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

Melina Kitsiou [a, b], Thomas Wantock [c], Gavin Sandison [c], Thomas Harle [c], Jorge Gutierrez-Merino [d], Oleksiy V. Klymenko [a], Kimon Andreas Karatzas [e] and Eirini Velliou [a,b*]

[a] *School of Chemistry and Chemical Engineering, University of Surrey, Guildford, GU2 7XH, UK.*

[b] *Centre for 3D models of Health and Disease, Division of Surgery and Interventional Science, University College London, London, W1W 7TY, UK.*

[c] *Fourth State Medicine Ltd, Longfield, Fernhurst, Haslemere, GU27 3HA, UK*

[d] *School of Biosciences and Medicine, University of Surrey, Guildford, GU2 7XH, UK.*

[e] *Department of Food and Nutritional Sciences, University of Reading, Reading, United Kingdom.*

*Corresponding author. E-mail address: *e.velliou@ucl.ac.uk*

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.

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

1. Introduction

Consumers increasingly demand food products that are processed using minimal and environmentally friendly methods (Dávila-Aviña et al., 2015; Pereira & Vicente, 2010). As a result, researchers and the food industry constantly look for novel sustainable ways to ensure microbiologically safe products via replacing chemical preservatives and antibiotics with natural antimicrobials. Fruit and vegetable by-products are a valuable source of natural antimicrobials that can also help to reduce food waste (Chandrasekaran, 2012; Costello et al., 2018, 2019, 2021a, 2021b; Sabater et al., 2020; Sharma et al., 2021).

Grape by-products, comprise roughly 20 % of the overall weight of the grape and are a substantial waste stream within the wine and juice industry (Oliveira et al., 2013; Özkan et al., 2004). The disposal of these by-products, including the skins, seeds, and stems of the fruit, can be challenging. Grape seed extract (GSE), a natural product derived from grape seeds, is a rich source of antioxidant and antimicrobial compounds, such as polyphenols (Chedea & Pop, 2019; Costa et al., 2022; Karnopp et al., 2017; Shrikhande, 2000). GSE is generally recognized as safe (GRAS) for use in food, but it is not yet commonly utilized as an antimicrobial agent.

The inactivation of bacteria by GSE has been linked to multiple modes of action, including the ability of polyphenols to permeate the bacterial cell walls and the potential of tannins to inactivate extracellular enzymes. Furthermore, the GSE compound shows the ability to form complexes with metal ions, leading to the removal of these ions from the bacterial environment (Corrales et al., 2009; Silván et al., 2013). The presence of metal ions is essential for pathogenic 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 $5x10^6$ 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.*

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

can easily interact with the cells on the surface of solid foods. However, some solid food products may have rougher surfaces, and this can be a hindering parameter for plasma treatment because it provides a natural protection for the bacteria that can attach within the available cavities (Surowsky et al., 2015). Additionally, some bacterial species and strains could be more susceptible to the treatment depending on their morphology, cell membrane and ability to form biofilms. For example, it has been observed that Gram-negative bacteria are more sensitive to plasma treatment in comparison with Gram-positive bacteria, because of their thinner cell wall (Mai-Prochnow et al., 2016). Other parameters that play a major role are the apparatus of CAP, the intensity and time of treatment and the initial microbial population present in the sample (Guo et al., 2015).

CAP treatment has shown some promising results for food decontamination. However, the plethora of parameters, affecting the outcome of the CAP treatment, are adding an element of variability when comparing results from different research groups. The magnitude of microbial inactivation ranges from no inhibition to several logs of reduction of the bacterial concentration, depending on the parameters of the treatment, the food properties such as the matrix, the water activity, pH and the sensitivity of the bacterial strain (Bahrami et al., 2020; Chizoba Ekezie et al., 2017; Choi et al., 2016; Niakousari et al., 2018; Sharma et al., 2014). For example, the microbial inactivation in meat products reported in literature ranges from 0.34-6.52 log CFU/g by changing one or more of the above parameters (Misra & Jo, 2017). More specifically, Choi et al. (2016) explored the inactivation of *L. monocytogenes* and *E. coli* by CAP (corona discharge, atmospheric air) on pork with initial microbial concentration $10^{7.5}$ CFU/g. After a 2 min treatment, it was observed that *E. coli* was reduced by 1.5 log CFU/g, while *L. monocytogenes* was inactivated by 1 log CFU/g. Additionally, Lee et al. (2011) noted a 4.73 log CFU/g reduction in *L. monocytogenes* by using different parameters of CAP treatment (dielectric barrier discharge, oxygen & nitrogen) applied in cooked chicken breast

(Lee et al., 2011). In another study, Smet et al. (2018) demonstrated higher CAP inactivation of cells grown planktonically as compared to cells grown on structured 3D models i.e., gelatin at 5% (w/v). Therefore, drawing general conclusions on the level of microbial inactivation is challenging. More work should be conducted, evaluating CAP individually or in combination with other technologies or natural antimicrobials i.e., in order to establish a hurdle technology and microorganism-specific approaches. In this way CAP could be approved as safe and utilised on a larger scale in production (Guo et al., 2015; Pankaj & Keener, 2017; Tewari & Juneja, 2007).

The principle of the hurdle technology approach on microbial safety, is the utilization of two or more methods/approaches/processes to enable microbial inactivation and consequently to ensure food safety (Bigi et al., 2023; Costello et al., 2021; Khan et al., 2017; Leistner, 2000; Liao et al., 2020; Velliou et al., 2011a, 2011b, 2012). Due to their mode of action, mild/alternative technologies can cause less damage and death to bacteria as compared to classic treatments, e.g., heat pasteurisation (Bahrami et al., 2020; Sunil et al., 2018; Tewari & Juneja, 2007). This can pose a challenge when employing these approaches, as they might not fully guarantee products that are microbiologically safe. However, the combination of these methods or technologies could potentially exert synergistic or additive effects against bacteria, thereby achieving a substantial microbial inactivation (>5 log CFU/ml), ensuring product safety (Millan-Sango et al., 2015; Mosqueda-Melgar et al., 2008). For example, one technology/treatment may be used to damage or increase the permeability of the bacterial cell wall while another could be used to interfere with the intracellular components. The trigger to develop such hurdle approaches has been to protect heat sensitive food products against bacterial growth with minimal processing aiming to maintain their quality (Costello al., 2021a; Khan et al., 2017; Leistner, 2000; Peleg, 2020). Therefore, developing hurdle approaches is

more pressing than ever, to meet the rising demand for minimally processed foods and sustainable production.

As previously mentioned, novel non-thermal technologies (NTTs) can be sometimes ineffective depending on external parameters such as the nature of the food, the CAP parameters and the type of microorganism (Costello et al., 2018, 2019, 2021; Dobrynin et al., 2011; Laroussi et al., 2011; Pereira & Vicente, 2010; Velliou et al., 2013). Furthermore, specifically natural antimicrobials derived from plants such as essential oils cannot be used in very high concentrations, as they might affect the organoleptic characteristics of the food product (Gutierrez et al., 2008; Mariod, 2016; Pateiro et al., 2021; Zhao et al., 2020).

Hence, combining NTTs with plant-derived antimicrobials as a hurdle approach could be a novel solution to increase the treatment efficacy and achieve food safety. However, to date, there is a very limited number of studies on such combined treatments and their mechanism of inactivation. In most cases, the current hurdle approaches involve the combination of established methods, such as heat treatment with chemical preservatives, or two NTTs combined together, or with heat treatment (Bermúdez-Aguirre et al., 2012). Additionally, the majority of studies combining NTTs with natural antimicrobials focus on the combination of NTTs with essential oils, rather than other natural antimicrobials derived by plants (Cui et al., 2016; Espina et al., 2014; Matan et al., 2014, 2015). For example, Matan et al. in 2014, studied the synergistic effect of radio frequency plasma with essential oils from clove, sweet basil and lime in concentrations of 0.5 to 2 % v/v. The most effective treatment was that combining plasma with clove oil (1 % v/v), which lead to a total microbial (*E. coli*, *S.* Typhimurium, *S. aureus*) inhibition on eggshells (Matan et al., 2014). Similarly, Cui et al. (2016b) studied the effect of the combination of cold nitrogen plasma (400 W) and thyme oil (0.05 % w/v) against *S.* Typhimurium and *S.* Enteritidis on eggshells achieving a total microbial inactivation (bacterial counts below detection limit), that lasted for 14 days at 3 different temperatures (4,

12, 25 °C) (Cui et al., 2016). The same year, Cui et al. (2016a) also showed that cold nitrogen plasma combined with *Helichrysum italicum* essential oil can inhibit *S. aureus* on food packaging. The microbial concentration decreased more than 5 log CFU/cm^2, in contrast with individual treatments that caused only 2 log CFU/cm^2 reduction of the microbial concentration (Cui et al., 2016).

