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Research Paper

Mature *Listeria monocytogenes* Biofilms Exhibit Reduced Susceptibility to Sanitizers – Relevance to the (Leafy Green) Fresh Food Supply Chain ☆

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ABSTRACT

Salads and herbs are important for a healthy diet but during their processing and packaging, they may be exposed to environmental contamination from foodborne pathogens. Of particular concern *is Listeria monocytogenes*, a Gram-positive, facultative anaerobe, found ubiquitously in the environment. *L. monocytogenes* can survive and grow under harsh conditions such as refrigeration temperatures, low oxygen or nutrient concentrations, which is why it is a problem in the fresh food supply chain. Infection with *L. monocytogenes* can result in listeriosis, potentially fatal in immunocompromised patients, pregnant women, newborn babies, and the elderly. This study aims to evaluate the efficacy of common sanitizing methods used in the fresh food supply chain, where biofilm formation has raised concerns, using appropriately developed laboratory models of *Listeria* biofilms. *L. monocytogenes* Scott A, *L. monocytogenes* CECT 936, and *L. innocua* NCTC 12210 biofilms were grown at 20 °C or 4 °C, on stainless steel coupons for 7 days, and treated with high concentrations of chlorine (up to 300 ppm) or peracetic acid (up to 500 ppm) on days 1, 3, 5, and 7. Coupons were then processed for culturable cell counts on treatment days, and imaged using episcopic differential interference microscopy, coupled with epi-fluorescence microscopy (EDIC/EF) on day 7 of growth. This determined that temperature affects biofilm growth on stainless steel, as biofilm growth reached ~8 $\log_{10}$ CFU/cm^2 at 20 °C, but was significantly lower at 4 °C (~4 $\log_{10}$ CFU/cm^2) – highlighting the importance of maintaining a cold chain. Chlorine and peracetic acid were shown to be effective at treating *Listeria* in the planktonic form but were not effective at treating aged biofilms at both temperatures and the high concentrations of sanitizers used. This work provides important information on sanitizing efforts in the fresh food supply chain, concerning factory temperature, processing surfaces, and the age of biofilm.

Food safety and security are increasingly important, as by 2050, the world's population will have exceeded 9 billion, meaning that 70% more food will be needed to sustain the growing population (Ghosh et al., 2024). Foodborne pathogens pose a major challenge to food safety, with approximately one in ten people falling ill every year after consuming contaminated food, resulting in 420,000 deaths globally (World Health Organisation, 2022). A major pathogen of concern is *Listeria monocytogenes,* a Gram-positive, facultative anaerobe, found ubiquitously in the environment, with the highest case mortality rate of all major foodborne pathogens (20–30%) (Huang et al., 2023; Koopmans et al., 2023). *L. monocytogenes* is the causative agent of listeriosis, a foodborne disease whereby transmission is through the consumption of contaminated food (Finn et al., 2023). High risk groups, such as the elderly, pregnant women, neonates, and those with a compromised immune system (Lourenco et al., 2022), are more likely to contract the invasive form of listeriosis which can result in various symptoms including sepsis, meningitis, encephalitis, and spontaneous abortion (Huang et al., 2023; Koopmans et al., 2023; Lourenco et al., 2022).

The ability of *L. monocytogenes* to survive and grow under stressful conditions, such as low temperatures and low nutrient or oxygen concentrations (Chlebicz & Śliżewska, 2018), is a concern for the fresh food industry, where ready-to-eat food such as salads and herbs is consumed without the final decontamination procedure of cooking. One

Abbreviations: BHIA, brain heart infusion agar; BHIB, brain heart infusion broth; PAA, peracetic acid/peroxyacetic acid; PI, propidium iodide; EDIC/EF, episcopic differential interference contrast and epifluorescence; eDNA, extracellular DNA.

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way, *L. monocytogenes* is able to survive, is by forming biofilms (Agustín & Brugnoni, 2018; Fagerlund et al., 2021), complex 3D communities of bacteria adhered to a surface and encapsulated in a self-produced matrix of extracellular polymeric substances (EPS). The EPS provides structure, allows for nutrient exchange and the sharing of genetic information, and also provides protection from external factors, such as shear forces and disinfection by biocide assimilation (Flemming et al., 2007). These factors mean that once biofilms become established in the fresh food supply chain, they are very hard to remove. *L. monocytogenes* biofilms have been shown to exist for years in the same factory (Fagerlund et al., 2021; Ferreira et al., 2014; Tompkin, 2002), which can lead to recurrent outbreaks, due to its potential in contaminating food (Fagerlund et al., 2021; Malley et al., 2015).

