Determination of the combined effect of grape seed extract and cold atmospheric plasma on foodborne pathogens and their environmental stress knockout mutants

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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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Abstract

The utilisation of hurdle approaches to ensure microbiological safety in the production of minimally processed food, while still adhering to sustainable practices, has great promise. Natural antimicrobials, such as grape seed extract (GSE), and novel non-thermal technologies, like cold atmospheric plasma (CAP), are appealing sustainable options to replace the traditional decontamination methods. The aim of this study was to explore the antimicrobial efficacy of GSE and CAP individually or in combination against *L. monocytogenes* and *E. coli* wild type (WT) and their isogenic mutants in environmental stress genes. More specifically, we examined the effects of 1 % (w/v) GSE, 4 min of CAP treatment, and their combined effect on *L. monocytogenes* 10403S WT and its isogenic mutants Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δ*gadD3,* as well as *E. coli* K12 and its isogenic mutants Δ*rpoS*, Δ*oxyR*, Δ*dnaK*. Additionally, the sequence of the combined treatments was tested. A synergistic effect was achieved for all strain of *L. monocytogenes* when exposure to GSE was followed by CAP treatment. However, the same effect was observed against the WT and mutant strains of *E. coli*, only when CAP treatment preceded exposure to GSE. Additionally, it was observed that *L. monocytogenes* Δ*sigB* was more sensitive to the individual GSE treatment and the combined GSE and CAP treatment, whereas Δ*gadD2* was more sensitive to CAP treatment*,* as compared to the rest of the mutants under study. Individual GSE exposure was unable to inhibit *E. coli* (both the WT and mutants), and individual CAP treatment resulted in higher inactivation of *E. coli* in comparison to *L. monocytogenes* with the strain Δ*rpoS* appearing the most sensitive among all studied strains. Our findings provide a step towards a better understanding of the mechanisms playing a role in tolerance/sensitivity of our model Gram-positive and Gram-negative bacteria towards GSE, CAP and their combination. Therefore, our results contribute to the development of more effective and targeted antimicrobial strategies for sustainable decontamination.

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50 1. Introduction

51   Consumers increasingly demand food products that are processed using minimal and

52 environmentally friendly methods (Dávila-Aviña et al., 2015; Pereira & Vicente, 2010). As a

53 result, researchers and the food industry constantly look for novel sustainable ways to ensure

54 microbiologically safe products via replacing chemical preservatives and antibiotics with

55 natural antimicrobials. Fruit and vegetable by-products are a valuable source of natural

56 antimicrobials that can also help to reduce food waste (Chandrasekaran, 2012; Costello et al.,

57 2018, 2019, 2021a, 2021b; Sabater et al., 2020; Sharma et al., 2021).

58   Grape by-products, comprise roughly 20 % of the overall weight of the grape and are a

59 substantial waste stream within the wine and juice industry (Oliveira et al., 2013; Özkan et al.,

60 2004). The disposal of these by-products, including the skins, seeds, and stems of the fruit, can

61 be challenging. Grape seed extract (GSE), a natural product derived from grape seeds, is a rich

62 source of antioxidant and antimicrobial compounds, such as polyphenols (Chedea & Pop, 2019;

63 Costa et al., 2022; Karnopp et al., 2017; Shrikhande, 2000). GSE is generally recognized as

64 safe (GRAS) for use in food, but it is not yet commonly utilized as an antimicrobial agent.

65   The inactivation of bacteria by GSE has been linked to multiple modes of action, including

66 the ability of polyphenols to permeate the bacterial cell walls and the potential of tannins to

67 inactivate extracellular enzymes. Furthermore, the GSE compound shows the ability to form

68 complexes with metal ions, leading to the removal of these ions from the bacterial environment

69 (Corrales et al., 2009; Silván et al., 2013). The presence of metal ions is essential for pathogenic

70 bacteria as it enables the preservation of protein structure and function, hence they are a critical-

limiting factor for their successful growth and survival. Consequently, the binding of these metal ions by GSE results in bacterial inhibition (Begg, 2019).

Previous studies using the agar diffusion method have provided evidence of significant antibacterial efficacy against Gram-positive bacteria including *Listeria monocytogenes, Bacillus cereus, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermidis and Mycobacterium smegmatis* (Baydar et al., 2006; Corrales et al., 2009; Silva et al., 2018). Additionally, Sivarooban et al. (2007) while studying the microbial dynamics of *L. monocytogenes* (initial load 5×10^6 CFU/ml) observed an inhibition of 2 log CFU/ml after 24 h of 1 % (w/v) GSE treatment in Tryptone Soy Broth supplemented with Yeast Extract (TSBYE) (Sivarooban et al., 2007). However, there are contradictory results in literature, on the GSE antimicrobial activity against Gram-negative bacteria. For example, Corrales et al. (2009) reported that in agar diffusion tests, 1 % (w/v) GSE was unable to inactivate *E. coli* and *S.* Typhimurium, whereas Baydar et al. (2006) observed inhibition of both bacteria using the same methodology (Baydar et al., 2006; Corrales et al., 2009). In previous work of our group the microbial dynamics of *L. monocytogenes* and its isogenic mutant Δ*sigB*, *E. coli* and *S.* Typhimurium treated with GSE in TSBYE were explored. We showed that GSE inactivated *L. monocytogenes* by 3 log CFU/ml at 1 % (w/v) GSE. Additionally, a mutant in *sigB,* a gene encoding the central stress gene regulator was more sensitive. On average, there was a 0.6 log CFU/ml difference in the surviving population between the WT and Δ*sigB*. *E. coli* and *S.* Typhimurium were more tolerant to GSE in comparison to *L. monocytogenes*. More specifically, for those Gram negative-bacteria a growth inhibition was observed (Kitsiou et al., 2023a). *To the best of our knowledge there are no other studies exploring the antimicrobial efficacy of GSE using functional genomics (use of isogenic mutants) in environmental stress genes of bacteria of importance in food safety.*

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CAP is a non-thermal emerging technology with multiple applications such as inactivation of microorganisms, wound healing, and cancer treatment (Bourke et al., 2017; Costello et al., 2021; El Kadri et al., 2021; Gilmore et al., 2018; Patange et al., 2019; Yan et al., 2020). After solid, liquid and gas, plasma has been described as the fourth state of matter. Plasma is achieved by ionizing a gaseous mixture composed of neutral molecules, electrons, positive and negative ions (Mandal et al., 2018; Niemira, 2012; Thirumdas et al., 2014). To create plasma, energy is applied to the gas to break the bonds between electrons and atoms resulting in the formation of charged particles. Most used ways to supply energy for plasma formation is electricity, heat, or by using lasers. The collision of gas particles in the plasma generates numerous highly reactive species such as high energy UV photons, charged particles including electrons and ions, oxygen reactive species (ROS), nitrogen reactive species (RNS) and hydrogen peroxide (Guo et al., 2015; Mandal et al., 2018; Niemira, 2012; Pankaj et al., 2018; Thirumdas et al., 2014).

The exact mode of action of the microbial inactivation of CAP is still elusive. Proposed mechanisms are the destruction of the cell wall, DNA damage, lipid peroxidation and protein dysfunction (Guo et al., 2015; Niemira, 2012; Pankaj & Keener, 2017). Moreover, CAP could possibly reduce the metabolic activity of the cell resulting in growth inhibition, possible loss of pathogenicity and prevention of biofilm formation by destroying the extracellular polymeric substances (Bourke et al., 2018; Gilmore et al., 2018).

The effectiveness of CAP depends on several parameters. The state of the substrate i.e., whether liquid or solid, the water activity and the structure of food are among the most important factors (Chizoba Ekezie et al., 2017; Guo et al., 2015). Liquids are presented in the literature as less favourable substrates for significant inactivation of microorganisms (>5 log CFU/ml), as the ionised gas needs to diffuse in the liquid to inactivate cells (Costello et al., 2021; Mandal et al., 2018; Smet et al., 2018; Surowsky et al., 2015). On the contrary, gases

120 can easily interact with the cells on the surface of solid foods. However, some solid food

121 products may have rougher surfaces, and this can be a hindering parameter for plasma treatment

122 because it provides a natural protection for the bacteria that can attach within the available

123 cavities (Surowsky et al., 2015). Additionally, some bacterial species and strains could be more

124 susceptible to the treatment depending on their morphology, cell membrane and ability to form

125 biofilms. For example, it has been observed that Gram-negative bacteria are more sensitive to

126 plasma treatment in comparison with Gram-positive bacteria, because of their thinner cell wall

127 (Mai-Prochnow et al., 2016). Other parameters that play a major role are the apparatus of CAP,

128 the intensity and time of treatment and the initial microbial population present in the sample

129 (Guo et al., 2015).

130 CAP treatment has shown some promising results for food decontamination. However, the

131 plethora of parameters, affecting the outcome of the CAP treatment, are adding an element of

132 variability when comparing results from different research groups. The magnitude of microbial

133 inactivation ranges from no inhibition to several logs of reduction of the bacterial

134 concentration, depending on the parameters of the treatment, the food properties such as the

135 matrix, the water activity, pH and the sensitivity of the bacterial strain (Bahrami et al., 2020;

136 Chizoba Ekezie et al., 2017; Choi et al., 2016; Niakousari et al., 2018; Sharma et al., 2014).

137 For example, the microbial inactivation in meat products reported in literature ranges from

138 0.34-6.52 log CFU/g by changing one or more of the above parameters (Misra & Jo, 2017).

139 More specifically, Choi et al. (2016) explored the inactivation of *L. monocytogenes* and *E. coli*

140 by CAP (corona discharge, atmospheric air) on pork with initial microbial concentration $10^{7.5}$

141 CFU/g. After a 2 min treatment, it was observed that *E. coli* was reduced by 1.5 log CFU/g,

142 while *L. monocytogenes* was inactivated by 1 log CFU/g. Additionally, Lee et al. (2011) noted

143 a 4.73 log CFU/g reduction in *L. monocytogenes* by using different parameters of CAP

144 treatment (dielectric barrier discharge, oxygen & nitrogen) applied in cooked chicken breast

6

145   (Lee et al., 2011). In another study, Smet et al. (2018) demonstrated higher CAP inactivation

146   of cells grown planktonically as compared to cells grown on structured 3D models i.e., gelatin

147   at 5% (w/v). Therefore, drawing general conclusions on the level of microbial inactivation is

148   challenging. More work should be conducted, evaluating CAP individually or in combination

149   with other technologies or natural antimicrobials i.e., in order to establish a hurdle technology

150   and microorganism-specific approaches. In this way CAP could be approved as safe and

151   utilised on a larger scale in production (Guo et al., 2015; Pankaj & Keener, 2017; Tewari &

152   Juneja, 2007).

153   The principle of the hurdle technology approach on microbial safety, is the utilization of

154   two or more methods/approaches/processes to enable microbial inactivation and consequently

155   to ensure food safety (Bigi et al., 2023; Costello et al., 2021; Khan et al., 2017; Leistner, 2000;

156   Liao et al., 2020; Velliou et al., 2011a, 2011b, 2012). Due to their mode of action,

157   mild/alternative technologies can cause less damage and death to bacteria as compared to

158   classic treatments, e.g., heat pasteurisation (Bahrami et al., 2020; Sunil et al., 2018; Tewari &

159   Juneja, 2007). This can pose a challenge when employing these approaches, as they might not

160   fully guarantee products that are microbiologically safe. However, the combination of these

161   methods or technologies could potentially exert synergistic or additive effects against bacteria,

162   thereby achieving a substantial microbial inactivation (>5 log CFU/ml), ensuring product

163   safety (Millan-Sango et al., 2015; Mosqueda-Melgar et al., 2008). For example, one

164   technology/treatment may be used to damage or increase the permeability of the bacterial cell

165   wall while another could be used to interfere with the intracellular components. The trigger to

166   develop such hurdle approaches has been to protect heat sensitive food products against

167   bacterial growth with minimal processing aiming to maintain their quality (Costello al., 2021a;

168   Khan et al., 2017; Leistner, 2000; Peleg, 2020). Therefore, developing hurdle approaches is

169 more pressing than ever, to meet the rising demand for minimally processed foods and

170 sustainable production.