To date, as previously described, GSE have not been extensively studied in terms of their antimicrobial properties nor in combination with other NTTs as a hurdle approach. The only reported combined approach of GSE as a microbial inactivation treatment was with nisin, a natural antimicrobial peptide produced by certain strains of *Lactococcus lactis* (Costello et al., 2018, 2019; Liu & Hansen, 1990; Sivarooban et al., 2008; Thanjavur et al., 2022; Zhao et al., 2020). More specifically, Zhao et al. (2020) reported that the simultaneous treatment of nisin (2000 IU/ml) and GSE (1 % w/v) in a liquid broth, was able to reduce the concentration of the bacterial pathogen *L. monocytogenes* by 5 logs after 10 min of treatment, whereas their separate use could not inhibit *L. monocytogenes* more than 2 log CFU/g (Zhao et al., 2020). A similar synergistic effect of the above combination was reported by another study where *L. monocytogenes* was completely inhibited after 12 h in the presence of 6400 IU/ml nisin and 1% w/v GSE (Sivarooban et al., 2007). The proposed synergistic mechanism of microbial inactivation was common in these studies. Nisin acted on the cell wall surface by forming large pores which allowed the GSE to diffuse in the cytoplasm causing further cell damage (Sivarooban et al., 2008; Zhao et al., 2020).

From the above studies, it can be concluded that, using hurdle approaches to deliver safe food products is a very promising strategy which has not been thoroughly studied nor 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).

2.2. Grape seed extracts (GSE)

This study utilised commercially available grape seed extract (GSE) from Bulk, UK. The GSE powder contained a minimum concentration of 95% oligomeric proanthocyanidin. Consequently, the powder is predominantly comprised oligomeric proanthocyanidins. To prepare the GSE solution, the powder was dissolved in Tryptic Soy Broth with 0.6% Yeast Extract (TSBYE) at a concentration of 1% w/v and subsequently autoclaved. The autoclaved TSBYE+GSE was stirred overnight to ensure thorough homogenization. The chosen GSE concentration was selected based on results from our previous study in TSBYE broth. The finding of our study showed that 1% w/v concentration of GSE significantly inactivated *L. monocytogenes* WT and its isogenic Δ*sigB* mutant in TSBYE, resulting in a 3 log CFU/ml reduction after 24 h (Kitsiou et al., 2023a).

2.3. CAP experimental set-up

The CAP apparatus utilised in this investigation was developed and supplied by Fourth State Medicine Ltd. The configuration of the device has been previously described in previous work of the group (El Kadri et al., 2021; Kitsiou et al., 2023b). Briefly, the generator of CAP in this apparatus was a dielectric barrier discharge in a remote and enclosed configuration, whereby the plasma source was contained in an electrically-shielded enclosure and separated from the treatment target by a tube, with no direct line of sight. The gas used for ionization was compressed air (25 °C, 3 bars), and its flow rate (0-5 L/min) was controlled by a needle valve and a flow meter mounted on the enclosure. The chemical composition of the plasma output varies based on the input air flow rate For example, at flow rate 1 L/min more reactive nitrogen species (RNS– primarily NO_x compounds, NO_2 and NO) are produced in comparison with higher flow rates at which the air flow is enriched with more reactive oxygen species (ROS– primarily O_3). At the used flow rate of the experiment (1 L/min) the concentration of ROS was

approximately 320 ppm (Kitsiou et al., 2023b). Additionally, data collected by Fourth State Medicine Ltd showed that the concentration of NOx was approx. 100-200 ppm and NO_z, compounds (mixture of N_2O, HONO, and other compounds, alongside $O_{3)}$ was approximately 200-300 ppm (Fourth State Medicine Ltd, 2023).

2.4. Combined treatment: CAP and GSE

To assess the combined treatment of GSE and CAP in liquid, *L. monocytogenes* WT and its isogenic mutants *(*Δ*sigB,* Δ*gadD1,* Δ*gadD2,* Δ*gadD3)* and *E. coli* and its isogenic mutants *(*Δ*oxyR,* Δ*dnaK,* Δ*rpoS)* were inoculated in TSBYE with 1% (w/v) GSE (Figure 1). The initial microbial population was 10^5 CFU/ml. Prior to CAP treatment, the samples were treated with GSE at 37 °C for 2 h. The treatment time in the presence of GSE was selected based on our previously published results in liquid nutrient medium (TSBYE) (Kitsiou et al., 2023a). These prior finding showed that following a 2 h treatment the cell population *L. monocytogenes* WT exhibited no significant reduction and its knockout mutant (Δ*sigB*) was decreased by 0.3 log CFU/ml (Kitsiou et al., 2023a). Therefore, the aim of the GSE treatment, for this study, was to cause a slight reduction in microbial population and create a state of stress by subjecting the cells to GSE. Thereafter, the sample was centrifuged at 5000 rpm for 10 min (Megafuge 16R, ThermoFisher, USA), the supernatant was discarded, and the pellet was resuspended in 20 ml PBS. To enumerate the viable population of the 2 h GSE treatment, the spread-plate method was followed using TSAYE non-selective media. Sub-lethally injured cells exhibit an inability to grow on selective media, while they are capable of normal growth on nonselective media (Shao et al., 2023). Therefore, to identify the number of cells that were sub-lethally damaged, the samples were also plated into selective media i.e., Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar for *L. monocytogenes* or Violet Red Bile

Glucose (VRBG) agar (Oxoid Ltd, UK) for *E. coli*. The number of injured cells was calculated based on the following equation (Busch & Donnelly, 1992) :

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

For the CAP treatment experiments, 300 μL of PBS containing the pre-treated with GSE and/or untreated cells were transferred in 12-well plate. The samples were exposed to CAP at 1 L/min flow rate for 4 min. The flow rate of the CAP treatment was determined through initial experiments (results not shown), which demonstrated that lower flow rates in the liquid carrier, enriched with RNS, resulted in more effective inactivation. Additionally, the duration of the CAP treatment was selected in order to induce a slight decrease in the microbial population, therefore allowing the investigation of the potential synergistic effects of the GSE and CAP treatment. The survival of the microbial population and sublethal injury after the treatment was assessed using the spread plate technique as described above. Additionally, for *E. coli*, the treatment sequence was reversed. Initially, the cells were subjected to CAP treatment, and subsequently, they were exposed to GSE (Figure 1). The parameters used for both treatments remained unchanged. This approach was implemented specifically for *E. coli* to explore the potential synergistic or altered effects resulting from the reversed treatment sequence. The decision resulted from the decreased antimicrobial efficacy, in comparison with the efficacy against *L. monocytogenes*, observed when *E. coli* was treated with GSE followed by CAP (see results section).

2.5. Statistical analysis

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

3. Results

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

To the authors' best knowledge this is the first study investigating the impact of the combined antimicrobial effect of GSE with a novel non-thermal technology such as CAP in a liquid carrier on L. monocytogenes and E. coli and their isogenic mutants in environmental stress

genes. Therefore, this study provides valuable insights into the microbial mechanisms of stress response to this combined treatment.

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

Figures 2 and 3 show the level of microbial inactivation caused by individual and combined GSE and CAP treatments, for all tested strains of *L. monocytogenes* (WT, Δ*sigB*, Δ*gadD1*, Δ*gadD2*, Δ*gadD3*). Figure 2 presents the data arranged by treatment while in Figure 3 the results are organised by strain of *L. monocytogenes*. Overall, the combined treatment of 1 % (w/v) GSE for 2 h followed by 4 min of CAP treatment at flow rate 1 L/min had a good synergistic effect against all strains of *L. monocytogenes*. After the individual GSE treatment, i.e., a 2 h exposure to 1 % (w/v) GSE, there was no significant decrease in the population of *L. monocytogenes* WT (Figure 3a). For most mutant strains of *L. monocytogenes*, the cell concentration was reduced by an average of 0.4 CFU/ml following a 2 h exposure to GSE ($p > 0.05$) (Figure 2 & 3b-e). Additionally, the sublethal injury assessment showed that the GSE treatment led to a greater percentage of sub-lethally injured cells among the mutant strains (Figure 4b & 5). The cells of Δ*sigB* and Δ*gadD1* were the most sensitive, as all the microbial population was sub-lethally injured (100 %) after 2 h in the presence of GSE. The WT strain exhibited the lowest percentage of sub-lethal injury following the individual GSE treatment, which was approximately 60 % (Figure 4 & 5). The high yield of sub-lethally injured cells for all *L. monocytogenes* strains emphasises the great potential of GSE as a sustainable solution for decontamination.

After the individual CAP treatment (4 min, 1 L/min), all strains of *L. monocytogenes* except the mutant strain Δ*gadD2* were inhibited by an average of 0.5 log CFU/ml. The inactivation of *L. monocytogenes* Δ*gadD2* mutant was higher as compared to all other mutant strains, with an

approximate reduction of 1.7 log CFU/ml ($p < 0.05$) (Figure 2a & 3c). Additionally, when measuring the sublethal population it was observed that all mutants had higher percentage of sub-lethally injured cells, in comparison to the WT, with the highest percentage of 24% belonging to Δ*gadD2* (Figure 4a & 5). These results indicate that the *gadD2* might have a significant role in the tolerance of *L. monocytogenes* to CAP treatment.