Oxidizing agents such as chlorine and peracetic acid are often used throughout fresh food processing factories as part of their disinfection processes, as these chemicals are relatively low cost and have little impact on the quality of fresh produce (Castro-Ibáñez et al., 2017). However, they have been shown to have varying results on treating *L. monocytogenes* contamination, especially when in biofilms (Aryal & Muriana, 2019; Korany et al., 2018; Olszewska & Diez-Gonzalez, 2021). As well as regular cleaning, maintenance of a cold chain throughout the fresh food supply chain is vital to reduce growth of potential microbial contaminants (Castro-Ibáñez et al., 2017; Francis et al., 2012). Despite this, temperatures can fluctuate from ~4°C upward toward ~20 °C in certain areas of the factory (Piercey et al., 2016). This study aimed to investigate the efficacy of sanitizers commonly used in the fresh food supply chain on treating *Listeria* biofilms, at different levels of establishment and growth temperature.

There are 14 *L. monocytogenes* serotypes (Muchaamba et al., 2022). The majority of listeriosis outbreaks globally are associated with serotype 4b, although serotype prevalence varies by region. *L. monocytogenes* Scott A is a well-characterized strain of serotype 4b notable for its high virulence (Muchaamba et al., 2022; Swaminathan & Gerner-Smidt, 2007). Listeriosis infections have also been linked to 1/2a and 1/b serotypes, but much less frequently (Swaminathan & Gerner-Smidt, 2007; Tompkin, 2002). Subsequently, three *Listeria* strains were used in this study*: L. monocytogenes* Scott A (serotype 4b), originally isolated from a cheese-associated outbreak in Massachusetts and widely used in biofilm research (Briers et al., 2011); *L. monocytogenes* CECT 936 (serotype 1/2b), a clinically relevant reference strain, frequently used in food safety studies (Ballesteros et al., 2011; Rodríguez-Melcón et al., 2025); and *L. innocua* NCTC 12210, a nonpathogenic isolate from English-produced cheese (UKHSA, 2003). Strains were selected due to their ubiquity in nature and relevance to foodborne disease outbreaks. Despite being avirulent, *L. innocua* was selected for its biofilm-forming ability (Costa et al., 2018; Perni et al., 2006) – biofilms in the fresh food supply chain will most likely be polymicrobial (Alvarez-Ordóñez et al., 2019) and reduced susceptibility to sanitization from avirulent strains may afford protection to virulent strains.

Previous studies have investigated the growth and protective effect of *L. monocytogenes* biofilms at room temperatures (Banire & Jia, 2014; Hua et al., 2019; Korany et al., 2018), but few have investigated biofilm maturity as a measure of sanitizer tolerance. This study offers a comprehensive investigation including multiple species, sanitizers, and temperatures, all relevant to the fresh food supply chain of leafy greens. The results of this study could provide important improvements in sanitization procedures in the food industry and therefore reduce the numbers of future foodborne disease outbreaks.

Materials and methods

Bacterial strains and culture preparation. *L. monocytogenes* Scott A was obtained from the Spanish Type Culture Collection, and *L. monocytogenes* CECT 936 and *L. innocua* NCTC 12210 were obtained from the University of Southampton Culture Collection. These strains were selected to represent clinically important *L. monocytogenes* lineages (4b and 1/2b) as well as a nonpathogenic reference strain. Cryopreserved cultures of each strain were revived by streak plating onto brain heart infusion agar (BHIA, Sigma-Aldrich) and then incubated overnight at 37 °C. For each experiment, a single colony was used to inoculate 10 mL of brain heart infusion broth (BHIB, Sigma-Aldrich), again incubated overnight at 37 °C to generate planktonic cultures for downstream experiments.