171 As previously mentioned, novel non-thermal technologies (NTTs) can be sometimes

172 ineffective depending on external parameters such as the nature of the food, the CAP

173 parameters and the type of microorganism (Costello et al., 2018, 2019, 2021; Dobrynin et al.,

174 2011; Laroussi et al., 2011; Pereira & Vicente, 2010; Velliou et al., 2013). Furthermore,

175 specifically natural antimicrobials derived from plants such as essential oils cannot be used in

176 very high concentrations, as they might affect the organoleptic characteristics of the food

177 product (Gutierrez et al., 2008; Mariod, 2016; Pateiro et al., 2021; Zhao et al., 2020).

178 *Hence, combining NTTs with plant-derived antimicrobials as a hurdle approach could be a*

179 *novel solution to increase the treatment efficacy and achieve food safety.* However, to date,

180 there is a very limited number of studies on such combined treatments and their mechanism of

181 inactivation. In most cases, the current hurdle approaches involve the combination of

182 established methods, such as heat treatment with chemical preservatives, or two NTTs

183 combined together, or with heat treatment (Bermúdez-Aguirre et al., 2012). Additionally, the

184 majority of studies combining NTTs with natural antimicrobials focus on the combination of

185 NTTs with essential oils, rather than other natural antimicrobials derived by plants (Cui et al.,

186 2016; Espina et al., 2014; Matan et al., 2014, 2015). For example, Matan et al. in 2014, studied

187 the synergistic effect of radio frequency plasma with essential oils from clove, sweet basil and

188 lime in concentrations of 0.5 to 2 % v/v. The most effective treatment was that combining

189 plasma with clove oil (1 % v/v), which lead to a total microbial (*E. coli*, *S.* Typhimurium, *S.*

190 *aureus*) inhibition on eggshells (Matan et al., 2014). Similarly, Cui et al. (2016b) studied the

191 effect of the combination of cold nitrogen plasma (400 W) and thyme oil (0.05 % w/v) against

192 *S.* Typhimurium and *S.* Enteritidis on eggshells achieving a total microbial inactivation

193 (bacterial counts below detection limit), that lasted for 14 days at 3 different temperatures (4,

194 12, 25 °C) (Cui et al., 2016). The same year, Cui et al. (2016a) also showed that cold nitrogen

195 plasma combined with *Helichrysum italicum* essential oil can inhibit *S. aureus* on food

196 packaging. The microbial concentration decreased more than 5 log CFU/cm^2, in contrast with

197 individual treatments that caused only 2 log CFU/cm^2 reduction of the microbial concentration

198 (Cui et al., 2016).

199 To date, as previously described, GSE have not been extensively studied in terms of their

200 antimicrobial properties nor in combination with other NTTs as a hurdle approach. The only

201 reported combined approach of GSE as a microbial inactivation treatment was with nisin, a

202 natural antimicrobial peptide produced by certain strains of *Lactococcus lactis* (Costello et al.,

203 2018, 2019; Liu & Hansen, 1990; Sivarooban et al., 2008; Thanjavur et al., 2022; Zhao et al.,

204 2020). More specifically, Zhao et al. (2020) reported that the simultaneous treatment of nisin

205 (2000 IU/ml) and GSE (1 % w/v) in a liquid broth, was able to reduce the concentration of the

206 bacterial pathogen *L. monocytogenes* by 5 logs after 10 min of treatment, whereas their separate

207 use could not inhibit *L. monocytogenes* more than 2 log CFU/g (Zhao et al., 2020). A similar

208 synergistic effect of the above combination was reported by another study where *L.*

209 *monocytogenes* was completely inhibited after 12 h in the presence of 6400 IU/ml nisin and

210 1% w/v GSE (Sivarooban et al., 2007). The proposed synergistic mechanism of microbial

211 inactivation was common in these studies. Nisin acted on the cell wall surface by forming large

212 pores which allowed the GSE to diffuse in the cytoplasm causing further cell damage

213 (Sivarooban et al., 2008; Zhao et al., 2020).

214 *From the above studies, it can be concluded that, using hurdle approaches to deliver safe*

215 *food products is a very promising strategy which has not been thoroughly studied nor*

216 *understood, especially for fruit by-products, i.e., such as GSE, and other NTTs like CAP.*

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

2. Materials and methods

2.1. Inoculum preparation

Stock cultures of *L. monocytogenes* 10403S WT*,* Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δg*adD3*, and *E. coli* K12 WT, Δ*rpoS*, Δ*dnaK*, Δ*oxyR* were stored in Tryptone Soy Broth (TSB, Oxoid Ltd, UK) supplemented with 15% glycerol at -80 °C. **Table 1** provides an overview of the strains and mutants utilised in this study, highlighting their relevance in this study. The inoculum preparation took place as previously described (Costello et al., 2018, 2019, 2021a, 2021b; Kitsiou et al., 2023a, 2023b; Velliou et al., 2010, 2011a, 2011b, 2012, 2013). More specifically, a loopful of thawed culture was inoculated in 20 ml TSB supplemented with 0.6% w/v of Yeast Extract (Oxoid Ltd, UK) (TSBYE) and cultured for 9.5 h in a shaking incubator at 37 °C and 175 rpm. Thereafter, 20 μl were transferred in 20 ml TSBYE and cultured for another 15 h until early stationary phase was reached (approximately 10^9 CFU/ml).

242 *2.2. Grape seed extracts (GSE)*

243   This study utilised commercially available grape seed extract (GSE) from Bulk, UK. The

244 GSE powder contained a minimum concentration of 95% oligomeric proanthocyanidin.

245 Consequently, the powder is predominantly comprised oligomeric proanthocyanidins. To

246 prepare the GSE solution, the powder was dissolved in Tryptic Soy Broth with 0.6% Yeast

247 Extract (TSBYE) at a concentration of 1% w/v and subsequently autoclaved. The autoclaved

248 TSBYE+GSE was stirred overnight to ensure thorough homogenization. The chosen GSE

249 concentration was selected based on results from our previous study in TSBYE broth. The

250 finding of our study showed that 1% w/v concentration of GSE significantly inactivated *L.*

251 *monocytogenes* WT and its isogenic $\Delta sigB$ mutant in TSBYE, resulting in a 3 log CFU/ml

252 reduction after 24 h (Kitsiou et al., 2023a).

253

254 *2.3. CAP experimental set-up*

255   The CAP apparatus utilised in this investigation was developed and supplied by Fourth State

256 Medicine Ltd. The configuration of the device has been previously described in previous work

257 of the group (El Kadri et al., 2021; Kitsiou et al., 2023b). Briefly, the generator of CAP in this

258 apparatus was a dielectric barrier discharge in a remote and enclosed configuration, whereby

259 the plasma source was contained in an electrically-shielded enclosure and separated from the

260 treatment target by a tube, with no direct line of sight. The gas used for ionization was

261 compressed air (25 °C, 3 bars), and its flow rate (0-5 L/min) was controlled by a needle valve

262 and a flow meter mounted on the enclosure. The chemical composition of the plasma output

263 varies based on the input air flow rate For example, at flow rate 1 L/min more reactive nitrogen

264 species (RNS– primarily NO_x compounds, NO_2 and NO) are produced in comparison with

265 higher flow rates at which the air flow is enriched with more reactive oxygen species (ROS–

266 primarily O_3). At the used flow rate of the experiment (1 L/min) the concentration of ROS was

267   approximately 320 ppm (Kitsiou et al., 2023b). Additionally, data collected by Fourth State

268   Medicine Ltd showed that the concentration of NOx was approx. 100-200 ppm and NO_z,

269   compounds (mixture of N_2O, HONO, and other compounds, alongside $O_{3)}$ was approximately

270   200-300 ppm (Fourth State Medicine Ltd, 2023).

271

272   *2.4. Combined treatment: CAP and GSE*

273   To assess the combined treatment of GSE and CAP in liquid, *L. monocytogenes* WT and its

274   isogenic mutants *(ΔsigB, ΔgadD1, ΔgadD2, ΔgadD3)* and *E. coli* and its isogenic mutants

275   *(ΔoxyR, ΔdnaK, ΔrpoS)* were inoculated in TSBYE with 1% (w/v) GSE (Figure 1). The initial

276   microbial population was 10^5 CFU/ml. Prior to CAP treatment, the samples were treated with

277   GSE at 37 °C for 2 h. The treatment time in the presence of GSE was selected based on our

278   previously published results in liquid nutrient medium (TSBYE) (Kitsiou et al., 2023a). These

279   prior finding showed that following a 2 h treatment the cell population *L. monocytogenes* WT

280   exhibited no significant reduction and its knockout mutant (Δ*sigB*) was decreased by 0.3 log

281   CFU/ml (Kitsiou et al., 2023a). Therefore, the aim of the GSE treatment, for this study, was to

282   cause a slight reduction in microbial population and create a state of stress by subjecting the

283   cells to GSE. Thereafter, the sample was centrifuged at 5000 rpm for 10 min (Megafuge 16R,

284   ThermoFisher, USA), the supernatant was discarded, and the pellet was resuspended in 20 ml

285   PBS. To enumerate the viable population of the 2 h GSE treatment, the spread-plate method

286   was followed using TSAYE non-selective media. Sub-lethally injured cells exhibit an inability

287   to grow on selective media, while they are capable of normal growth on nonselective media

288   (Shao et al., 2023). Therefore, to identify the number of cells that were sub-lethally damaged,

289   the samples were also plated into selective media i.e., Polymyxin Acriflavin Lithium-chloride

290   Ceftazidime Esculin Mannitol (PALCAM) agar for *L. monocytogenes* or Violet Red Bile

291 Glucose (VRBG) agar (Oxoid Ltd, UK) for *E. coli*. The number of injured cells was calculated

292 based on the following equation (Busch & Donnelly, 1992) :

293

294 $$\% \, Injured \, cells = [1 - \frac{Count \, on \, selective \, agar}{Count \, on \, non-selective \, agar}(\frac{CFU}{ml})] \times 100 \qquad (1)$$

295

296   For the CAP treatment experiments, 300 μL of PBS containing the pre-treated with GSE

297 and/or untreated cells were transferred in 12-well plate. The samples were exposed to CAP at

298 1 L/min flow rate for 4 min. The flow rate of the CAP treatment was determined through initial

299 experiments (results not shown), which demonstrated that lower flow rates in the liquid carrier,

300 enriched with RNS, resulted in more effective inactivation. Additionally, the duration of the

301 CAP treatment was selected in order to induce a slight decrease in the microbial population,

302 therefore allowing the investigation of the potential synergistic effects of the GSE and CAP

303 treatment. The survival of the microbial population and sublethal injury after the treatment was

304 assessed using the spread plate technique as described above. Additionally, for *E. coli*, the

305 treatment sequence was reversed. Initially, the cells were subjected to CAP treatment, and

306 subsequently, they were exposed to GSE (Figure 1). The parameters used for both treatments

307 remained unchanged. This approach was implemented specifically for *E. coli* to explore the

308 potential synergistic or altered effects resulting from the reversed treatment sequence. The

309 decision resulted from the decreased antimicrobial efficacy, in comparison with the efficacy

310 against *L. monocytogenes,* observed when *E. coli* was treated with GSE followed by CAP (see

311 results section).

312

313

314   *2.5. Statistical analysis*

315   At least two independent biological experiments with three replicate samples were

316   conducted for all conditions under study. When comparing two mean values, a t-test was used

317   to confirm statistical significance ($p < 0.05$) while for multiple comparisons, a two-way

318   ANOVA followed by Tukey's HSD post hoc was used to confirm statistically significant ($p <$

319   0.05) differences between independent experimental groups. In the plots below, the mean value

320   is presented with error bars representing the standard deviation. In cases where the viable cell

321   count was below the detection limit (<10 CFU/ml) in the general and selective media the

322   number of viable and sub-lethally damaged cells was set to 1 log CFU/ml and/or 100%,

323   respectively. All statistical analysis was performed using GraphPad Prim and Microsoft Excel.

324

325   **3. Results**

326   As previously mentioned, to investigate the combined effect of grape seed extract (GSE)

327   and cold atmospheric plasma on *L. monocytogenes, E. coli* and their isogenic mutants

328   (mentioned in section 2.1) in TSBYE, the pathogens were firstly treated with 1 % w/v GSE for

329   2 h. Thereafter, the cells were treated with CAP for 4 min at 1 L/min flow rate. Finally, the

330   viable and sublethal populations of the individual and combined treatments were measured, to

331   enable a meaningful comparison between the wild types and their isogenic mutants.