As previously mentioned, the combined treatment of GSE (1 % w/v, 2 h) and CAP (1 L/min, 4 min) had a great synergistic effect against all strains of *L. monocytogenes.* As can be seen in Figures 2 and 3, the viable population of *L. monocytogenes* WT after the combined treatment was equal to 3.2 log CFU/ml i.e., the combined treatment led to a 2.5 log CFU/ml reduction when compared to untreated controls (Figure 2c & 3a). Additionally, the microbial inactivation of most mutant strains (Δ*gadD1*, Δ*gadD2*, Δ*gadD3*) was comparable to the WT strain with an average reduction of 2.3 log CFU/ml. *L. monocytogenes* Δ*sigB* was the only mutant strain for which a higher level of microbial inactivation (3.2 log CFU/ml) was observed in comparison to all other *L. monocytogenes* strains. ($p < 0.05$) (Figure 2c). However, when assessing the extend of sub-lethal injury (Figure 4c & 5), it was noted that cells of all strains could not grow on selective medium (PALCAM) i.e., most cells of all strains were in the state of sub-lethal injury indicating the great antimicrobial efficacy of this hurdle approach.

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

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

420 **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

inactivation, in comparison to the WT strain, was *L. monocytogenes* Δ*sigB* ($p > 0.05$) (Figure 2b). However, all mutant strains of *L. monocytogenes* (Δ*sigB,* Δ*gadD1*, Δ*gadD2*, Δ*gadD3*) demonstrated higher percentage of sublethal injury as compared to the WT (Figure 4b & 5), indicating that GSE causes significant damage to those mutants, and it is a promising agent for the design of antimicrobial strategies. Additionally, *L. monocytogenes* Δ*sigB* showed a slightly increased sensitivity to CAP treatment when compared to the WT, as demonstrated in the evaluation of sublethal injury (Figure 2a). The higher antimicrobial effect of GSE against Δ*sigB* can be explained by the fact that SigB (σ^B) regulates the general stress response of Gram-positive bacteria like *L. monocytogenes* (Abee, 1999; Guerreiro et al., 2020). More specifically, the gene regulator SigB plays a crucial role in controlling the expression of more than 100 genes involved in various stress responses (see also Table 1) therefore plays a major role in the resistance of *L. monocytogenes* to various treatments (Abee, 1999; Y. Liu et al., 2019; NicAogáin & O'Byrne, 2016; O'Byrne & Karatzas, 2008). Results showing the effect of SigB in a treatment are important as they suggest that at least one of the genes controlled by SigB plays a role in the resistance to this stress. This narrows our investigation regarding the specific mechanisms that contribute to the resistance under a certain stress. SigB has been reported to exhibit increased expression in *L. monocytogenes* as a response to stress, significantly contributing to the adaptability of the bacterium to various types of stress including heat, acid, and osmotic stress. (Boura et al., 2016; Cheng et al., 2015; O'Byrne & Karatzas, 2008; Raengpradub et al., 2008). However, for oxidative stress, the results existing in the literature are contradicting (Boura et al., 2016; Patange, O'Byrne, et al., 2019). For example, Patange et al. (2019) showed that the mutant in *sigB* was more susceptible when exposed to CAP treatment (directly applied dielectric barrier discharge, sealed container, 1-5 min) in comparison to the WT. However, in a study by Boura et al. (2016), Δ*sigB* was more tolerant to oxidative stress (H_2O_2 treatment) than the WT. The latter authors demonstrated that the discrepancies were due

to different oxygen levels during growth, with presence of SigB resulting in high sensitivity to oxidative stress under aerobic conditions and the opposite effect under anaerobic conditions. In our results, the percentage of sub-lethally damaged cells of Δ*sigB* (grown in aerobic condition) was higher in comparison to control, but the viable count was not significantly different in comparison to the WT (Figure 2a, 3b, 4a).

Additionally, to date, the studies on the contribution of SigB in the tolerance to natural antimicrobial treatments are very limited, and there is absence of studies examining its impact to GSE treatment (Begley et al., 2006; Palmer et al., 2009). According to the limited studies on natural antimicrobials (other than GSE), SigB impacts the antimicrobial resistance to certain bacteriocins like nisin and lacticin 3147 (Begley et al., 2006; O'Byrne & Karatzas, 2008) which is in accordance to our results on the sensitivity of Δ*sigB* to GSE (Kitsiou et al., 2023a).

As previously mentioned, there was no significant difference in the microbial inactivation (viable count) between the Δ*gadD1, D2* and WT strains after the GSE treatment (Figure 2b) which can be explained by the fact the GAD system has been primary linked to contribute to acid stress responses (Table 1). However, it was shown that the percentage of sublethal injury of Δ*gadD1, D2 and D3* mutants was higher following the GSE treatment in comparison to the WT (Figure 4b, & 5). After CAP treatment, Δ*gadD2* exhibited the highest level of inactivation in terms of viable count among all strains after CAP treatment (Figure 2a & 3d). Additionally, the absence of *gadD1* and *gadD3* did not result to a higher level of inactivation as compared to *L. monocytogenes* WT after CAP treatment (Figure 2a, 3a, 3c, 3e). The GAD system is crucial for the viability of *L. monocytogenes* under acid stress as is responsible for maintaining the cellular pH in certain optimal range for survival and growth. It comprises 5 proteins, or 3 depending on the strain. Although all strains possess both *gadT2D2* and *gadD3,* the *gadD1T1* operon is missing from serotype 4 *L. monocytogenes* strains (Cotter et al., 2005). Two of the 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 Δ*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* Δ*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

system in oxidative stress (H_2O_2) in 3 strains of *L. monocytogenes* namely EGD-e, LO28 and 10403S. The study demonstrated that *gadD3 and gadD2* play a role in oxidative stress resistance of EGD-e, *gadD1* in LO28 while no role of the GAD system was found in 10403S (Boura et al., 2020). Therefore, overall, several components of the GAD system play a role in oxidative stress while this can depend on the strain, the stage of growth and other environmental conditions such as the type of medium. The CAP treatment utilised in this study, generates reactive oxygen species (ROS) leading to oxidative stress and nitrogen reactive species (RNS), that might result in microbial inactivation (Kitsiou et al., 2023). Hence, the increased microbial inactivation of Δ*gadD2*, observed in this study, may be attributed to the RNS or at the different ROS present in the CAP output species, in comparison to H_2O_2 treatment (Figure 2d). In addition, the lack of response of the GAD system to the CAP treatment might also be related to the stage of growth or the medium used which play an important role (Karatzas et al., 2012). RNS are very reactive and have the ability to modify DNA, lipids, and proteins (Chautrand et al., 2022) while they can also reduce the intracellular pH. The conversion of glutamate to γ-aminobutyric acid (GABA) carried out by the GAD system, might have an indirect role as a cellular defence mechanism against the RNS (Feehily & Karatzas, 2013; Feehily et al., 2014; Karatzas et al., 2012). Additionally, similar to nisin, CAP treatment can cause pore formation leading to the release of ATP. Therefore, the GadD2, which has been shown to be the dominant gene in the GAD system of *L. monocytogenes* 10403S, might help in sustaining the intracellular ATP levels (Aktop et al., 2023; Begley et al., 2010; Feehily et al., 2014). As a result, the absence of this gene might increase the sensitivity of *L. monocytogenes* 10403S to CAP treatment, as shown by our results (Figure 2a & Figure 3d).

In the evaluation of the efficacy of the individual treatments on WT *E. coli* K12 and its mutants *(*Δ*rpoS,* Δ*oxyR,* Δ*dnaK*), it was observed that the individual GSE treatment was inefficient in reducing the microbial population, for all strains under study (Figure 6b & 7).

This is in accordance with a previous published study of our group, showing that 1 % (w/v) did not inhibit both tested Gram-negative wild type strains of *E. coli* and *S.* Typhimurium (Kitsiou et al., 2023a). This trend was expected as it is known in literature that Gram-negative bacteria have a higher level of resistance to natural antimicrobials, as compared to Gram-positive bacteria (Gyawali & Ibrahim, 2014; Kao et al., 2010; Quinto et al., 2019). This difference/resistance, arises from the presence of an outer lipid membrane, which acts as a protective barrier, limiting the penetration of antimicrobial compounds (Corrales et al., 2009). Additionally, during the mild GSE treatment stress adaptation mechanisms could be activated, which help *E. coli* to overcome the imposed stressor (Bearson et al., 2009; Wang et al., 2019; Ziuzina et al., 2015).