Biofilm assays. Experiments were performed in triplicate. Overnight cultures were pelleted using a centrifuge (Heraeus Megafuge, Thermo Scientific) at 4,000*g* for 10 min at room temperature. The growth medium was decanted, and the pellet was resuspended in 10 mL phosphate-buffered saline (PBS, Oxoid) using a vortex mixer. Centrifugation and resuspension were repeated again to remove remaining growth medium and then diluted to ~10^5 CFU/mL into 20% BHIB. Two milliliters of diluted culture was added to each well of a 12-well plate, each containing an autoclaved stainless steel coupon (1 cm^2; 2B finish), and the plates were incubated overnight at either room temperature (~20 °C) or refrigeration temperature (4 °C) to produce static, single species biofilms. Humidity of both environments was recorded at 53 ± 3% using a Fisherbrand Traceable humidity monitor (Fisher Scientific). Spent medium was removed from each well and replaced with 2 mL of fresh 20% BHIB every day for 7 days. Plates were housed within a plastic container to minimize contamination from the laboratory environment.

Chlorine treatment. Biofilms were treated with chlorine on days 1, 3, 5, or 7. A 5000 ppm stock concentration of chlorine was prepared by dissolving one Haz-Tab (St John's Ambulance, UK) in 200 mL of dH_2O. On treatment days, spent medium was removed carefully from the wells and replaced with PBS; chlorine was added from the stock chlorine solution to make final concentrations of 0 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2. Concentrations were checked using semiquantitative colorimetric chlorine test strips, which measured free chlorine (0–500 mg/L Cl_2) (McQuant, Merck). The 12-well plates were then placed on a gyratory rocker (ST9, Bibby Stuart) at 40 rpm, to ensure that the chlorine was well dispersed across the biofilms. After 10 min, sodium thiosulfate (anhydrous) (Fisher Chemical) was added to quench the chlorine and stop it from having further effect. A 0.0161 M stock concentration of sodium thiosulfate was made by dissolving 399.26 mg in 100 mL of dH_2O, and sufficient stock was added to each biofilm to quench the chlorine treatment (equivalent to ~4.08 μl per ppm Cl_2 in a 2 mL sample). Quenching was carried out for 10 min on a gyratory rocker at 40 rpm and was confirmed using chlorine test strips (no color change meant all chlorine had been quenched).

Peracetic acid treatment. The same protocol was followed as with the chlorine-treated biofilms; however, a 1,000 ppm stock of peracetic acid was prepared by dissolving one Peracide tablet (CK Consumables, UK) in 500 mL of dH_2O. Biofilms were treated with 0 ppm, 25 ppm, 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm of peracetic acid on days 1, 3, 5, or 7 – concentrations were validated using semiquantitative colorimetric peracetic acid test strips (0–500 mg/L) (Johnson J-QUANT, Gem Scientific, UK). Peracetic acid was also quenched after 10 min with sodium thiosulfate.

Culturable cell counts. To quantify biofilm growth, stainless steel coupons were vortexed for 2 min in 1 mL PBS with approximately 100 sterilized 2 mm glass beads (Hecht Karl™, Fisher Scientific), serially diluted with PBS and plated onto BHIA in triplicate to quantify culturable cell numbers. The limit of detection was 200 CFU/mL.

Planktonic experiments. Overnight cultures were pelleted as described before and diluted to ~10^7 CFU/mL in PBS. Resuspended overnight cultures were then treated with chlorine or peracetic acid for 10 min before quenching with sodium thiosulfate, as performed

before. Cultures were then serially diluted in PBS and plated out onto BHIA.

Epifluorescence microscopy. Biofilms were grown at both room temperature and refrigeration temperature and were treated with chlorine or peracetic acid on day 7, as described before. After treatment, chlorine was removed and replaced with 1 mL of LIVE/DEAD™ BacLight™ Bacterial Viability Kit (ThermoFisher Scientific), prepared at a concentration of 3 μl/mL of fluorophores SYTO 9 and propidium iodide (PI). Biofilms were stained for 20 min in the dark, the coupons were moved to wells containing 2 mL PBS for 3 min to wash off residual dye, and allowed to air dry in the dark. The coupons were examined with episcopic differential interference microscopy/epifluorescence microscopy (EDIC/EF) (Keevil, 2003) on a Nikon Eclipse ME600 microscope (Best Scientific, Swindon, UK), using long working distance objectives. All images were taken using a 100x air objective (1,000× mag); three biological repeats per treatment concentration were carried out, with five images taken per coupon. Representative images were processed in ImageJ (version 1.53 t).