332   Furthermore, examining both the individual treatments of GSE and CAP and their combination,

333   allowed for precise evaluation of each treatment's impact on the isogenic mutant strains as well

334   as the evaluation of their combined effect.

335   *To the authors' best knowledge this is the first study investigating the impact of the combined*

336   *antimicrobial effect of GSE with a novel non-thermal technology such as CAP in a liquid*

337   *carrier on L. monocytogenes and E. coli and their isogenic mutants in environmental stress*

338 *genes. Therefore, this study provides valuable insights into the microbial mechanisms of stress*

339 *response to this combined treatment.*

340

341 **3.1 The effect of GSE and CAP against L. monocytogenes WT and its isogenic mutants.**

342   Figures 2 and 3 show the level of microbial inactivation caused by individual and combined

343 GSE and CAP treatments, for all tested strains of *L. monocytogenes* (WT, Δ*sigB*, Δ*gadD1*,

344 Δ*gadD2*, Δ*gadD3*). Figure 2 presents the data arranged by treatment while in Figure 3 the

345 results are organised by strain of *L. monocytogenes*. Overall, the combined treatment of 1 %

346 (w/v) GSE for 2 h followed by 4 min of CAP treatment at flow rate 1 L/min had a good

347 synergistic effect against all strains of *L. monocytogenes*. After the individual GSE treatment,

348 i.e., a 2 h exposure to 1 % (w/v) GSE, there was no significant decrease in the population of *L.*

349 *monocytogenes* WT (Figure 3a). For most mutant strains of *L. monocytogenes,* the cell

350 concentration was reduced by an average of 0.4 CFU/ml following a 2 h exposure to GSE ($p >$

351 0.05) (Figure 2 & 3b-e). Additionally, the sublethal injury assessment showed that the GSE

352 treatment led to a greater percentage of sub-lethally injured cells among the mutant strains

353 (Figure 4b & 5). The cells of Δ*sigB* and Δ*gadD1* were the most sensitive, as all the microbial

354 population was sub-lethally injured (100 %) after 2 h in the presence of GSE. The WT strain

355 exhibited the lowest percentage of sub-lethal injury following the individual GSE treatment,

356 which was approximately 60 % (Figure 4 & 5). The high yield of sub-lethally injured cells for

357 all *L. monocytogenes* strains emphasises the great potential of GSE as a sustainable solution

358 for decontamination.

359   After the individual CAP treatment (4 min, 1 L/min), all strains of *L. monocytogenes* except

360 the mutant strain Δ*gadD2* were inhibited by an average of 0.5 log CFU/ml. The inactivation of

361 *L. monocytogenes* Δ*gadD2* mutant was higher as compared to all other mutant strains, with an

362 approximate reduction of 1.7 log CFU/ml ($p < 0.05$) (Figure 2a & 3c). Additionally, when

363 measuring the sublethal population it was observed that all mutants had higher percentage of

364 sub-lethally injured cells, in comparison to the WT, with the highest percentage of 24%

365 belonging to $\Delta gadD2$ (Figure 4a & 5). These results indicate that the *gadD2* might have a

366 significant role in the tolerance of *L. monocytogenes* to CAP treatment.

367   As previously mentioned, the combined treatment of GSE (1 % w/v, 2 h) and CAP (1 L/min,

368 4 min) had a great synergistic effect against all strains of *L. monocytogenes.* As can be seen in

369 Figures 2 and 3, the viable population of *L. monocytogenes* WT after the combined treatment

370 was equal to 3.2 log CFU/ml i.e., the combined treatment led to a 2.5 log CFU/ml reduction

371 when compared to untreated controls (Figure 2c & 3a). Additionally, the microbial inactivation

372 of most mutant strains ($\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$) was comparable to the WT strain with an

373 average reduction of 2.3 log CFU/ml. *L. monocytogenes* $\Delta sigB$ was the only mutant strain for

374 which a higher level of microbial inactivation (3.2 log CFU/ml) was observed in comparison

375 to all other *L. monocytogenes* strains. ($p < 0.05$) (Figure 2c). However, when assessing the

376 extend of sub-lethal injury (Figure 4c & 5), it was noted that cells of all strains could not grow

377 on selective medium (PALCAM) i.e., most cells of all strains were in the state of sub-lethal

378 injury indicating the great antimicrobial efficacy of this hurdle approach.

379

380 *3.2. Combined treatment of GSE and CAP against E. coli WT and its isogenic mutants.*

381   For the inactivation of *E. coli,* the same treatments as *L. monocytogenes* were performed. In

382 addition, the combined treatment in reverse sequence, i.e., CAP treatment followed by GSE,

383 was examined due to the observed inefficient microbial inactivation of *E. coli* by the initial

384 sequence of the combined treatment.

385   The individual GSE treatment was unable to reduce the population of *E. coli* WT, Δ*oxyR*,

386 Δ*rpoS*, Δ*dnaK* (Figure 6b & 7). The inability of GSE to inactivate all strains of *E. coli* was also

387 observed in the sub-lethally damaged microbial population, were the percentage of sub-lethally

388 damaged cells after the GSE treatment was similar to that of the control ($p > 0.05$) (Figure 8b

389 & 9).

390   The individual CAP treatment was more effective against *E. coli* in comparison to *L.*

391 *monocytogenes* (Figure 2 and 6)*.* As can be seen in Figures 6 and 7, the microbial inactivation

392 of *E. coli* WT and Δ*oxyR* were similar and on average 1.4 log CFU/ml ($p > 0.05$). When

393 subjected to CAP treatment, the mutant strain *E. coli* Δ*dnaK* showed increased inactivation in

394 comparison to *E. coli* WT and Δ*oxyR* resulting in a reduction of 2.3 log CFU/ml (Figure 6a &

395 7c). However, the count of sub-lethally injured cells of *E. coli* Δ*oxyR* showed a higher

396 percentage of sub-lethally damaged cells (67 %) as compared to *E. coli* WT (average of 31.7

397 %) (Figure 8). Overall, our results show that both mutant strains *E. coli* Δ*oxyR* and Δ*dnaK,* are

398 more sensitive to CAP treatment than the WT. The most significant reduction in microbial

399 concentration following CAP treatment was observed in *E. coli* Δ*rpoS* with a population

400 decrease of 3.8 log CFU/ml (Figure 6a & 7b) with the surviving population being 100% sub-

401 lethally injured (Figure 8a & 9b).

402   For the combined treatment of GSE and CAP, when treating the cells with GSE followed

403 by CAP, no synergistic or additive effects were observed against any of the strains of *E. coli*

404 under study, as shown in Figure 6c & 7. The results indicated that there was an increase in the

405 tolerance to CAP treatment after a 2 h exposure to 1% (w/v) GSE, as seen by the viable counts

406 (Figure 7). However, it was noted that nearly all cells of the *E. coli* Δ*oxyR* and Δ*rpoS* strains

407 were in a sub-lethal injury physiological state, indicating that the combined treatment affected

408 those mutants inducing injury, but did not affect the overall cell viability (Figure 8c, 9b, 9c).

409 Moreover, while *E. coli* Δ*dnaK* showed increased tolerance to CAP treatment after GSE

410   treatment, it did not demonstrate an equivalent level of tolerance as compared to the WT strain

411   (Figure 6c, 7a, 7d). As previously mentioned, due to the inability of the combined treatment of

412   CAP and GSE to inactivate *E. coli*, the reversed combined treatment was investigated.

413   Interestingly, in a combined CAP/GSE treatment where the samples were first treated with

414   CAP followed by a 2 h exposure to GSE, all strains of *E. coli* were completely inactivated (~5

415   log CFU/ml reduction as compared to the controls; Figure 6d). This suggests that the sequence

416   of the CAP/GSE treatments can have a detrimental effect on the microbial inactivation *E. coli*.

417   As the combined treatment of GSE and CAP achieved total inactivation of all strains *E. coli*,

418   there was no scope to enumerate the sub-lethally damaged population.

419

4. Discussion

421   In this study the antimicrobial effect of grape seed extract (GSE, 1 % w/v, 2 h), cold

422   atmospheric plasma (CAP, 1 L/min, 4 min) and their combination against *L. monocytogenes,*

423   *E. coli* and their isogenic mutants in environmental stress genes was systematically explored.

424   To the best of our knowledge this is the first study combining natural antimicrobials with non-

425   thermal technologies like CAP in a controlled liquid system against *L. monocytogenes*, *E. coli*

426   along with functional genomics work (usage of targeted knockout mutants) to identify

427   mechanisms of resistance and modes of action. Overall, our results show that most mutant

428   strains were more susceptible to the individual and combined treatments than the wild type

429   (WT) strains. Additionally, the sequence of the combined treatment played a significant role

430   on the efficacy of the combined treatment against *E. coli.*

431   *4.1 Individual GSE and CAP treatments*

432   For the individual GSE treatment against *L. monocytogenes* and its isogenic mutants, after

433   2 h of exposure to GSE, the only mutant strain having significant difference in the microbial

434 inactivation, in comparison to the WT strain, was *L. monocytogenes* Δ*sigB* (p > 0.05) (Figure

435 2b). However, all mutant strains of *L. monocytogenes* (Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δ*gadD3*)

436 demonstrated higher percentage of sublethal injury as compared to the WT (Figure 4b & 5),

437 indicating that GSE causes significant damage to those mutants, and it is a promising agent for

438 the design of antimicrobial strategies. Additionally, *L. monocytogenes* Δ*sigB* showed a slightly

439 increased sensitivity to CAP treatment when compared to the WT, as demonstrated in the

440 evaluation of sublethal injury (Figure 2a). The higher antimicrobial effect of GSE against Δ*sigB*

441 can be explained by the fact that SigB (σ^B) regulates the general stress response of Gram-

442 positive bacteria like *L. monocytogenes* (Abee, 1999; Guerreiro et al., 2020). More specifically,

443 the gene regulator SigB plays a crucial role in controlling the expression of more than 100

444 genes involved in various stress responses (see also Table 1) therefore plays a major role in the

445 resistance of *L. monocytogenes* to various treatments (Abee, 1999; Y. Liu et al., 2019;

446 NicAogáin & O'Byrne, 2016; O'Byrne & Karatzas, 2008). Results showing the effect of SigB

447 in a treatment are important as they suggest that at least one of the genes controlled by SigB

448 plays a role in the resistance to this stress. This narrows our investigation regarding the specific

449 mechanisms that contribute to the resistance under a certain stress. SigB has been reported to

450 exhibit increased expression in *L. monocytogenes* as a response to stress, significantly

451 contributing to the adaptability of the bacterium to various types of stress including heat, acid,

452 and osmotic stress. (Boura et al., 2016; Cheng et al., 2015; O'Byrne & Karatzas, 2008;

453 Raengpradub et al., 2008). However, for oxidative stress, the results existing in the literature

454 are contradicting (Boura et al., 2016; Patange, O'Byrne, et al., 2019). For example, Patange et

455 al. (2019) showed that the mutant in *sigB* was more susceptible when exposed to CAP treatment

456 (directly applied dielectric barrier discharge, sealed container, 1-5 min) in comparison to the

457 WT. However, in a study by Boura et al. (2016), Δ*sigB* was more tolerant to oxidative stress

458 (H_2O_2 treatment) than the WT. The latter authors demonstrated that the discrepancies were due

459   to different oxygen levels during growth, with presence of SigB resulting in high sensitivity to

460   oxidative stress under aerobic conditions and the opposite effect under anaerobic conditions.

461   In our results, the percentage of sub-lethally damaged cells of Δ*sigB* (grown in aerobic

462   condition) was higher in comparison to control, but the viable count was not significantly

463   different in comparison to the WT (Figure 2a, 3b, 4a).

464   Additionally, to date, the studies on the contribution of SigB in the tolerance to natural

465   antimicrobial treatments are very limited, and there is absence of studies examining its impact

466   to GSE treatment (Begley et al., 2006; Palmer et al., 2009). According to the limited studies on

467   natural antimicrobials (other than GSE), SigB impacts the antimicrobial resistance to certain

468   bacteriocins like nisin and lacticin 3147 (Begley et al., 2006; O'Byrne & Karatzas, 2008) which

469   is in accordance to our results on the sensitivity of Δ*sigB* to GSE (Kitsiou et al., 2023a).

