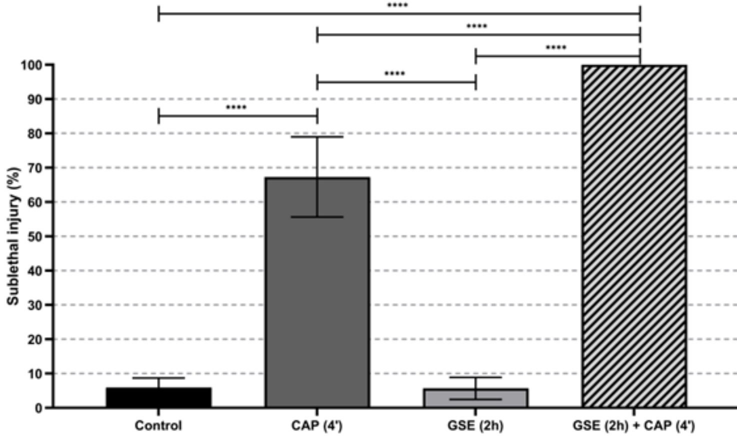
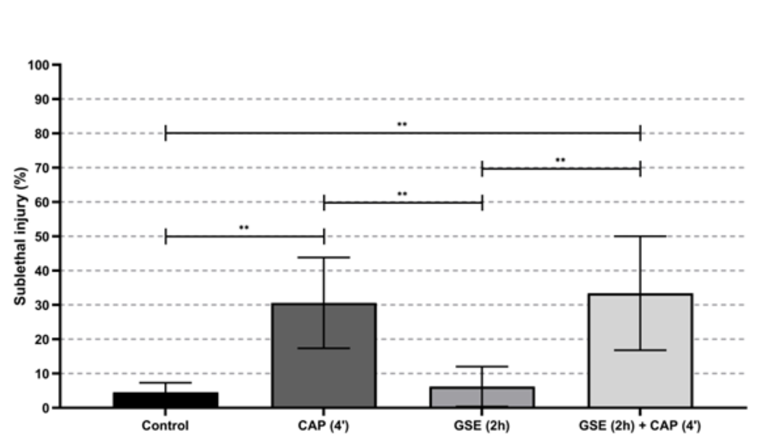
1273 **Figure 9**









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