Statistical analysis. The number of bacterial cells recovered from the stainless steel coupons was converted to $\log_{10}(CFU/cm^2 + 1)$. For each treatment and timepoint, three biological repeats were analyzed as independent measures in GraphPad Prism (version 10.5.0(774)) using nonparametric testing (Kruskal-Wallis) followed by Dunn's post-hoc tests. Analyses were performed separately for each day of biofilm growth and for planktonic assays. Each treatment was compared only to its corresponding control within the same timepoint, rather than across all groups, and therefore, *p*-values were not adjusted. Uncorrected Dunn's posthoc test was also used to compare control biofilms grown at 20 °C and 4 °C. All graphs were produced in GraphPad Prism.

Results

L. monocytogenes Scott A, *L. monocytogenes* CECT 936, and *L. innocua* all formed biofilms when grown at 20 °C, with significantly higher numbers of culturable cell counts than biofilms grown at 4 °C (Figs. 1–3). Room temperature biofilms were established by day 3 of growth (Fig. 1), reaching >7 log CFU/cm^2, and were stable at these numbers for days 5 and 7 of growth. However, refrigeration temperature biofilms were consistently ~3 log lower than room temperature biofilms at days 3, 5, and 7 of growth.

After 1 day of growth, all 3 *Listeria* biofilms show tolerance to treatment with chlorine and peracetic acid when grown at room temperature (Figs. 1–3). Conversely, there was a significant reduction in CFU counts in day 1 refrigeration temperature biofilm in *L. monocytogenes* Scott A and *L. innocua*, with the greatest reduction from the highest treatment concentrations. There was a 2-log reduction in CFU counts in day 1 *L. monocytogenes* Scott A biofilms when treated with 200 ppm and 300 ppm Cl_2, and a 2-log reduction when treated with 500 ppm PAA (Fig. 1a + b), and similar trends were seen in the other two strains grown at 4 °C. There were significant reductions in day 1, *L. innocua* biofilms grown at 4 °C, with 2-log reductions at 200 ppm and 300 ppm Cl_2, and >2-log reductions at 300 ppm, 400 ppm, and 500 ppm of PAA. A similar pattern was observed in *L. monocytogenes* CECT 936 4 °C biofilms, with >2-log reductions in CFU counts when treated with 150 ppm, 200 ppm and 300 ppm Cl_2 (Fig. 2a). PAA produced a 2-log reduction at 200 ppm PAA and 3-log reductions at concentrations 300 ppm, 400 ppm, and 500 ppm PAA (Fig. 2b). This pattern of sanitizer tolerance was also observed with the *Listeria* strains at lower, more industrially relevant concentrations (Supplementary Figs. 1–3).

There was an increase in CFU counts of day 3 *L. monocytogenes* Scott A refrigeration biofilms when treated with chlorine (Fig. 1), at all of the treatment concentrations. Furthermore, older (day 5 and day 7) refrigeration temperature *L. monocytogenes* Scott A biofilms were tolerant to both chlorine and peracetic acid treatment (Fig. 1).

It is evident that when *Listeria* forms a biofilm, it exhibits reduced susceptibility to both chlorine and peracetic acid treatment, as planktonic results (Fig. 4) show complete eradication of *L. monocytogenes* Scott A at 50 ppm Cl_2 and 25 ppm PAA. *L. monocytogenes* CECT 936 is eradicated at 50 ppm Cl_2, and *L. innocua* at 25 ppm Cl_2 and 50 ppm PAA. Despite the multiple comparisons in Figures 1–3 showing significant reductions when compared to the control, the CFU counts remain stable for each of the treatment concentrations – showing the favorable growth of *Listeria* when in a biofilm, and can be seen at both temperatures.

Epifluorescent microscopy was carried out on established day 7 biofilms and representative images (Figs. 1–3) of SYTO 9 and PI costaining of biofilms depict dense biofilm coverage on biofilms grown at 20 °C than biofilms grown at 4 °C, which supports the culturable cell counts. Microscopy images of the higher treatment concentrations depict larger amounts of PI staining, which is clearer in the room temperature biofilms; despite this, there is still a large amount of viable cells (complementing the culturable cell counts found). EDIC/EF micrographs were taken to illustrate the distribution of the *L. monocytogenes* growth on the stainless steel surface. The biofilm tended to grow in cracks and troughs across the steel surface (Fig. 5).