470   As previously mentioned, there was no significant difference in the microbial inactivation

471   (viable count) between the Δ*gadD1, D2* and WT strains after the GSE treatment (Figure 2b)

472   which can be explained by the fact the GAD system has been primary linked to contribute to

473   acid stress responses (Table 1). However, it was shown that the percentage of sublethal injury

474   of Δ*gadD1, D2 and D3* mutants was higher following the GSE treatment in comparison to the

475   WT (Figure 4b, & 5). After CAP treatment, Δ*gadD2* exhibited the highest level of inactivation

476   in terms of viable count among all strains after CAP treatment (Figure 2a & 3d). Additionally,

477   the absence of *gadD1* and *gadD3* did not result to a higher level of inactivation as compared to

478   *L. monocytogenes* WT after CAP treatment (Figure 2a, 3a, 3c, 3e). The GAD system is crucial

479   for the viability of *L. monocytogenes* under acid stress as is responsible for maintaining the

480   cellular pH in certain optimal range for survival and growth. It comprises 5 proteins, or 3

481   depending on the strain. Although all strains possess both *gadT2D2* and *gadD3*, the *gadD1T1*

482   operon is missing from serotype 4 *L. monocytogenes* strains (Cotter et al., 2005). Two of the

483   proteins namely GadT1 and GadT2 are glutamate/GABA antiporters while GadD1, GadD2,

GadD3 are glutamate decarboxylases (Cheng et al., 2015; Conor Feehily et al., 2014; Karatzas et al., 2012; Ryan et al., 2008). The five corresponding proteins are encoded in three transcriptional units, namely *gadD1T1*, *gadT2D2*, and *gadD3*. Previous studies have shown that the *gadT2D2* locus has a significant impact on the survival of *L. monocytogenes* in highly acidic environments, whereas the *gadD1T1* locus has been observed to promote growth in moderately acidic conditions (Feehily & Karatzas, 2013; Feehily et al., 2014; Karatzas et al., 2012). Additionally, it has been shown that GadD2 might be the dominant gene within the GAD system of *L. monocytogenes* 10403S (Feehily et al., 2014; Karatzas et al., 2012). The full functionality of the GAD system in stress adaptation has not yet been completely elucidated and the studies exploring its role to other treatments like natural antimicrobial or oxidative stress are extremely limited. For instance, Begley et al. (2010) observed that $\Delta gadD1$ in *L. monocytogenes* LO28 exhibited increased susceptibility to nisin treatment in BHI broth at a concentration of 300 μg/ml, when compared to the WT strain (Begley et al., 2010). Nisin's mechanism of inactivation is based on its ability to bind to Lipid II, a precursor involved in the synthesis of peptidoglycan of the cell wall. This binding process hinders the cell wall synthesis resulting to pore formation in the cell membrane and ultimately causes release of the cell's intracellular content and ATP (Begley et al., 2010; Costello et al., 2019; Yusuf, 2018). Begley et al. (2010), proposed that under specific circumstances, the presence of *gadD1* may play a role in increasing the intracellular ATP pools, therefore increasing the resistance to nisin. It is possible that, similarly, there is a GAD system-mediated mechanism protecting cells from GSE and its absence results in increased percentage of sub-lethally damaged cells, as indicated by our results (Figure 4b, 5c, 5d, 5e).

As previously mentioned, for the individual CAP treatment, *L. monocytogenes* $\Delta gadD2$ was the most sensitive strain with the highest microbial inactivation and the highest percentage of sub-lethally damaged cells (Figure 2a & 3c). Boura et al. (2020) investigated the role of GAD

509   system in oxidative stress (H_2O_2) in 3 strains of *L. monocytogenes* namely EGD-e, LO28 and

510   10403S. The study demonstrated that *gadD3 and gadD2* play a role in oxidative stress

511   resistance of EGD-e, *gadD1* in LO28 while no role of the GAD system was found in 10403S

512   (Boura et al., 2020). Therefore, overall, several components of the GAD system play a role in

513   oxidative stress while this can depend on the strain, the stage of growth and other environmental

514   conditions such as the type of medium. The CAP treatment utilised in this study, generates

515   reactive oxygen species (ROS) leading to oxidative stress and nitrogen reactive species (RNS),

516   that might result in microbial inactivation (Kitsiou et al., 2023). Hence, the increased microbial

517   inactivation of $\Delta gadD2$, observed in this study, may be attributed to the RNS or at the different

518   ROS present in the CAP output species, in comparison to H_2O_2 treatment (Figure 2d). In

519   addition, the lack of response of the GAD system to the CAP treatment might also be related

520   to the stage of growth or the medium used which play an important role (Karatzas et al., 2012).

521   RNS are very reactive and have the ability to modify DNA, lipids, and proteins (Chautrand et

522   al., 2022) while they can also reduce the intracellular pH. The conversion of glutamate to γ-

523   aminobutyric acid (GABA) carried out by the GAD system, might have an indirect role as a

524   cellular defence mechanism against the RNS (Feehily & Karatzas, 2013; Feehily et al., 2014;

525   Karatzas et al., 2012). Additionally, similar to nisin, CAP treatment can cause pore formation

526   leading to the release of ATP. Therefore, the GadD2, which has been shown to be the dominant

527   gene in the GAD system of *L. monocytogenes* 10403S, might help in sustaining the intracellular

528   ATP levels (Aktop et al., 2023; Begley et al., 2010; Feehily et al., 2014). As a result, the

529   absence of this gene might increase the sensitivity of *L. monocytogenes* 10403S to CAP

530   treatment, as shown by our results (Figure 2a & Figure 3d).

531   In the evaluation of the efficacy of the individual treatments on WT *E. coli* K12 and its

532   mutants *(ΔrpoS, ΔoxyR, ΔdnaK)*, it was observed that the individual GSE treatment was

533   inefficient in reducing the microbial population, for all strains under study (Figure 6b & 7).

534   This is in accordance with a previous published study of our group, showing that 1 % (w/v) did

535   not inhibit both tested Gram-negative wild type strains of *E. coli* and *S.* Typhimurium (Kitsiou

536   et al., 2023a). This trend was expected as it is known in literature that Gram-negative bacteria

537   have a higher level of resistance to natural antimicrobials, as compared to Gram-positive

538   bacteria (Gyawali & Ibrahim, 2014; Kao et al., 2010; Quinto et al., 2019). This

539   difference/resistance, arises from the presence of an outer lipid membrane, which acts as a

540   protective barrier, limiting the penetration of antimicrobial compounds (Corrales et al., 2009).

541   Additionally, during the mild GSE treatment stress adaptation mechanisms could be activated,

542   which help *E. coli* to overcome the imposed stressor (Bearson et al., 2009; Wang et al., 2019;

543   Ziuzina et al., 2015).

544   The efficacy of the individual CAP treatment was found to be higher against all strains of

545   *E. coli* when compared to its effectiveness against most strains of *L. monocytogenes* (Figure 2a

546   and 6a). It has been observed that Gram-negative bacteria are more sensitive to plasma

547   treatment in comparison to Gram-positive bacteria (Aktop et al., 2023; Lee et al., 2006; Cindy

548   Smet et al., 2018). For example, Smet et al., (2018) examined the inactivation of Gram-positive

549   *L. monocytogenes* and Gram-negative *S.* Typhimurium by CAP (directly applied dielectric

550   barrier discharge, mixture of 4 L/min helium and 40 ml/min oxygen) and observed that *L.*

551   *monocytogenes* was more tolerant to the CAP treatment. The microbial inactivation of *S.*

552   Typhimurium after 10 min of CAP treatment was approx. 2 log CFU/ml. However, the

553   population of *L. monocytogenes* was reduced by less than 0.5 log CFU/ml (Smet et al., 2018).

554   One of the contributing factors to this difference is the structural characteristics of their cell

555   walls. The thinner peptidoglycan layer in Gram-negative bacteria allows reactive species, such

556   as ROS and RNS generated by CAP, to penetrate more easily into the bacterial cell and cause

557   damage to essential cellular components i.e., proteins and nucleic acids (Mai-Prochnow et al.,

558   2016; Misra & Jo, 2017; Pankaj & Keener, 2017; Smet et al., 2017). However, the sensitivity

559　　to CAP can still vary among different bacterial species of the same cell structure or strains of

560　　the same species. Other factors affecting the sensitivity are the physiological state of the cells

561　　and the initial microbial population existing in the sample (El Kadri et al., 2021; Guo et al.,

562　　2015).

563　　　　For the individual CAP treatment, when comparing the different strains of *E. coli*, the most

564　　sensitive mutant strain was *E. coli* Δ*rpoS* followed by Δ*dnaK* (Figure 6a, 7b, 7d). In addition,

565　　despite having a similar number of viable cells after CAP treatment (Figure 6a & 7c), the *E.*

566　　*coli* Δ*oxyR* strain had a much higher percentage of sub-lethally injured cells as compared to the

567　　WT strain (Figure 8a & 9c). The high sensitivity of *E. coli* Δ*rpoS* can be explained by the fact

568　　that in Gram-negative bacteria like *E. coli,* the general stress response is regulated by the RpoS

569　　(σ^s; see also Table 1*)*. Similarly to SigB for Gram-positive bacteria, RpoS is an alternative

570　　sigma factor responsible for the expression of >50 genes involved in stress adaptation of Gram-

571　　negative bacteria (Battesti et al., 2011; Hengge-Aronis, 1996; Yousef & Juneja, 2002).

572　　However, the genes affected by the central stress gene sigma factor are not the same in Gram-

573　　positive and Gram-negative bacteria and there are differences between species and strains of

574　　the same species (Ferreira et al., 2004; Venturi, 2003). The above could explain the discrepancy

575　　in the effect of SigB between *L. monocytogenes* and *E. coli*.

576　　　　According to our results, DnaK could have an impact on the sensitivity of *E. coli* to CAP

577　　treatment (Figure 6a & 7d). This is due to the existence of another sigma factor, namely RpoH,

578　　which regulates the expression of genes that are involved in the heat shock response, such as

579　　chaperones and heat shock proteins like DnaK. DnaK is a chaperone that helps in the folding

580　　of proteins and prevents protein aggregation under heat stress and/or other stresses. Therefore,

581　　it is crucial for the maintenance of the cellular protein homeostasis and in its absence the cells

582　　could become more sensitive to CAP treatment, as it causes protein denaturation (Arcari et al.,

583　　2020; Ding et al., 2022).　Other important gene regulators worth mentioning are OxyR and

584   SoxR (Guo et al., 2019; Storz et al., 1990) that respond to oxidative stress and subsequently

585   activate *soxS* and *sod* that are associated with reactive oxygen species (ROS) defence

586   mechanisms (Patil et al., 2011). Therefore, when cells are under oxidative stress, they produce

587   proteins that contribute to DNA repair or the free radicals elimination. The results of the current

588   study indicate that the transcriptional regulator OxyR plays an important role in the CAP

589   treatment tolerance of *E. coli*, as evidenced by the increase sublethal injury (Figure 5).

590   However, in the absence of *oxyR*, the presence of *soxS* is possibly sufficient for the cells to

591   cope with the oxidative stress caused by CAP treatment or cover for the absence of the former.

592   The sensitivity of the isogenic mutants of *E. coli (ΔrpoS*, *ΔoxyR*, *ΔdnaK)* to CAP treatment has

593   been reported in previous studies (Connolly et al., 2013; Han et al., 2016; Li et al., 2013; Perni

594   et al., 2007). The results of these studies are in accordance with the results of the current study,

595   suggesting that RpoS, OxyR and DnaK might play a role in the tolerance of *E. coli* to CAP

596   treatment. However, it is challenging to compare the level of inactivation due to various factors

597   that influence the efficiency of cold atmospheric plasma (CAP). These factors, as previously

598   stated, include the plasma source, the duration of treatment, the system on which it is

599   implemented and the treated level of microbial population (Bahrami et al., 2020; Chizoba

600   Ekezie et al., 2017; Costello al., 2021a; Niakousari et al., 2018; Sharma et al., 2014; Smet et

601   al., 2018). For example, Connolly et al. (2013) explored the inactivation of *E. coli* K12 and its

602   isogenic mutants Δ*soxR*, Δ*soxS*, Δ*oxyR*, Δ*rpoS* and Δ*dnaK* by treating cells of *E. coli* on agar

603   with CAP (dielectric barrier discharge, fixed volume of helium and air mixture) for 5 min.