The efficacy of the individual CAP treatment was found to be higher against all strains of *E. coli* when compared to its effectiveness against most strains of *L. monocytogenes* (Figure 2a and 6a). It has been observed that Gram-negative bacteria are more sensitive to plasma treatment in comparison to Gram-positive bacteria (Aktop et al., 2023; Lee et al., 2006; Cindy Smet et al., 2018). For example, Smet et al., (2018) examined the inactivation of Gram-positive *L. monocytogenes* and Gram-negative *S.* Typhimurium by CAP (directly applied dielectric barrier discharge, mixture of 4 L/min helium and 40 ml/min oxygen) and observed that *L. monocytogenes* was more tolerant to the CAP treatment. The microbial inactivation of *S.* Typhimurium after 10 min of CAP treatment was approx. 2 log CFU/ml. However, the population of *L. monocytogenes* was reduced by less than 0.5 log CFU/ml (Smet et al., 2018). One of the contributing factors to this difference is the structural characteristics of their cell walls. The thinner peptidoglycan layer in Gram-negative bacteria allows reactive species, such as ROS and RNS generated by CAP, to penetrate more easily into the bacterial cell and cause damage to essential cellular components i.e., proteins and nucleic acids (Mai-Prochnow et al., 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

SoxR (Guo et al., 2019; Storz et al., 1990) that respond to oxidative stress and subsequently activate *soxS* and *sod* that are associated with reactive oxygen species (ROS) defence mechanisms (Patil et al., 2011). Therefore, when cells are under oxidative stress, they produce proteins that contribute to DNA repair or the free radicals elimination. The results of the current study indicate that the transcriptional regulator OxyR plays an important role in the CAP treatment tolerance of *E. coli*, as evidenced by the increase sublethal injury (Figure 5). However, in the absence of *oxyR*, the presence of *soxS* is possibly sufficient for the cells to cope with the oxidative stress caused by CAP treatment or cover for the absence of the former. The sensitivity of the isogenic mutants of *E. coli (*Δ*rpoS*, Δ*oxyR*, Δ*dnaK)* to CAP treatment has been reported in previous studies (Connolly et al., 2013; Han et al., 2016; Li et al., 2013; Perni et al., 2007). The results of these studies are in accordance with the results of the current study, suggesting that RpoS, OxyR and DnaK might play a role in the tolerance of *E. coli* to CAP treatment. However, it is challenging to compare the level of inactivation due to various factors that influence the efficiency of cold atmospheric plasma (CAP). These factors, as previously stated, include the plasma source, the duration of treatment, the system on which it is implemented and the treated level of microbial population (Bahrami et al., 2020; Chizoba Ekezie et al., 2017; Costello al., 2021a; Niakousari et al., 2018; Sharma et al., 2014; Smet et al., 2018). For example, Connolly et al. (2013) explored the inactivation of *E. coli* K12 and its isogenic mutants Δ*soxR*, Δ*soxS*, Δ*oxyR*, Δ*rpoS* and Δ*dnaK* by treating cells of *E. coli* on agar with CAP (dielectric barrier discharge, fixed volume of helium and air mixture) for 5 min. After the treatment, the microbial inactivation of all strains was 1.5 log CFU/cm^2. However, it was noted that Δ*oxyR*, Δ*rpoS* and Δ*dnaK* had a much slower recovery compared to the WT strain indicating that these gene regulators impact the cell's repair mechanisms (Connolly et al., 2013). Additionally, Han et al. (2016) investigated the effects of CAP (dielectric barrier 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

617 *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

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

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

Δ*gadD2D3*, Δ*prfA*, Δ*rsbR*, Δ*lmo0799*, Δ*lmo0799-C56A*). In this study, it was shown that the susceptibility of various strains of *L. monocytogenes* bacteria to CAP treatment was enhanced by exposing them to cold stress. However, the efficacy of CAP treatment was shown to be comparable among the various strains, with the exception of the Δ*rsbR* mutant, which showed an increased inactivation after the combined cold stress and CAP treatment (Patange, O'Byrne, et al., 2019). After the combined acid stress and CAP treatment, all strains of *L. monocytogenes* were completely inactivated indicating a synergistic effect of the tested treatments. To the author's best knowledge there are no studies exploring the combined effect of CAP or natural antimicrobials with other treatments against *E. coli* and its mutants.

As previously stated, the total inactivation of *E. coli* was achieved through a sequenced treatment approach, starting with the application of CAP followed by GSE treatment (Figure 6d & 7). Previous studies have demonstrated that the order of antimicrobial treatments can influence their efficacy and the microbial response, depending on the cellular component they targeted (Chaplot et al., 2019; Costello et al., 2021b; Govaert et al., 2019; Liao et al., 2018). For instance, Chaplot et al. (2019) investigated the hurdle approach of CAP (dielectric barrier discharge, 6 min) and peracetic acid (100 ppm, 6min) against *S.* Typhimurium in raw poultry meat. The CAP treatment followed by peracetic acid resulted in a 3.8 log CFU/cm^2 reduction, however when the reversed order was applied, *S.* Typhimurium was inhibited by 2.5 log CFU/cm^2. In this study it was proposed that the release of active oxygen by peracetic acid, disrupted the sulfhydryl and sulphur bonds present in the cellular membrane resulting in the effective penetration of RONS in the cell and further inactivation caused by RONS interacting with the intracellular components (Chaplot et al., 2019). The proposed inactivation mechanism for the combined treatment against *L. monocytogenes* involves firstly the penetration of GSE in the bacterial cells and the interaction with their intracellular components (Begg, 2019; 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.

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Table legends

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

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

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

***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

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

***Figure 5*:** Quantification of sub-lethally injured cells (%) 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 2 h, (■) Combination of 1 % (w/v) GSE (2 h) and CAP treatment (4 min). In cases where the viable cell count in the selective media was below detection limit (<10 CFU/ml) the number of sublethal damaged cells was set to 100 % (bar with stripes). 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 $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

***Figure 6*:** Reduction (log CFU/ml) of the viable population *E. coli* K12 (WT and mutants) followed by (a) CAP (4 min), (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP treatment (4 min) (d) CAP (4 min) following with 1% (w/v) GSE treatment in TSBYE. In all plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*, (■) Δ*dnaK*. Data are normalised with respect to untreated controls for all conditions under study. In cases where the viable cell count in the selective media was below detection limit (<10 CFU/ml) the reduction is portrayed as total inactivation (bar with stripes). 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 $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

***Figure 7*:** Viable counts of *E. coli* K12 (a) WT, (b) Δ*rpoS* (c) Δ*oxyR*, (d) Δ*dnaK* 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 2 h, (■) Treatment with 1 % (w/v) GSE (2 h) following with CAP treatment (4 min), (■) Treatment with CAP (4 min) following with 1% (w/v) GSE treatment (2 h). In cases where the viable cell count was below the detection limit (<10 CFU/ml) the number was set to 1 log CFU/ml. 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 control and treated samples (* if $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

***Figure 8*:** Sublethal injury (%) of *E. coli* K12 (WT and mutants) induced by (a) CAP (4 min), (b) GSE (2h), (c) 1 % (w/v) GSE (2 h) following with CAP treatment (4 min) (d) CAP (4 min) following with 1% (w/v) GSE treatment in TSBYE. Data are normalised with respect to untreated controls for all conditions under study. In all plots, (■) WT, (■) Δ*rpoS*, (■) Δ*oxyR*, (■) Δ*dnaK*. In cases where the viable cell count in the selective media was below detection limit (<10 CFU/ml) the reduction is portrayed as total inactivation (bar with stripes). Each bar represents the average of two independent experiments with three technical replicates per experiment while error bars represent the standard deviation. Connecting lines with asterisks indicate significant differences between control and treated samples (* if $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

***Figure 9*:** Quantification of sub-lethally injured cells (%) of *E. coli* K12 (a) WT, (b) Δ*rpoS*, (c) Δ*oxyR*, (d) Δ*dnaK* 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, (■) treatment with 1% (w/v) GSE (2h) following with CAP treatment (4 min), (■) treatment 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 $0.01<p \leq 0.05$, ** if $0.001< p \leq 0.01$, *** if $0.0001< p \leq 0.001$, *** if $p \leq 0.0001$)