Discussion

The current study has examined the antimicrobial efficacy of two different sanitizers commonly used in the fresh food supply chain, chlorine and peracetic acid, on two pathogenic strains of *L. monocytogenes* Scott A and CECT 936, and avirulent *L. innocua* NCTC 12210. All biofilms were grown as single-species static biofilms, on stainless steel, at either room temperature (20 °C) or refrigeration temperature (4 °C). The static model was chosen to be the most representative as the majority of factory surfaces are not exposed to a continuous flow of fluid and also can be used to represent hard-to-clean areas where a biofilm can be left undisturbed for a period of time.

A comprehensive range of different treatment concentrations have been used: a lower range (0–100 ppm for both Cl_2 and PAA) (Supplementary Figs. 1–3) that is currently recommended for use in the production of fresh food (Petri et al., 2021; Ruiz-Cruz et al., 2007), and a higher concentration range (150–300 ppm for Cl_2 and 200–500 ppm for PAA) (Figs. 1–3). The higher concentrations investigated in this study were chosen after initial experiments found that all three bacterial biofilms were tolerant to the lower range of sanitizer concentrations.

This study shows that *L. monocytogenes* Scott A, *L. monocytogenes* CECT 936, and *L. innocua* not only form good biofilms when grown at room temperature but also are able to form biofilms at 4 °C. Culturable cell counts of biofilms grown at 4 °C were approximately 3-log lower than at 20 °C for all species tested, which highlights the importance of maintaining the cold chain throughout the production process. This is also evidenced in the microscopy images, which depict denser and thicker biofilms at 20 °C, and sparse and fewer cells in 4 °C biofilms. The data obtained from the culturable cell counts clearly show that *Listeria* is able to survive and persist after very high doses of both chlorine and peracetic acid, when in a biofilm. A potential reason for biofilm tolerance to sanitizers is the EPS, which acts as a physical barrier from the environment and has been shown to prevent sanitizer entry and sequester toxins (Flemming et al., 2007).

EDIC/EF images taken of SYTO 9 and PI costained 20 °C biofilms, depict greater amounts of red fluorescence coverage (bound PI) as the chlorine or peracetic acid concentration is increased. This implies that there is an increase in dead cells, as PI is understood to bind to the DNA of dead or damaged cells – contradicting the culturable cell counts reported here. However, PI has also been found to bind to extracellular DNA (eDNA) (Rosenberg et al., 2019), which is a major component of the EPS in biofilms, mediating bacterial attachment