604   After the treatment, the microbial inactivation of all strains was 1.5 log CFU/cm^2. However, it

605   was noted that Δ*oxyR*, Δ*rpoS* and Δ*dnaK* had a much slower recovery compared to the WT

606   strain indicating that these gene regulators impact the cell's repair mechanisms (Connolly et

607   al., 2013). Additionally, Han et al. (2016) investigated the effects of CAP (dielectric barrier

608   discharge, fixed volume of atmospheric air) on *E. coli* K12 using the same mutant strains as

609 our study i.e., *E. coli* Δ*rpoS*, Δ*oxyR*, and Δ*dnaK* genes. The cells were treated in a sealed

610 container for 1, 3, and 5 min and their inactivation levels were assessed after being stored for

611 0, 1, and 24 h at room temperature. The results demonstrated increased sensitivity of Δ*rpoS* to

612 CAP treatment whereas Δ*oxyR* did not show a sensitive phenotype until after 5 min of

613 treatment. In this study, the importance of *dnaK* was more apparent after analysing the viable

614 population after storage time, suggesting that its role is in contributing to the repair mechanism

615 rather than the immediate reaction right after CAP treatment (Han et al., 2016).

616

4.2 Combined GSE and CAP treatments

618 The combined treatment of GSE and CAP achieved a synergistic effect against all strains of

619 *L. monocytogenes* with the mutant strain Δ*sigB* to be the most sensitive to the combined

620 treatment (Figure 2c & 3b). However, for *E. coli* a synergistic effect was only achieved when

621 CAP preceded the GSE treatment (Figure 6c, 6d, 7). The combined effect of GSE and CAP has

622 been investigated in previous work from our group in which similar results were observed when

623 1 % (w/v) GSE was incorporated in various 3D *in vitro* models with varying rheological

624 properties. *L. monocytogenes* was treated with GSE on the surface of the 3D models for either

625 2 h and/or 8 h and treated with CAP for 2 min at flow 5 L/min (higher concentration of ROS

626 species). To the best of our knowledge, no other studies to date have investigated the

627 combination of GSE and CAP against *L. monocytogenes* and *E. coli* and their isogenic mutants.

628 However, prior research has investigated the combined use of CAP with different natural

629 antimicrobials. During these studies it was shown that employing a combined approach led to

630 more effective microbial inactivation compared to applying the treatments individually

631 (Costello 2021a; De la Ossa et al., 2021; Matan et al., 2014, 2015; Patange et al., 2019). For

632 example, De la Ossa et al. (2021) evaluated the synergistic effect of olive leaf extract (with a

total phenolic content of 100 mg/ml) and CAP treatment (using the same apparatus as utilised in this study, with a flow rate of 5 L/min for 1 min) in a liquid nutrient broth against exponential and stationary phase cells of *Listeria innocua, E. coli*, and *Staphylococcus aureus*. The combination of CAP and olive leaf extract resulted in total inactivation of exponential cells of all tested strains, while no inhibitory effects were observed with either treatment applied individually. Additionally, cells in stationary phase appeared to be more resistant to the combined treatment therefore the same synergistic effect was not observed. In another study, Costello et al. (2021a) investigated the hurdle strategy of nisin in sublethal concentration (35 IU/ml, 30 min) and CAP (directly applied dielectric barrier discharge, 4 L/min helium and 40 ml/min oxygen, 30 min) against *L. innocua* in/on liquid and solid like 3D *in vitro* models (1.5% w/v XG). Again, a combined effect was reported when the hurdle approach of CAP and nisin was tested, in comparison to the individual treatments (Costello et al., 2021a). Furthermore, the combination of CAP with other natural antimicrobials has been explored using real food products (Cui et al., 2016a, 2016b, 2017; Matan et al., 2015). For example, Matan et al. (2015), reported a synergistic effect of plasma (radio frequency 40W) and green tea extract (5% w/v) against *L. monocytogenes, E. coli,* and *S.* Typhimurium, on the surface of dragon fruit (10^6 CFU/g initial microbial concentration). More specifically, when the combined treatment of CAP and green tea extract was applied, complete inactivation was achieved for all bacterial strains. The individual plasma treatment caused a reduction in bacterial population by 1-1.5 log CFU/g depending on the strain. The individual treatment with green tea extract did not exhibit a significant antimicrobial effect against the tested Gram-negative bacteria, however *L. monocytogenes* was reduced by 1 log CFU/g (Matan et al., 2015).

There is only one study examining the combination of CAP (directly applied dielectric barrier discharge, atmospheric air, 1-5 min) with other treatments (4 °C and/or acetic acid at pH 4.0 for 1 h) against *L. monocytogenes* and its mutants (Δ*sigB,* Δ*gadD1,* Δ*gadD2,* Δ*gadD3,*

27

658 *ΔgadD2D3, ΔprfA, ΔrsbR, Δlmo0799, Δlmo0799-C56A*). In this study, it was shown that the

659 susceptibility of various strains of *L. monocytogenes* bacteria to CAP treatment was enhanced

660 by exposing them to cold stress. However, the efficacy of CAP treatment was shown to be

661 comparable among the various strains, with the exception of the Δ*rsbR* mutant, which showed

662 an increased inactivation after the combined cold stress and CAP treatment (Patange, O'Byrne,

663 et al., 2019). After the combined acid stress and CAP treatment, all strains of *L. monocytogenes*

664 were completely inactivated indicating a synergistic effect of the tested treatments. To the

665 author's best knowledge there are no studies exploring the combined effect of CAP or natural

666 antimicrobials with other treatments against *E. coli* and its mutants.

667 As previously stated, the total inactivation of *E. coli* was achieved through a sequenced

668 treatment approach, starting with the application of CAP followed by GSE treatment (Figure

669 6d & 7). Previous studies have demonstrated that the order of antimicrobial treatments can

670 influence their efficacy and the microbial response, depending on the cellular component they

671 targeted (Chaplot et al., 2019; Costello et al., 2021b; Govaert et al., 2019; Liao et al., 2018).

672 For instance, Chaplot et al. (2019) investigated the hurdle approach of CAP (dielectric barrier

673 discharge, 6 min) and peracetic acid (100 ppm, 6min) against *S*. Typhimurium in raw poultry

674 meat. The CAP treatment followed by peracetic acid resulted in a 3.8 log CFU/cm^2 reduction,

675 however when the reversed order was applied, *S*. Typhimurium was inhibited by 2.5 log

676 CFU/cm^2. In this study it was proposed that the release of active oxygen by peracetic acid,

677 disrupted the sulfhydryl and sulphur bonds present in the cellular membrane resulting in the

678 effective penetration of RONS in the cell and further inactivation caused by RONS interacting

679 with the intracellular components (Chaplot et al., 2019). The proposed inactivation mechanism

680 for the combined treatment against *L. monocytogenes* involves firstly the penetration of GSE

681 in the bacterial cells and the interaction with their intracellular components (Begg, 2019;

682 Corrales et al., 2009; Silván et al., 2013). According to our results GSE treatment causes a

683   moderate stress to the cells resulting in sublethal injury (>60 %), making them more susceptible

684   to CAP treatment, which targets other cellular structures (Figures 2b, 3, 4b, 5). Therefore, the

685   increased sensitivity of the Δ*sigB* strain to the combined treatment can be attributed to its higher

686   susceptibility to GSE treatment (Kitsiou et al., 2023a). The same combined effect could not

687   be observed when GSE followed by CAP treatment was tested against *E. coli* as the GSE

688   treatment, according to our viable and sublethal count, imposed a mild stress from which the

689   cells could easily adapt (Figures 6c and 8b). This suggests that the exposure of *E. coli* to GSE

690   could lead to a higher tolerance to the CAP treatment via cross-protection mechanisms. The

691   proposed mechanism for the total inactivation of *E. coli* by CAP followed by GSE treatment,

692   is the ability of CAP to cause cell wall disruption hence allowing increased penetration of the

693   GSE components in the cell (Guo et al., 2015; Niemira, 2012; Pankaj & Keener, 2017).

694

695   **Conclusion**

696   In this work we investigated the antimicrobial activity of grape seed extracts (GSE), cold

697   atmospheric plasma (CAP, a remote air plasma with an ozone-dominated RONS output) and

698   their combination against *L. monocytogenes, E. coli* and their environmental stress isogenic

699   mutants in liquid nutrient medium (TSBYE). More specifically, all bacteria under study were

700   treated with 1% (w/v) GSE for 2h, CAP at flow rate 1 l/min for 4 min and/or their combination.

701   The combined treatment was applied sequentially by exposing the cells first to GSE followed

702   by CAP. For *E. coli*, the treatment sequence was also reversed i.e., treating the cells with CAP

703   prior to GSE.

704   A synergistic effect was achieved when GSE and CAP treatments were combined to

705   inactivate *L. monocytogenes* (WT, Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δ*gadD3*) *and E. coli* (WT*,*

706   Δ*rpoS*, Δ*oxyR*, Δ*dnaK*). Specifically, GSE followed by CAP treatment effectively inactivated

all strains of *L. monocytogenes* with Δ*sigB* having the highest microbial inactivation. However, this combined treatment sequence did not exhibit the same efficacy against *E. coli*. Interestingly, when the reverse sequence was explored i.e., first applying CAP and then GSE, a total inactivation of all strains of *E. coli* was observed. For the individual treatments, *L. monocytogenes* Δ*sigB* was more sensitive to GSE treatment, *while L. monocytogenes* Δ*gadD2* was more susceptible to CAP treatment, as compared to all other *L. monocytogenes* strains under study. The individual GSE treatment did not inhibit *E. coli* (WT, Δ*rpoS*, Δ*oxyR*, Δ*dnaK*) after 2 h and the individual CAP treatment was more effective against *E. coli* Δ*rpoS* as compared to all other *E. coli* strains under study.

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

Officer, School of Chemistry and Chemical Engineering). Mutant strain Δ*sigB* was a gift from

Martin Wiedmann, Kathryn J. Boor and Conor P. O'Byrne.

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1199 **Table legends**

1200 *Table 1. Strains and isogenic mutants used in this study and function of deleted genes.*

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Figure legends

Figure 1: Experimental procedure of the individual and combined treatment of GSE and CAP

(created with BioRender.com).

*Figure 2***:** Reduction (log CFU/ml) of the viable population of *L. monocytogenes* 10403S (WT

and mutants) following (a) CAP (4 min), (b) GSE (2h), (c) GSE (2h) + CAP (4 min) treatment

in TSBYE. Data are normalised for each strain/condition with respect to untreated controls. In

all plots, (■) WT, (■) Δ*sigB*, (■) Δ*gadD1*, (■) Δ*gadD2*, (▫) Δ*gadD3*. Each bar represents the

average of two independent experiments with three technical replicates per experiments while

error bars represent 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 $p \le 0.0001$)

*Figure 3***:** Viable counts of *L. monocytogenes* 10403S (a) WT, (b) Δ*sigB*, (c) Δ*gadD1*, (d)

Δ*gadD2*, (e) Δ*gadD3* in TSBYE for all treatments under study. In all plots, (■) control

(untreated sample), (■) CAP treatment for 4 min at flow rate of 1 L/min, (■) 1% (w/v) GSE

treatment for 2h, (▫) Combination of 1% (w/v) GSE (2h) and CAP treatment (4 min). Each bar

represents the average of two independent experiments with three technical replicates per

experiment while error bars represent 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 $p \le 0.0001$)

*Figure 4***:** Sublethal injury (%) of *L. monocytogenes* 10403S (WT and mutants) induced by (a)

CAP (4 min), (b) GSE (2h), (c) GSE (2h) + CAP (4 min) treatment in TSBYE. In all plots, (■)

WT, (■) Δ*sigB*, (■) Δ*gadD1*, (■) Δ*gadD2*, (▫) Δ*gadD3*. Data are normalised with respect to

untreated samples for each condition under study. Each bar represents the average of two

independent experiments with three technical replicates per experiment. In cases where the

1243 viable cell count in the selective media was below detection limit (<10 CFU/ml) the number of

1244 sublethal damaged cells was set to 100 % (bar with stripes) while error bars represent standard

1245 deviation. Connecting lines with asterisks indicate significant differences between samples (*

1246 if $0.01 < p \leq 0.05$, ** if $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1247 *Figure 5*: Quantification of sub-lethally injured cells (%) of *L. monocytogenes* 10403S (a) WT,