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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. -*gadD1* active in moderately acidic condition -*gadD2* active in severe acidic condition -*gadD3* associated with the intracellular glutamic acid decarboxylase system (GADi).	Feehily et al., 2014; Karatzas et al., 2012
	Δ*gadD2*		
	Δg*adD3*		
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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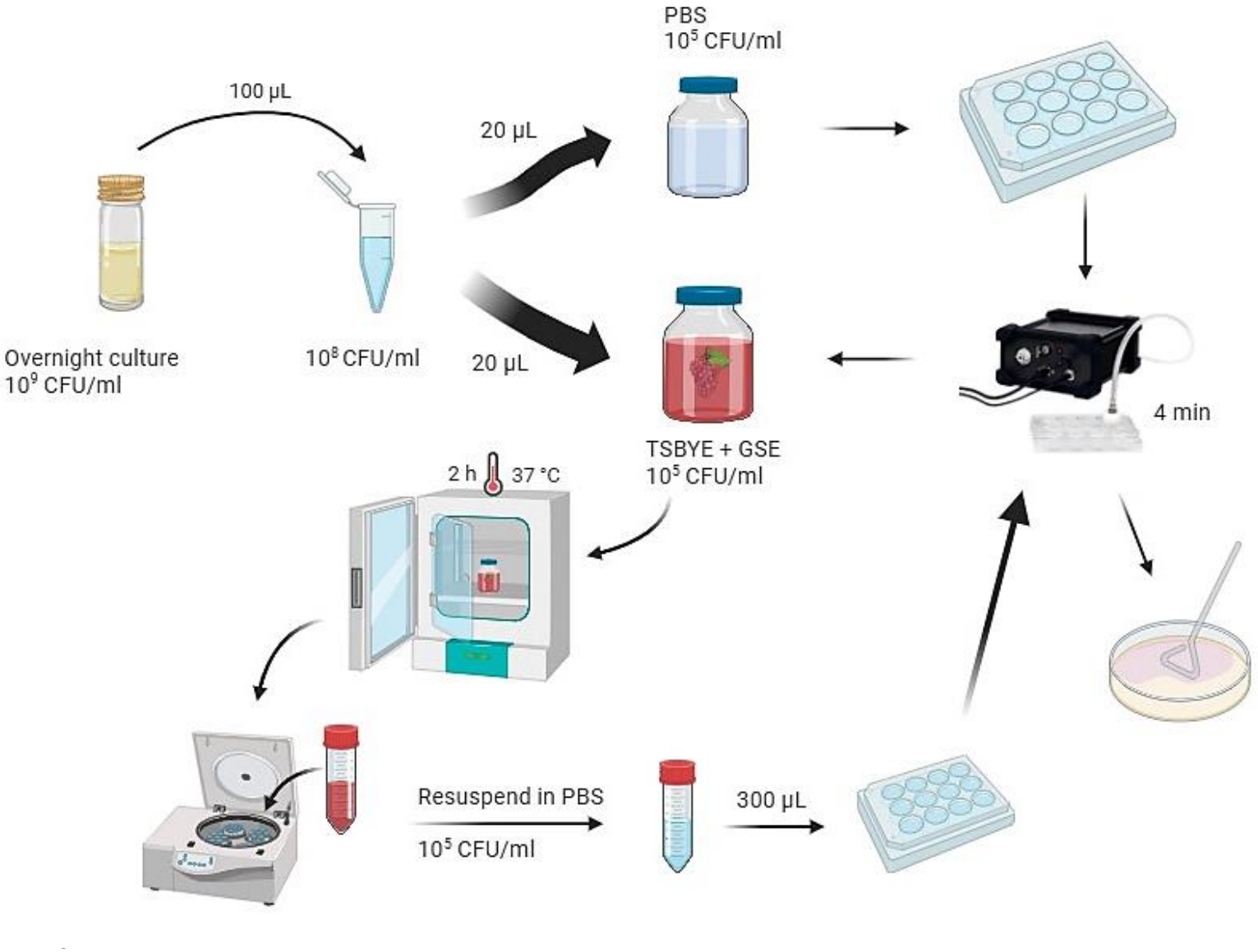

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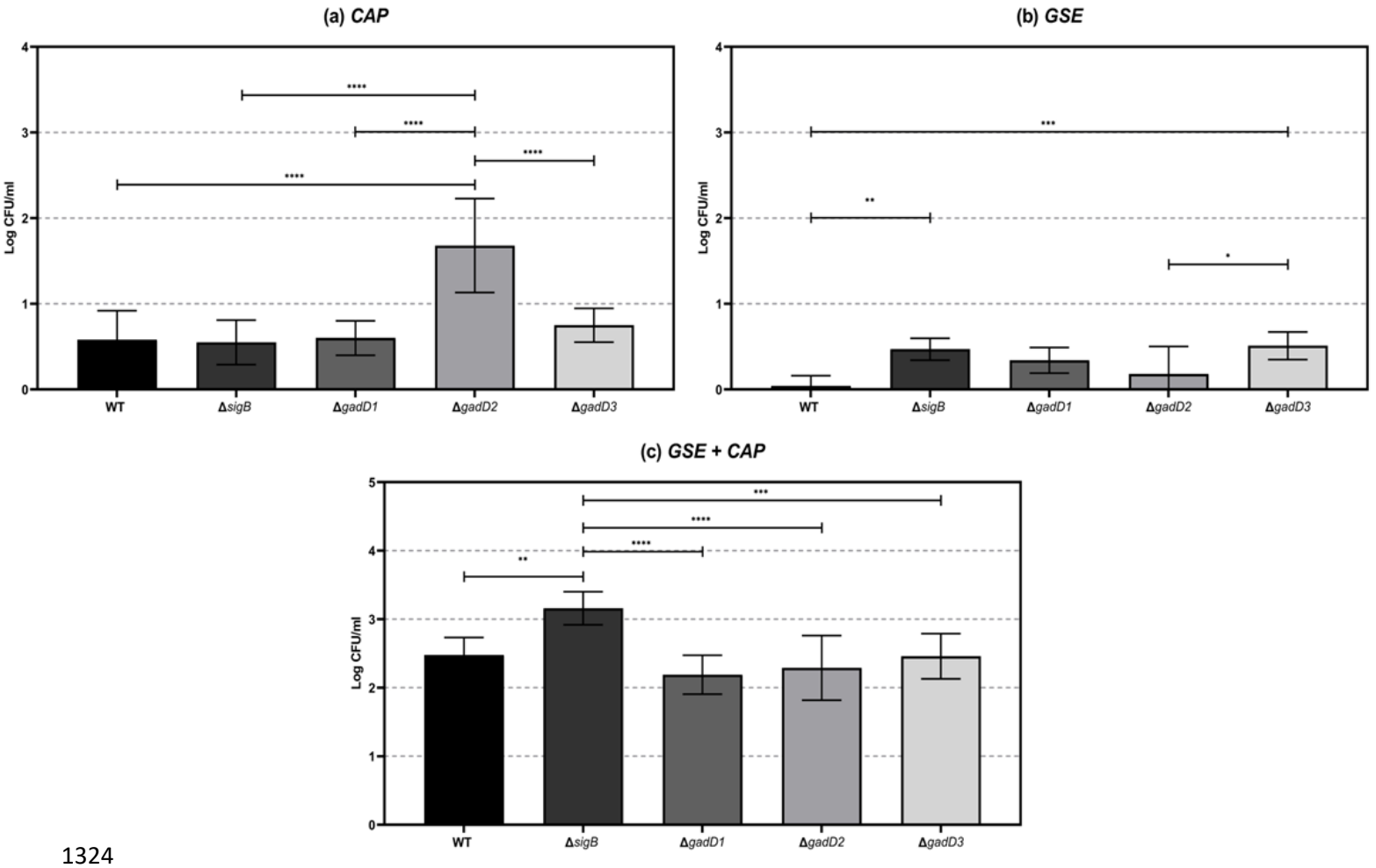
(a) CAP
Log CFU/ml
0
1
2
3
4
****
****
****
****
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3
(b) GSE
Log CFU/ml
0
1
2
3
4
***
**
*
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3
(c) GSE + CAP
Log CFU/ml
0
1
2
3
4
5
***
****
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**
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3