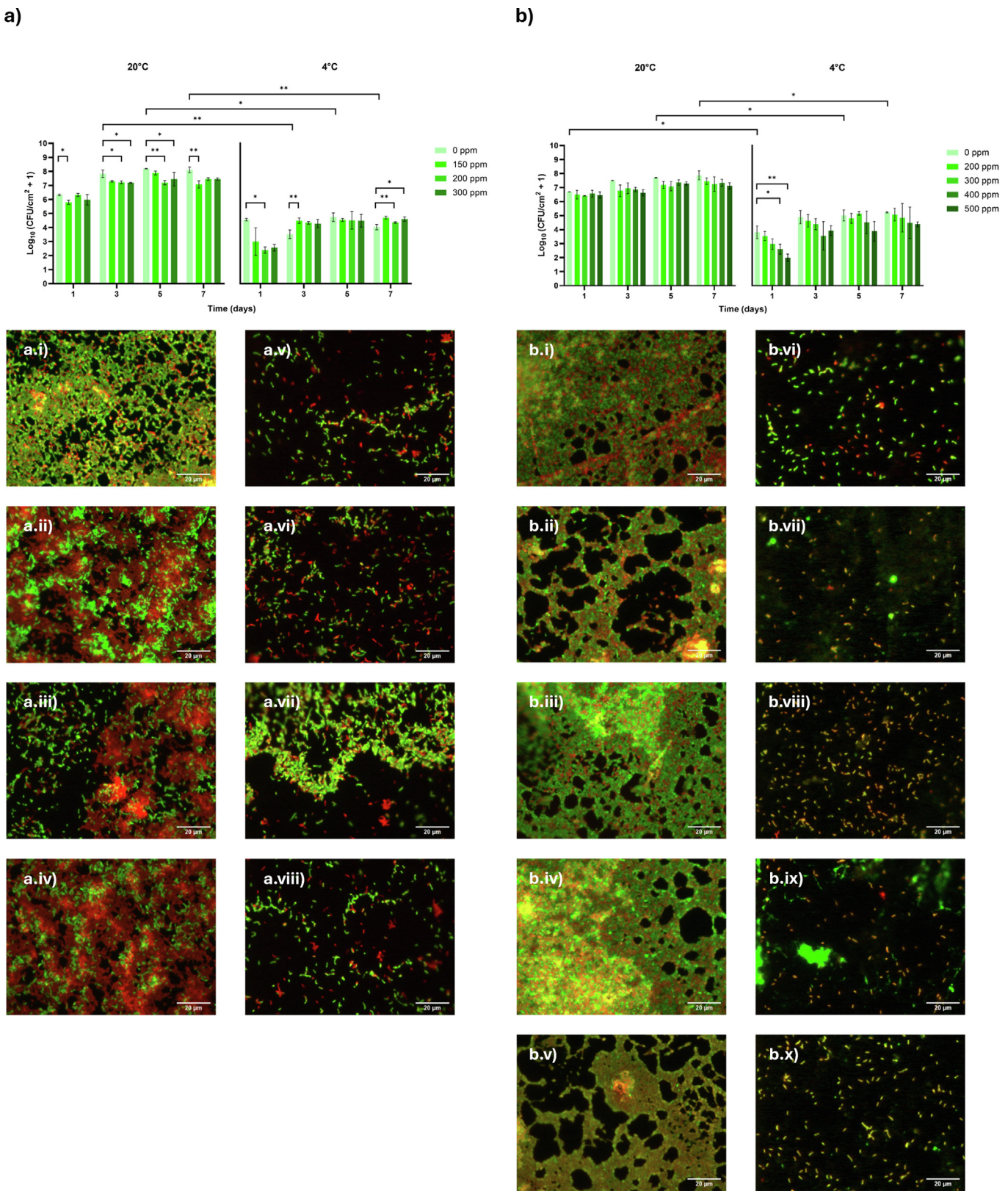

Figure 1. Culturable cell counts and EDIC microscopy of treated L. monocytogenes Scott A biofilms. Biofilms were grown at 20 °C or 4 °C, on 1 cm^2 stainless steel coupons for 7 days, and treated with either (a) chlorine or (b) peracetic acid on days 1, 3, 5, or 7. Culturability was enumerated by measuring colony-forming units (CFUs) after treatment. Error bars show ±SD for $n = 3$, and significance has been annotated with an asterisk (* = $p \leq 0.05$; ** = $p \leq 0.01$). The LIVE/DEAD® Bacterial Viability Kit (BacLightTM) has been used to distinguish between viable (green) and dead (red) cells and imaged using EDIC microscopy. (a.i–a.viii) Micrographs of chlorine-treated biofilms. (a.i–a.iv) Grown at 20 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively; (a.v–a.vii) Grown at 4 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively. (b.i–b.x) Micrographs of peracetic acid-treated biofilms. (b.i–b.v) Grown at 20 °C and treated with: 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively; (b.vi–b.x) Grown at 4 °C and treated with 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