1248 (b) Δ*sigB*, (c) Δ*gadD1*, (d) Δ*gadD2* (e) Δ*gadD3* in TSBYE for all treatments under study. In

1249 all plots, (■) control (untreated sample), (■) CAP treatment for 4 min at flow rate of 1 L/min,

1250 (■) 1 % (w/v) GSE treatment for 2 h, (■) Combination of 1 % (w/v) GSE (2 h) and CAP

1251 treatment (4 min). In cases where the viable cell count in the selective media was below

1252 detection limit (<10 CFU/ml) the number of sublethal damaged cells was set to 100 % (bar

1253 with stripes). Each bar represents the average of two independent experiments with three

1254 technical replicates per experiment while error bars represent standard deviation. Connecting

1255 lines with asterisks indicate significant differences between samples (* if $0.01 < p \leq 0.05$, ** if

1256 $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1257 *Figure 6*: Reduction (log CFU/ml) of the viable population *E. coli* K12 (WT and mutants)

1258 followed by (a) CAP (4 min), (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP

1259 treatment (4 min) (d) CAP (4 min) following with 1% (w/v) GSE treatment in TSBYE. In all

1260 plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*, (■) Δ*dnaK*. Data are normalised with respect to untreated

1261 controls for all conditions under study. In cases where the viable cell count in the selective

1262 media was below detection limit (<10 CFU/ml) the reduction is portrayed as total inactivation

1263 (bar with stripes). Each bar represents the average of two independent experiments with three

1264 technical replicates per experiment while error bars represent standard deviation. Connecting

1265 lines with asterisks indicate significant differences between samples (* if $0.01 < p \leq 0.05$, ** if

1266 $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1267 ***Figure 7*:** Viable counts of *E. coli* K12 (a) WT, (b) Δ*rpoS* (c) Δ*oxyR*, (d) Δ*dnaK* in TSBYE for

1268 all treatments under study. In all plots, (■) control (untreated sample), (■) CAP treatment for 4

1269 min at flow rate of 1 L/min, (■) 1 % (w/v) GSE treatment for 2 h, (■) Treatment with 1 % (w/v)

1270 GSE (2 h) following with CAP treatment (4 min), (■) Treatment with CAP (4 min) following

1271 with 1% (w/v) GSE treatment (2 h). In cases where the viable cell count was below the

1272 detection limit (<10 CFU/ml) the number was set to 1 log CFU/ml. Each bar represents the

1273 average of two independent experiments with three technical replicates per experiments while

1274 error bars represent the standard deviation. Connecting lines with asterisks indicate significant

1275 differences between control and treated samples (* if $0.01 < p \leq 0.05$, ** if $0.001 < p \leq 0.01$, ***

1276 if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1277 ***Figure 8*:** Sublethal injury (%) of *E. coli* K12 (WT and mutants) induced by (a) CAP (4 min),

1278 (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP treatment (4 min) (d) CAP (4 min)

1279 following with 1% (w/v) GSE treatment in TSBYE. Data are normalised with respect to

1280 untreated controls for all conditions under study. In all plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*,

1281 (■) Δ*dnaK*. In cases where the viable cell count in the selective media was below detection

1282 limit (<10 CFU/ml) the reduction is portrayed as total inactivation (bar with stripes). Each bar

1283 represents the average of two independent experiments with three technical replicates per

1284 experiment while error bars represent the standard deviation. Connecting lines with asterisks

1285 indicate significant differences between control and treated samples (* if $0.01 < p \leq 0.05$, ** if

1286 $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq 0.001$, *** if $p \leq 0.0001$)

1287 ***Figure 9*:** Quantification of sub-lethally injured cells (%) of *E. coli* K12 (a) WT, (b) Δ*rpoS*, (c)

1288 Δ*oxyR*, (d) Δ*dnaK* in TSBYE for all treatments under study. In all plots, (■) control (untreated

1289 sample), (■) CAP treatment for 4 min at flow rate of 1 L/min, (■) 1% (w/v) GSE treatment for

1290 2h, (■) treatment with 1% (w/v) GSE (2h) following with CAP treatment (4 min), (■) treatment

1291 with CAP (4 min) following with 1% (w/v) GSE treatment (2h). In cases where the viable cell

1292   count in the specific media was below detection limit (<10 CFU/ml) the number of sublethal

1293   damaged cells was set to 100% (indicated with stripes). Each bar represents the average of two

1294   independent experiments with three technical replicates per experiments while error bars

1295   represent the standard deviation. Connecting lines with asterisks indicate significant

1296   differences between samples (* if $0.01 < p \leq 0.05$, ** if $0.001 < p \leq 0.01$, *** if $0.0001 < p \leq$

1297 0.001, *** if $p \leq 0.0001$)

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1313   **List of Tables**

Microorganism	Deleted gene/ Mutant strain	Gene function	Reference
L. monocytogenes 10403S	Δ*sigB*	Central stress (heat, acid, osmotic stress) gene regulator in *L. monocytogenes*.	Boura et al., 2016; O'Byrne & Karatzas, 2008; Raengpradub et al., 2008
	Δ*gadD1*	Encode glutamate decarboxylases which are part of the GAD system responsible for the pH homeostasis within the cell.	Feehily et al., 2014; Karatzas et al., 2012
	Δ*gadD2*	-*gadD1* active in moderately acidic condition	
	Δg*adD3*	-*gadD2* active in severe acidic condition -*gadD3* associated with the intracellular glutamic acid decarboxylase system (GADi).	
E. coli K12	Δ*rpoS*	Responsible for the general response of *E. coli* and the expression of over 50 genes involved in stress adaptation	Battesti et al., 2011; Baba et al., 2006; Hengge-Aronis, 1996; Yousef & Juneja, 2002
	Δ*oxyR*	Encodes transcriptional regulators that respond to oxidative stress	Baba et al., 2006; Guo et al., 2019; Storz et al., 1990
	Δ*dnaK*	Chaperone that helps in the folding of proteins and prevents protein aggregation under heat stress and/or other stresses (oxidative, osmotic).	Baba et al., 2006; Kim et al., 2021; Meury & Kohiyama, 1991; Schramm et al., 2017; Tilly et al., 1983

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100 μL
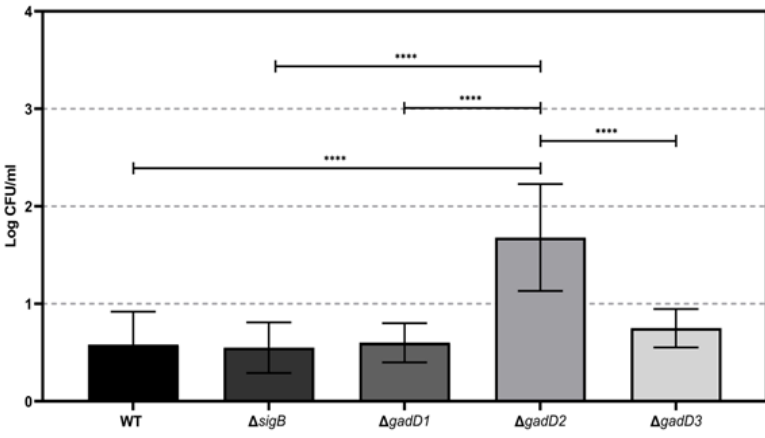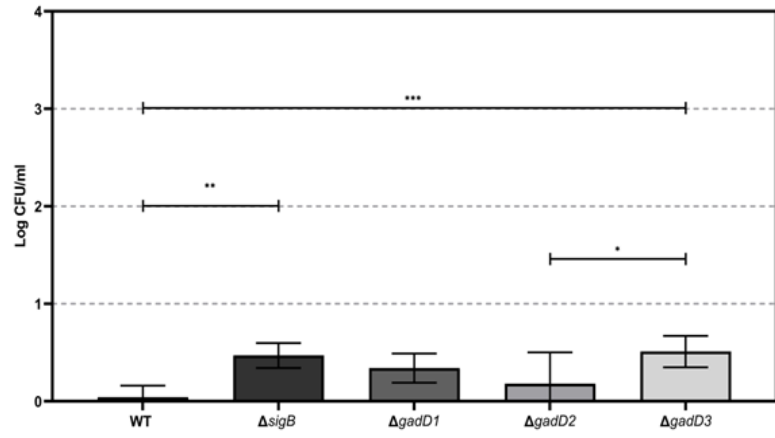
Overnight culture
10^9 CFU/ml

10^8 CFU/ml

20 μL

20 μL

PBS
10^5 CFU/ml

TSBYE + GSE
10^5 CFU/ml

2 h 37 °C

4 min

Resuspend in PBS
10^5 CFU/ml

300 μL

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(a) *CAP*

(b) *GSE*

(c) *GSE + CAP*
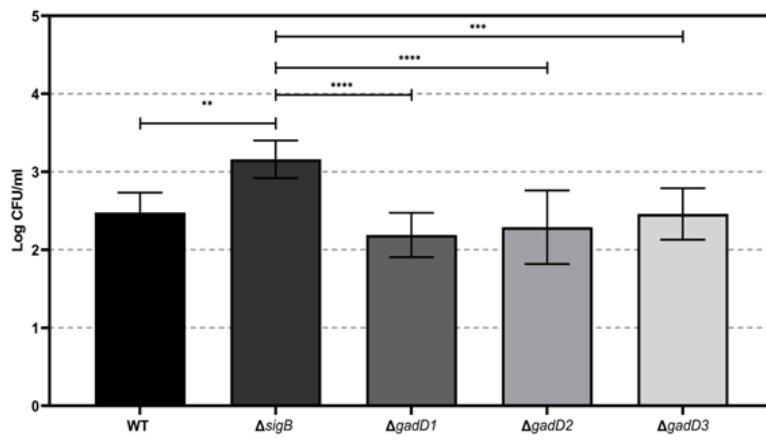
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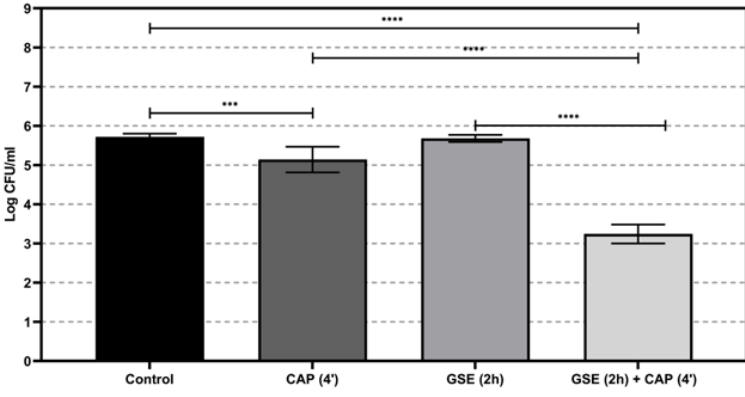
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(a) *L. monocytogenes* WT