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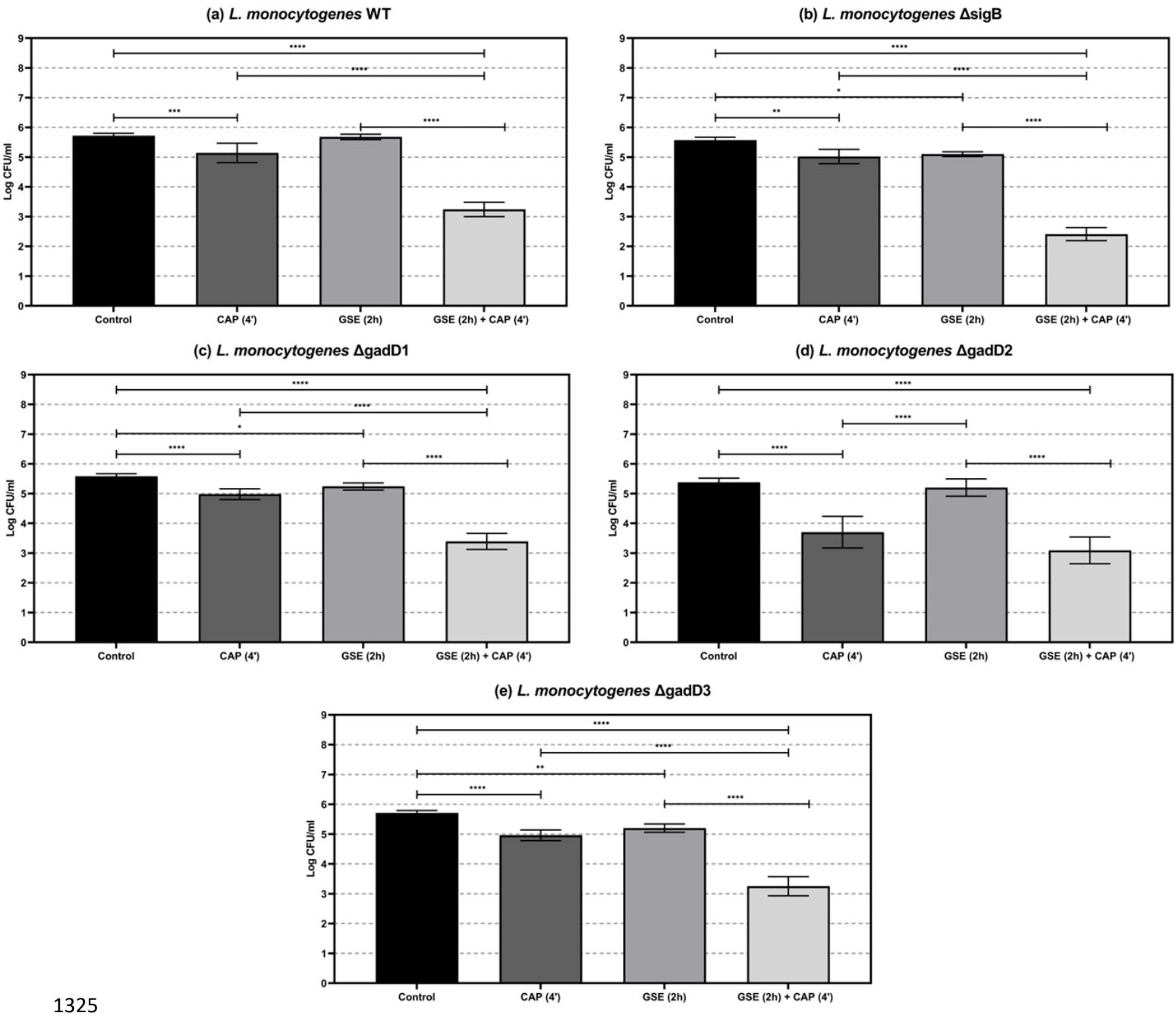
(a) L. monocytogenes WT
(b) L. monocytogenes ΔsigB
(c) L. monocytogenes ΔgadD1
(d) L. monocytogenes ΔgadD2
(e) L. monocytogenes ΔgadD3
Log CFU/ml
Control
CAP (4')
GSE (2h)
GSE (2h) + CAP (4')

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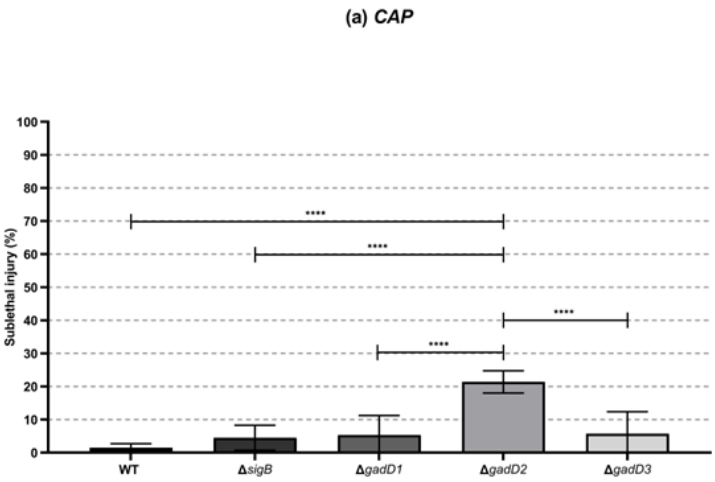
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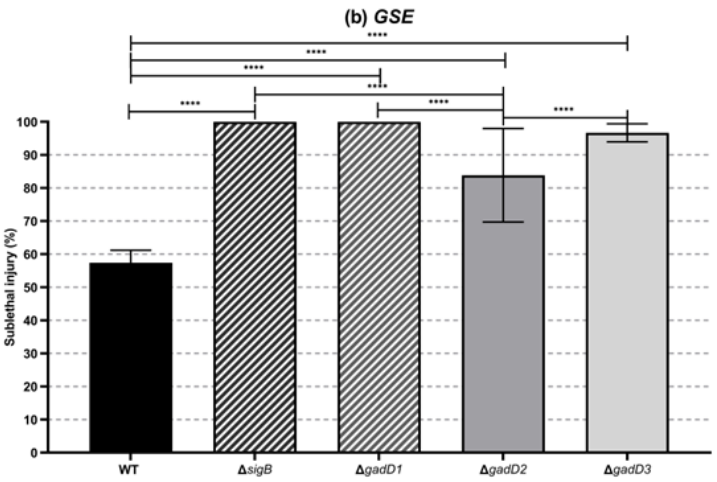
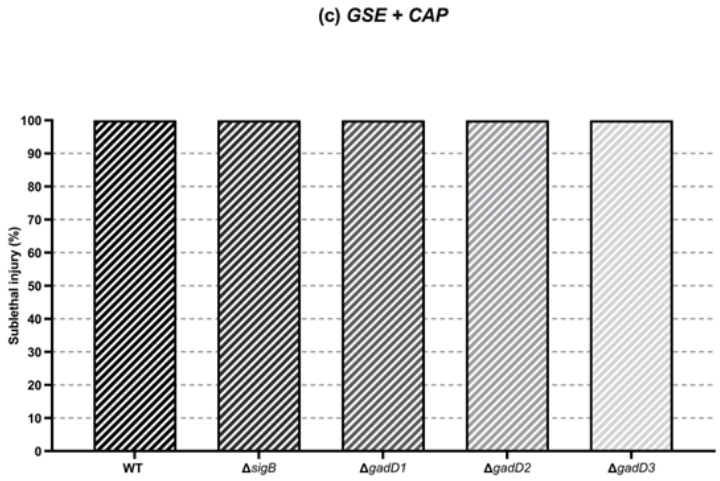
Sublethal injury (%)
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3


(b) GSE
Sublethal injury (%)
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3


(c) GSE + CAP
Sublethal injury (%)
WT
ΔsigB
ΔgadD1
ΔgadD2
ΔgadD3

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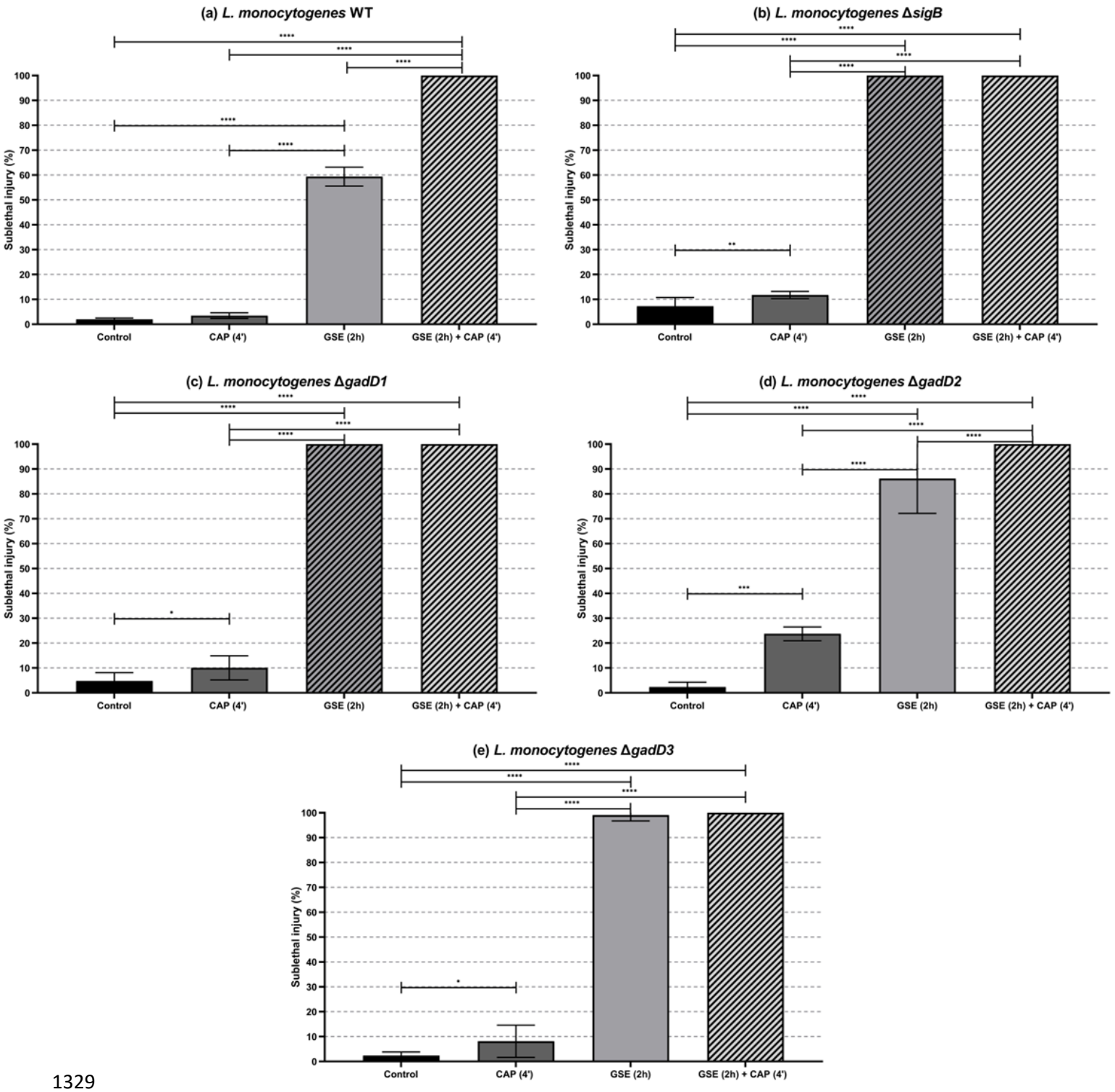
(a) L. monocytogenes WT
(b) L. monocytogenes ΔsigB
(c) L. monocytogenes ΔgadD1
(d) L. monocytogenes ΔgadD2
(e) L. monocytogenes ΔgadD3
Sublethal injury (%)
Control
CAP (4')
GSE (2h)
GSE (2h) + CAP (4')