(b) *L. monocytogenes* ΔsigB
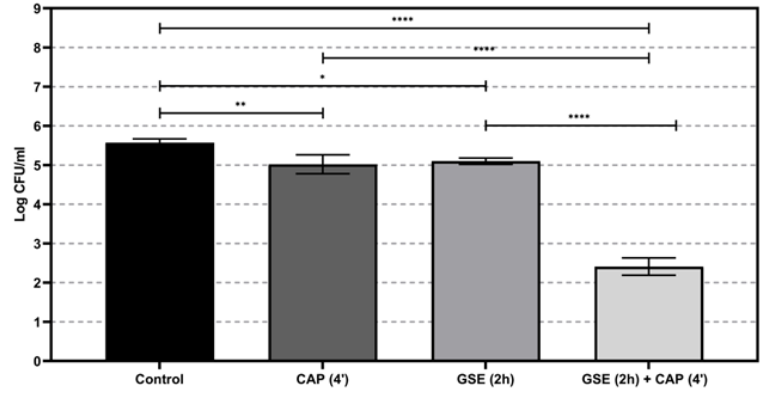
(c) *L. monocytogenes* ΔgadD1
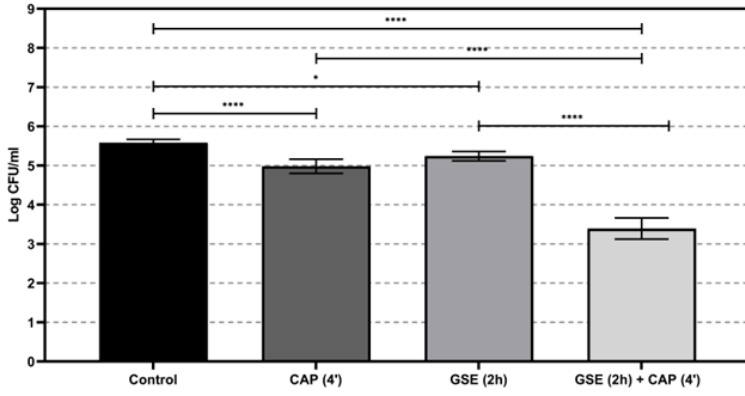
(d) *L. monocytogenes* ΔgadD2
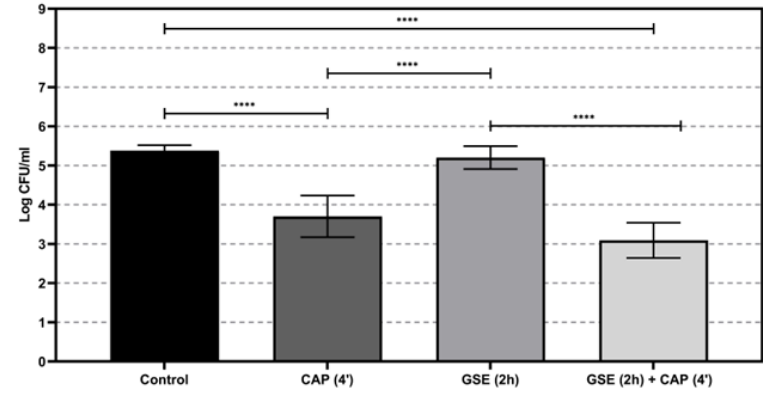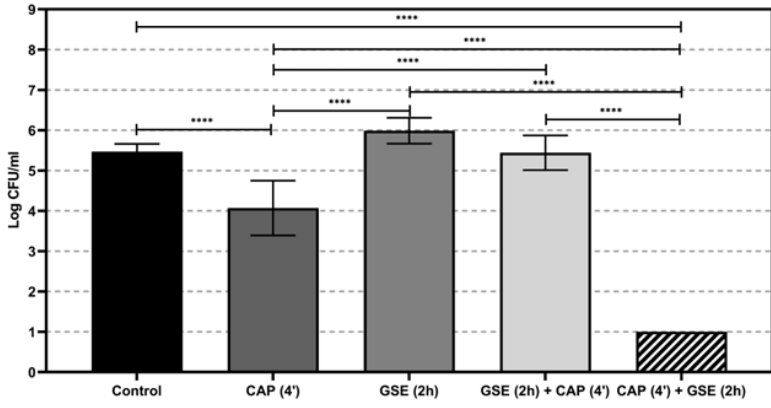
(e) *L. monocytogenes* ΔgadD3
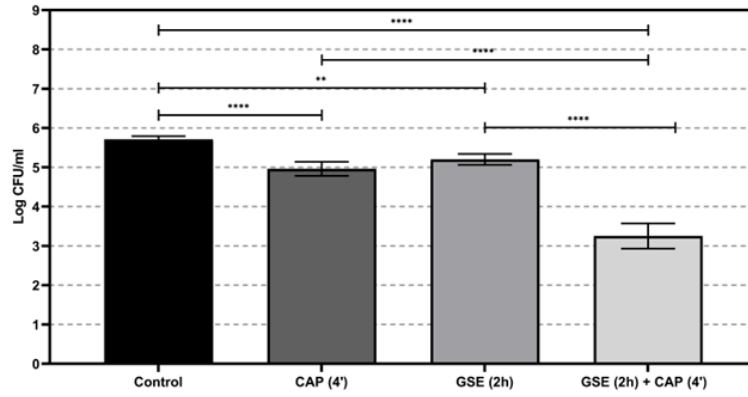
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(a) CAP
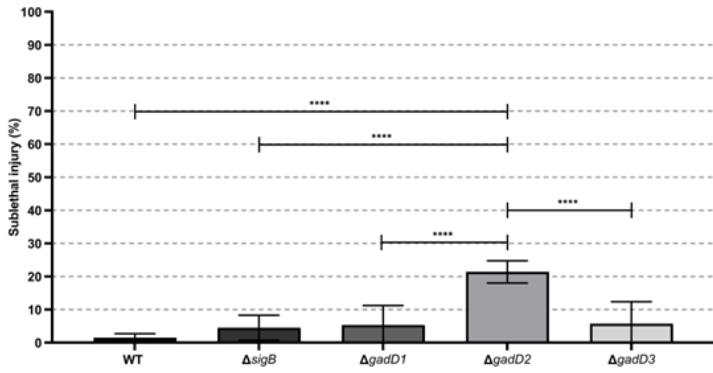
(b) GSE
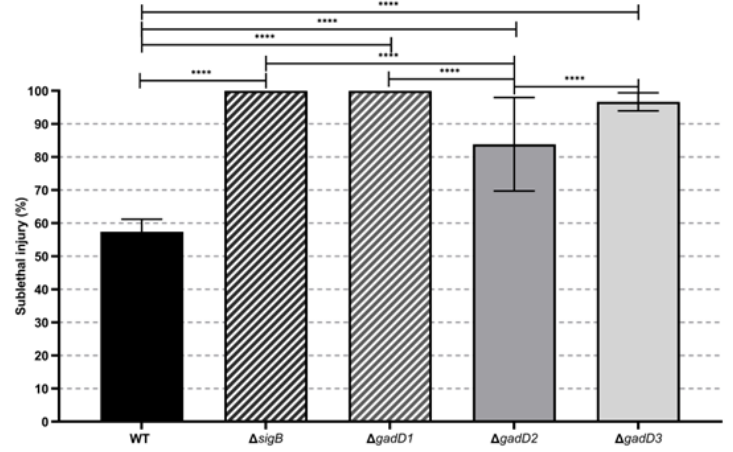
(c) GSE + CAP
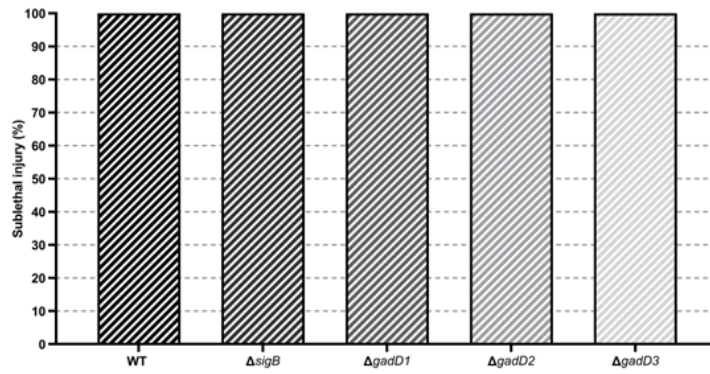
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(a) *L. monocytogenes* WT
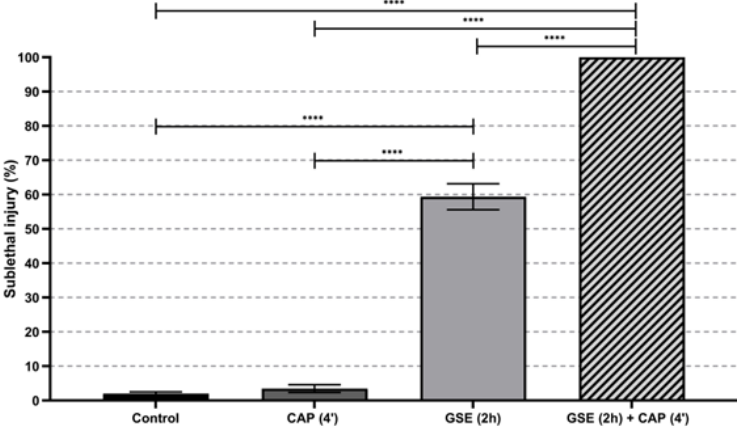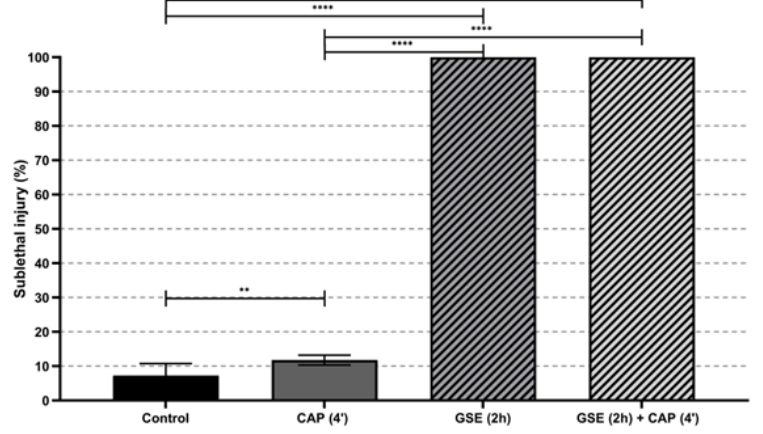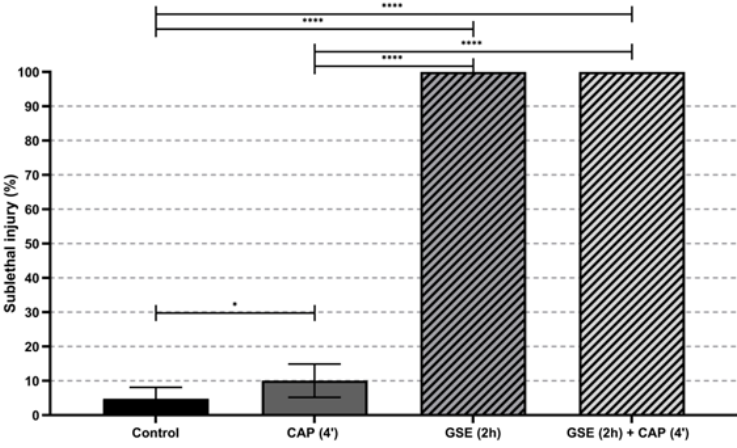
(b) *L. monocytogenes* Δ*sigB*