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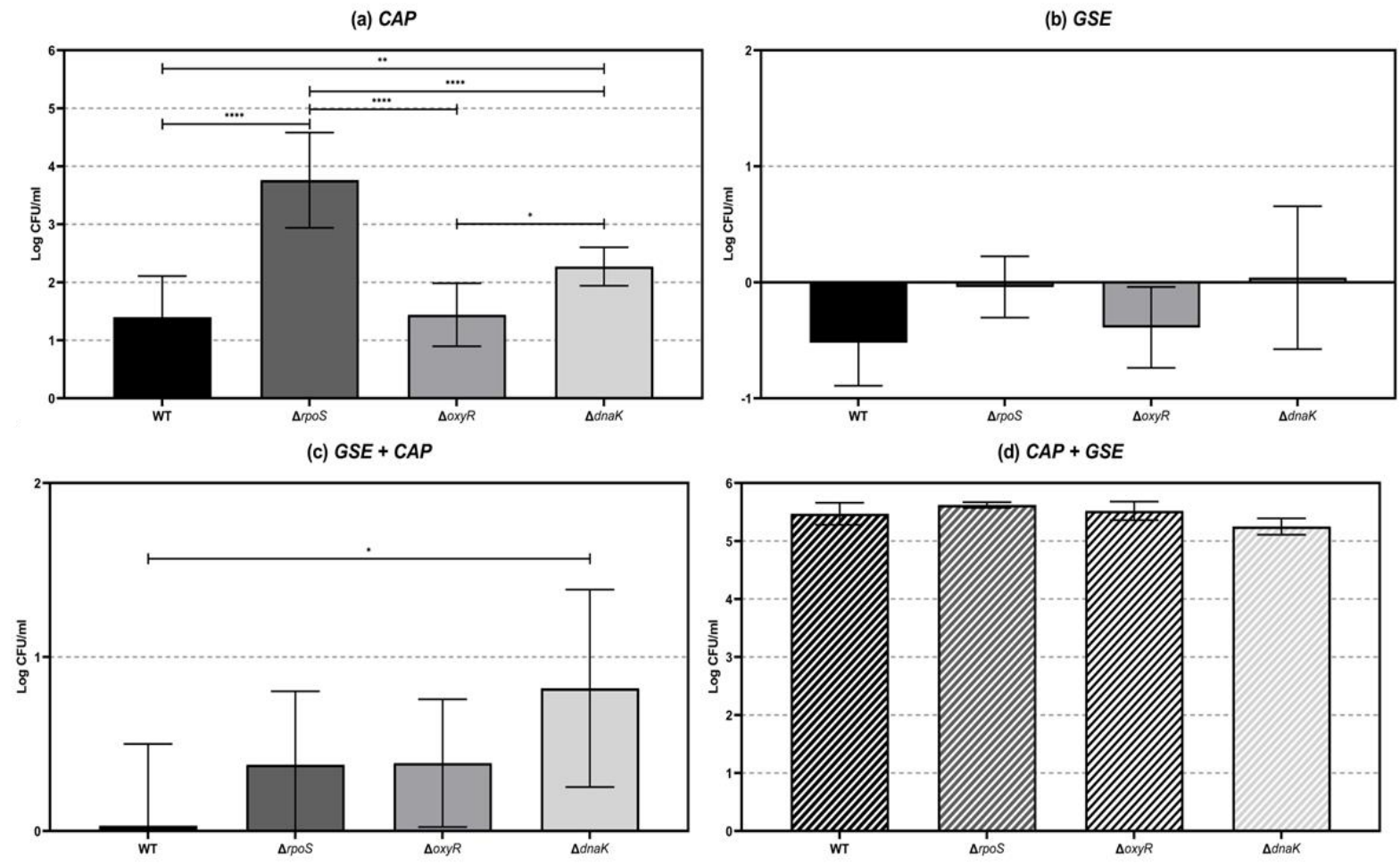
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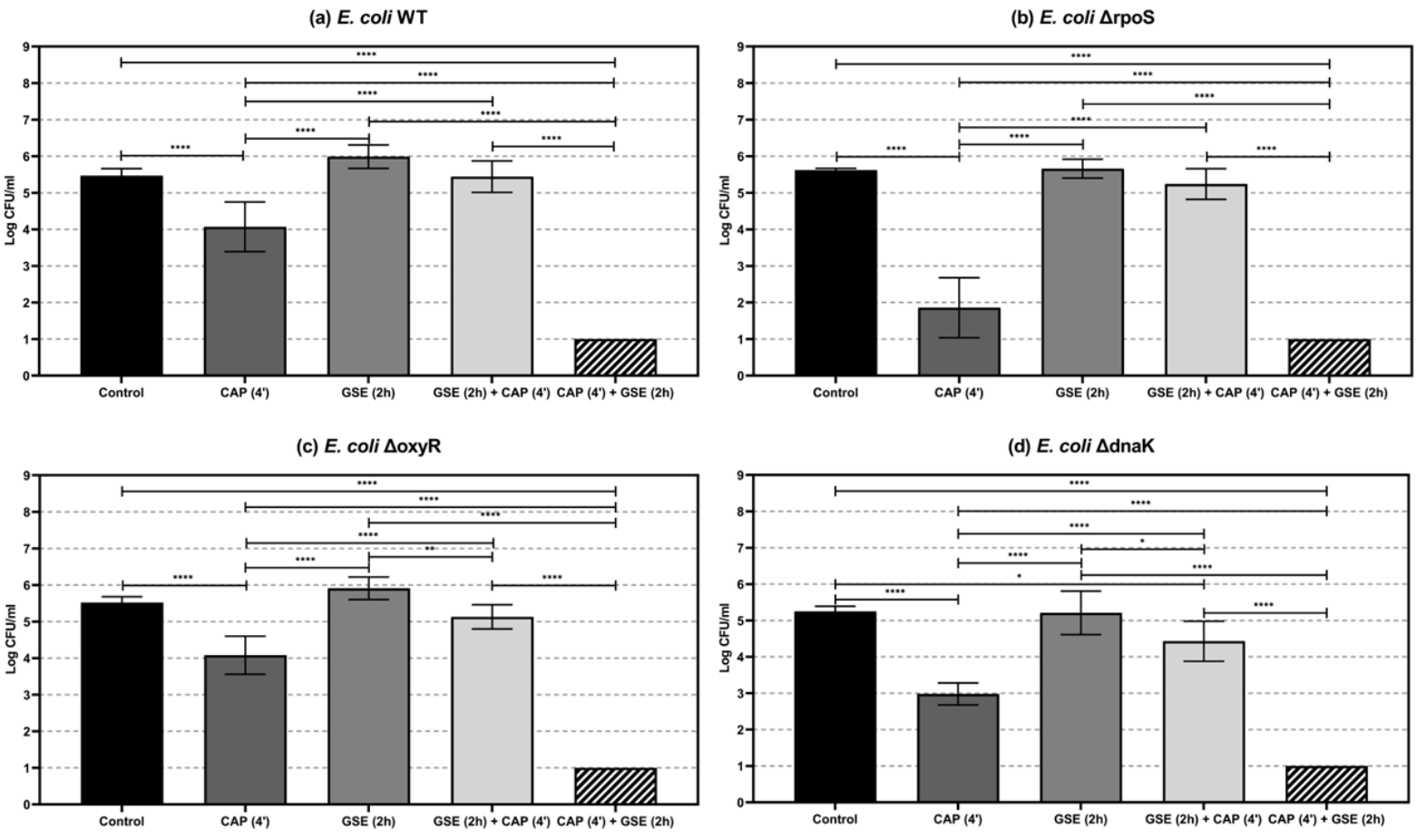
Log CFU/ml
WT
ΔrpoS
ΔoxyR
ΔdnaK
(b) GSE
Log CFU/ml
WT
ΔrpoS
ΔoxyR
ΔdnaK
(c) GSE + CAP
Log CFU/ml
WT
ΔrpoS
ΔoxyR
ΔdnaK
(d) CAP + GSE
Log CFU/ml
WT
ΔrpoS
ΔoxyR
ΔdnaK