(c) *L. monocytogenes* Δ*gadD1*

(d) *L. monocytogenes* Δ*gadD2*

(e) *L. monocytogenes* Δ*gadD3*

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(a) *E. coli* WT

(b) *E. coli* ΔrpoS

(c) *E. coli* ΔoxyR
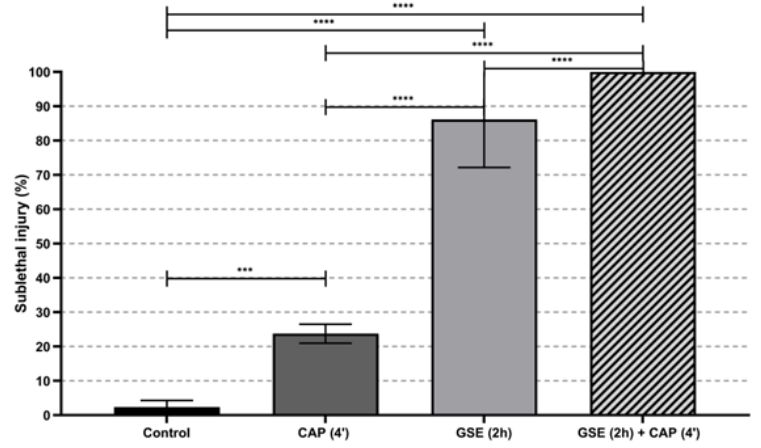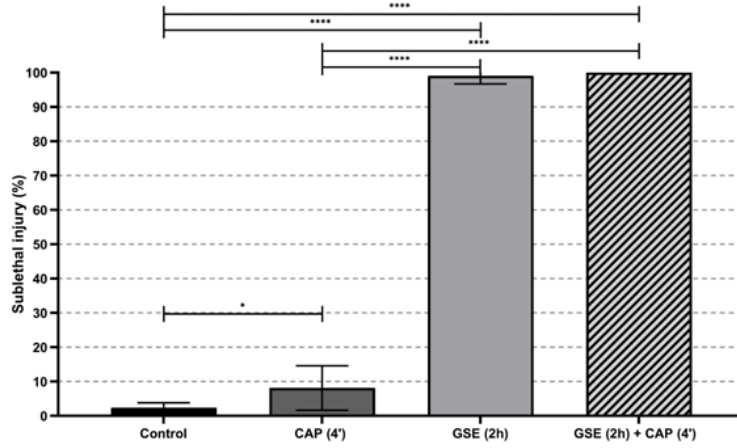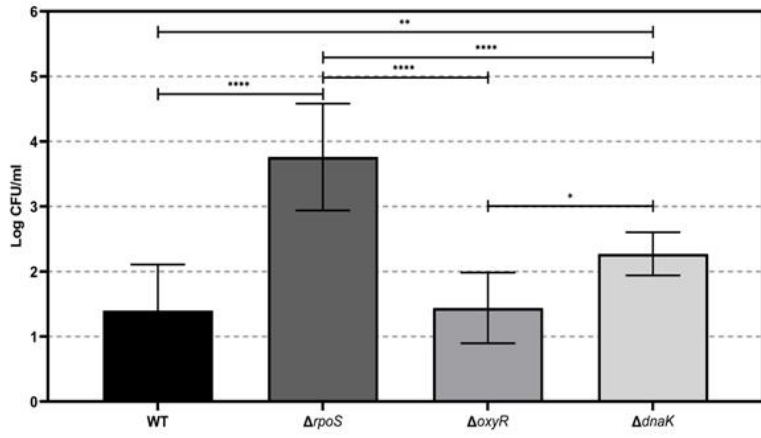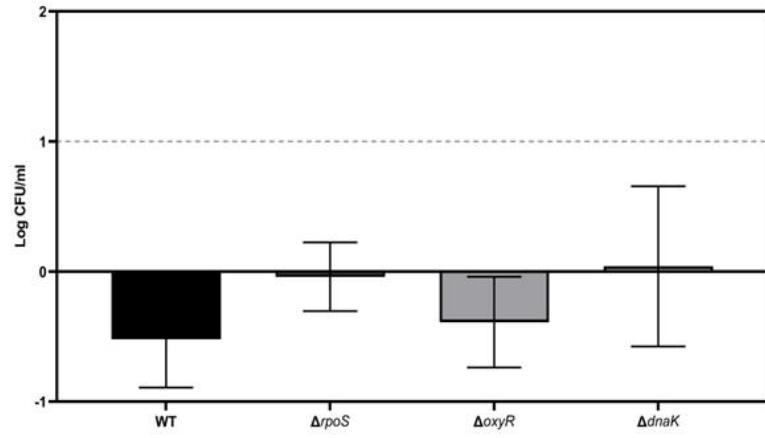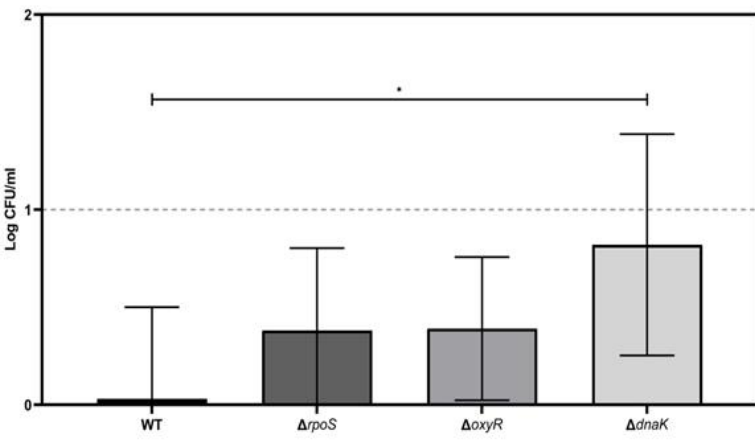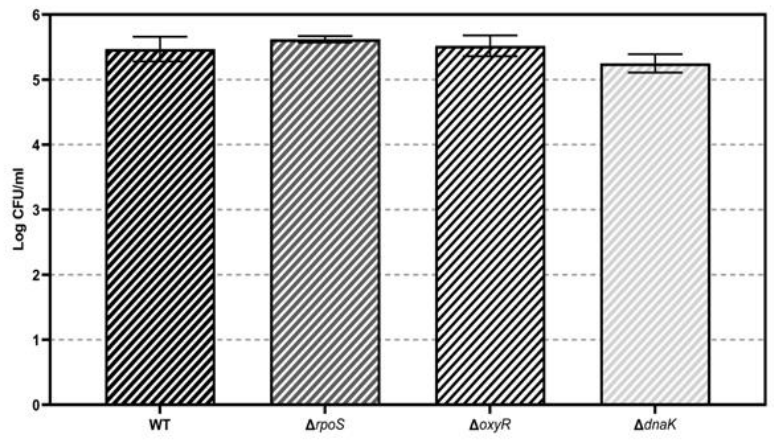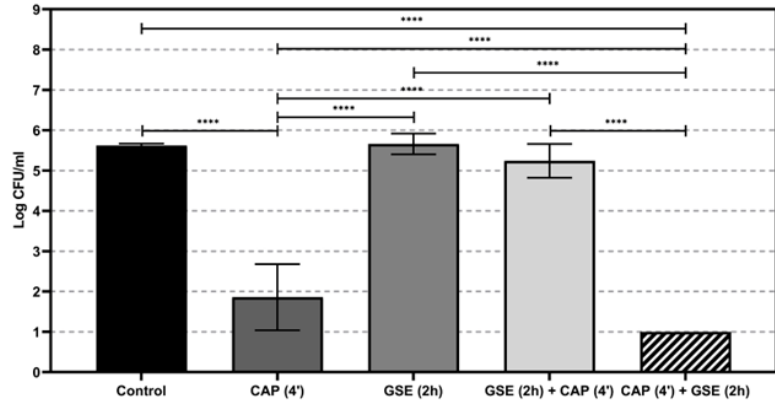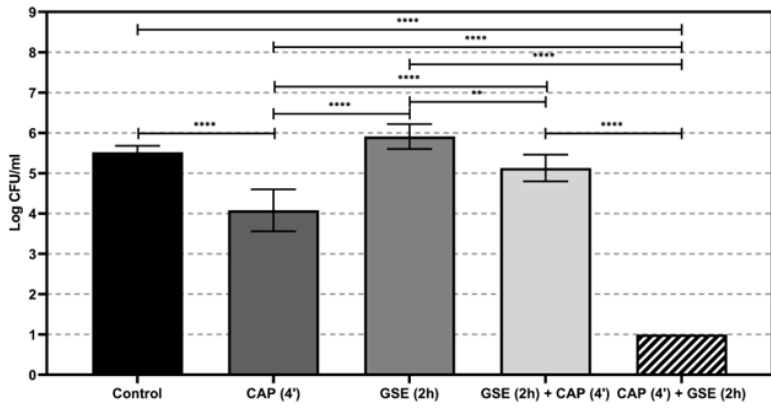
(d) *E. coli* ΔdnaK
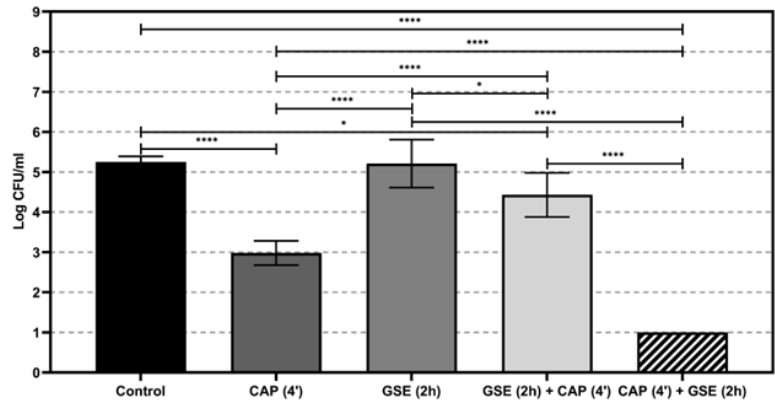
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1334
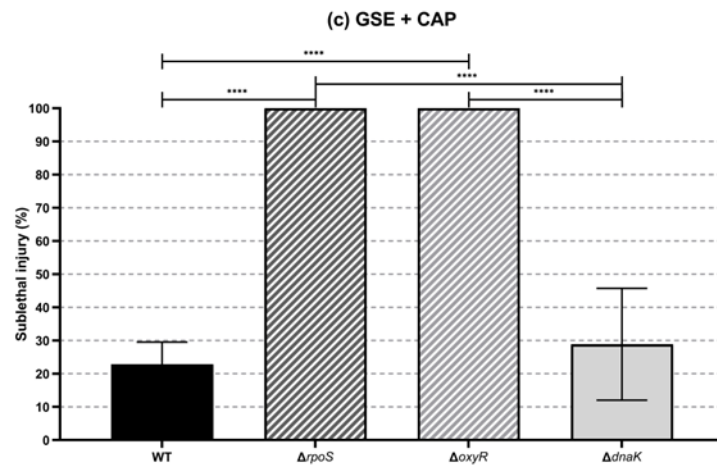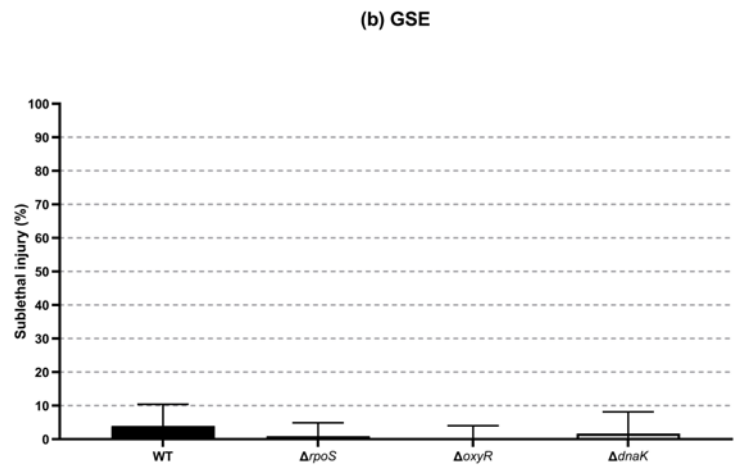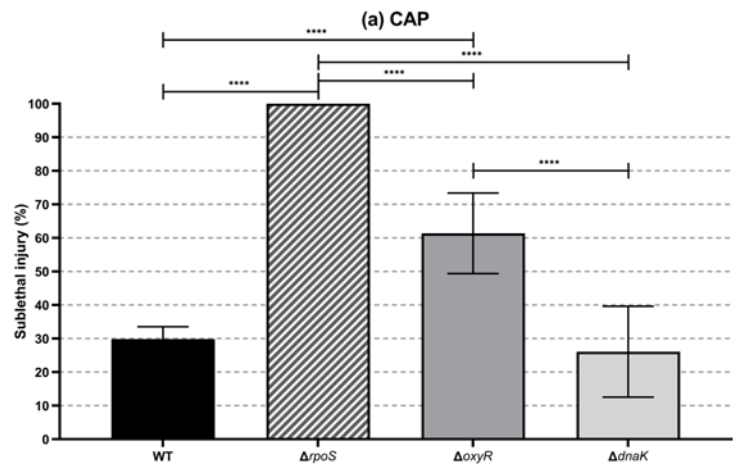
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(a) *E. coli* WT

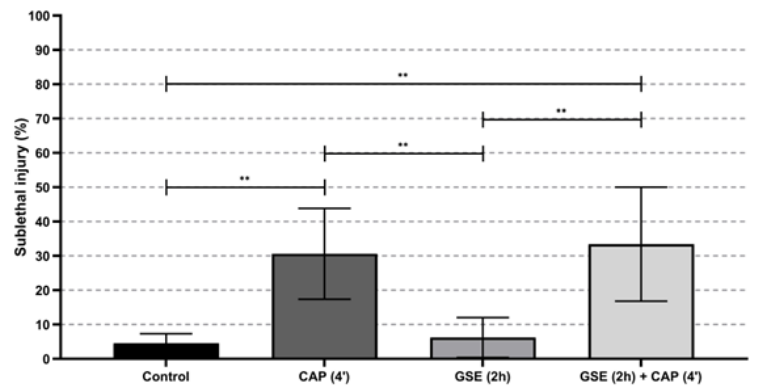(b) *E. coli* Δ*rpoS*

(c) *E. coli* Δ*oxyR*

(d) *E. coli* Δ*dnaK*

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