(a) E. coli WT
(b) E. coli ΔrpoS
(c) E. coli ΔoxyR
(d) E. coli ΔdnaK
Log CFU/ml
Control
CAP (4')
GSE (2h)
GSE (2h) + CAP (4')
CAP (4') + GSE (2h)

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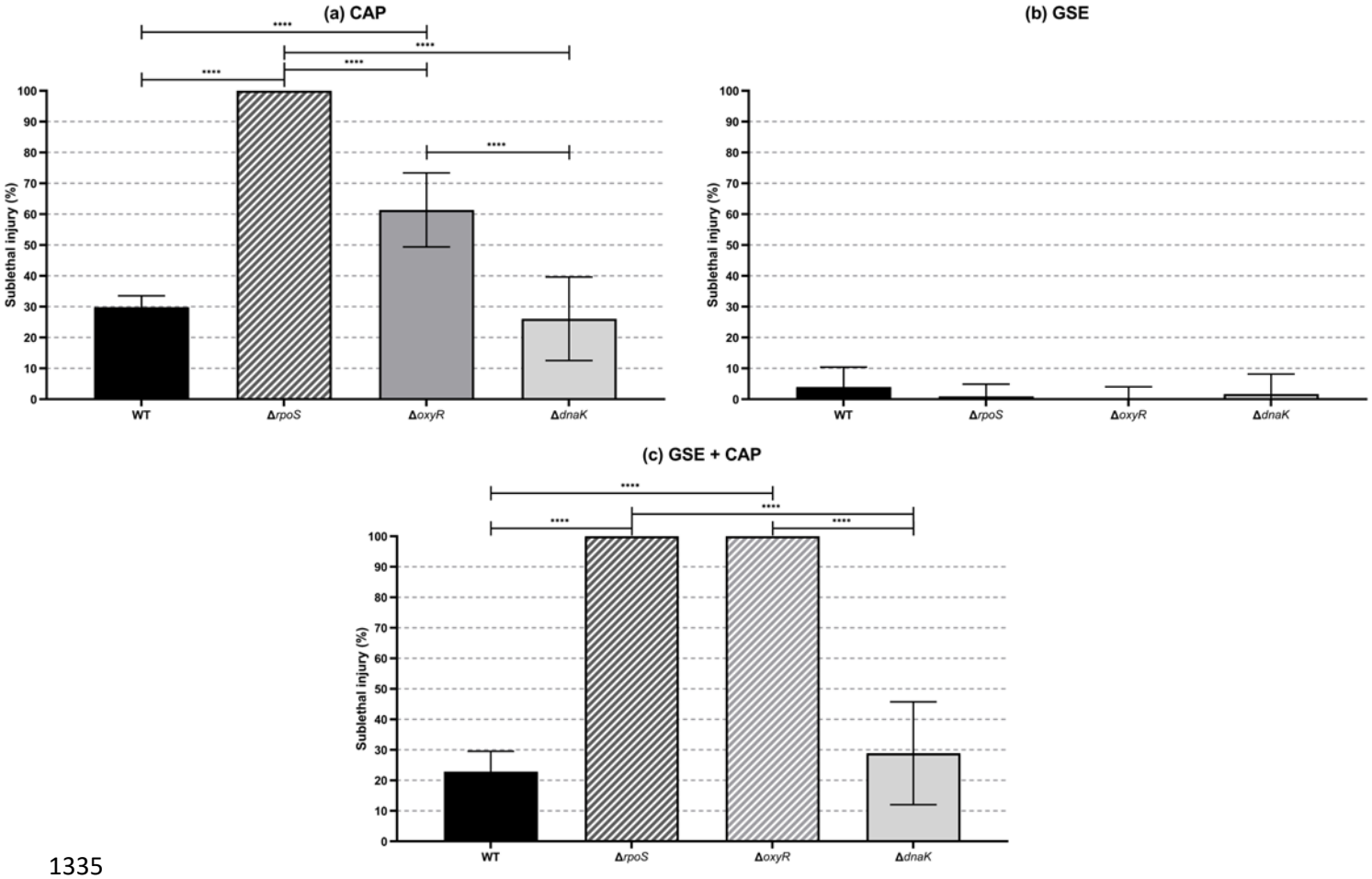

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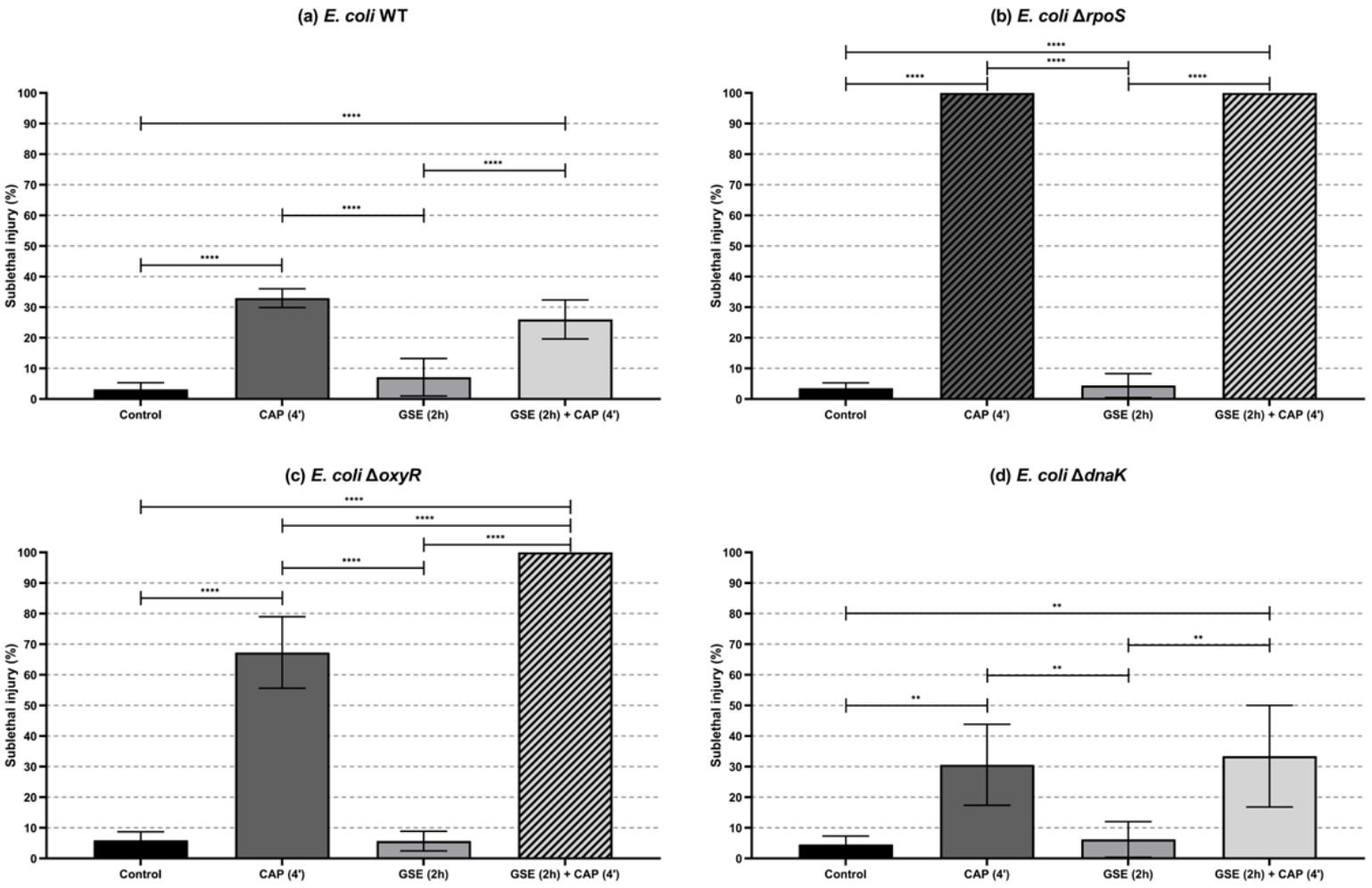
(a) E. coli WT
(b) E. coli ΔrpoS
(c) E. coli ΔoxyR
(d) E. coli ΔdnaK
Sublethal injury (%)
Control
CAP (4')
GSE (2h)
GSE (2h) + CAP (4')
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1339