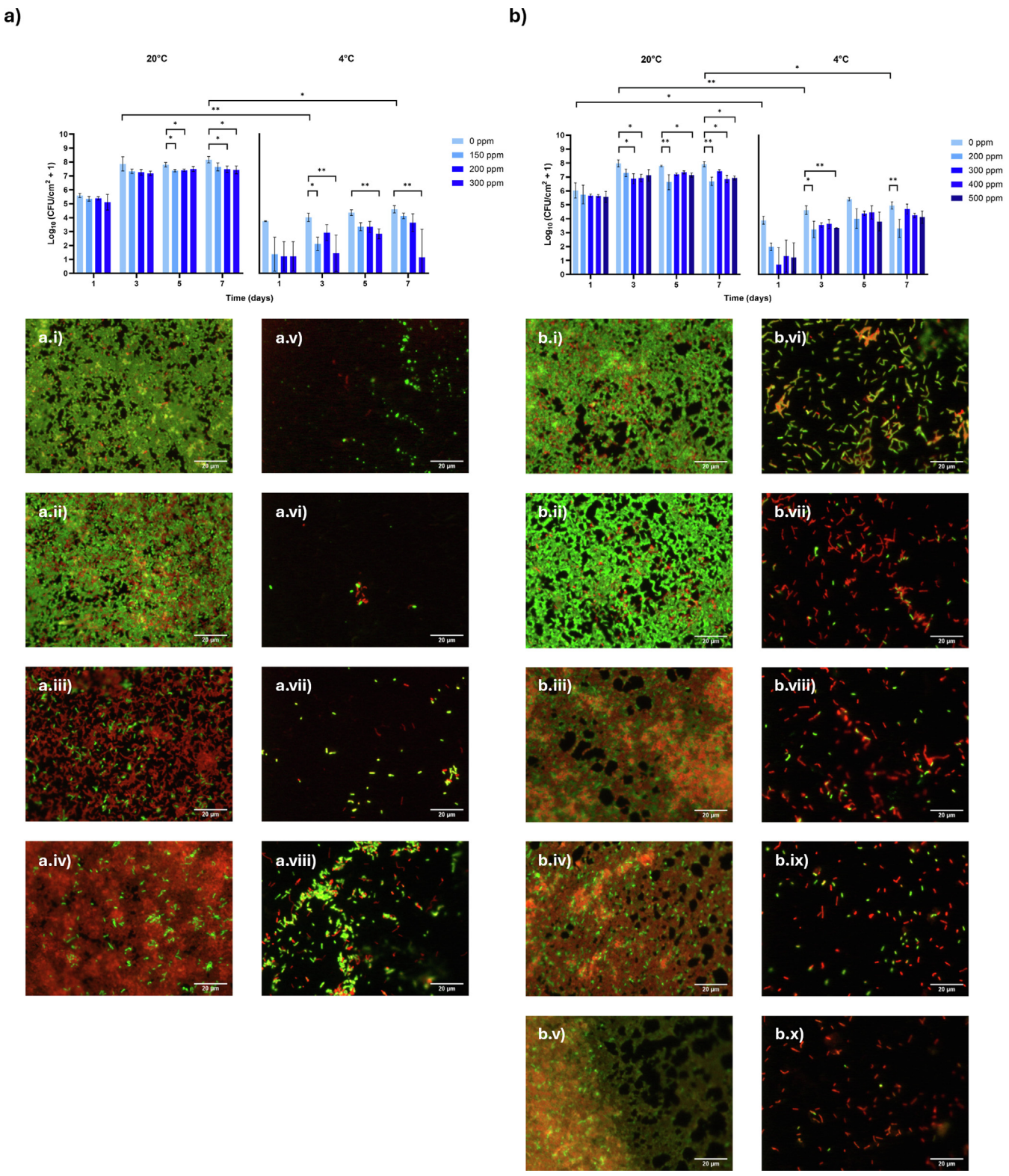

Figure 2. Culturable cell counts and EDIC microscopy of treated L. monocytogenes CECT 936 biofilms. Biofilms were grown at 20 °C or 4 °C, on 1 cm2 stainless steel coupons for 7 days, and treated with either a) chlorine or b) peracetic acid on days 1, 3, 5, or 7. Culturability was enumerated by measuring colony-forming units (CFUs) after treatment. Error bars show ±SD for $n = 3$, and significance has been annotated with an asterisk (*$p = \leq 0.05$; ** = $p \leq 0.01$). The LIVE/DEAD® Bacterial Viability Kit (BacLightTM) has been used to distinguish between viable (green) and dead (red) cells and imaged using EDIC microscopy. (a.i–a.viii) Micrographs of chlorine-treated biofilms. (a.i–a.iv) Grown at 20 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively; (a.v–a.vii) Grown at 4 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively. (b.i–b.x) Micrographs of peracetic acid-treated biofilms. (b.i–b.v) Grown at 20 °C and treated with: 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively; (b.vi–b.x) Grown at 4 °C and treated with 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

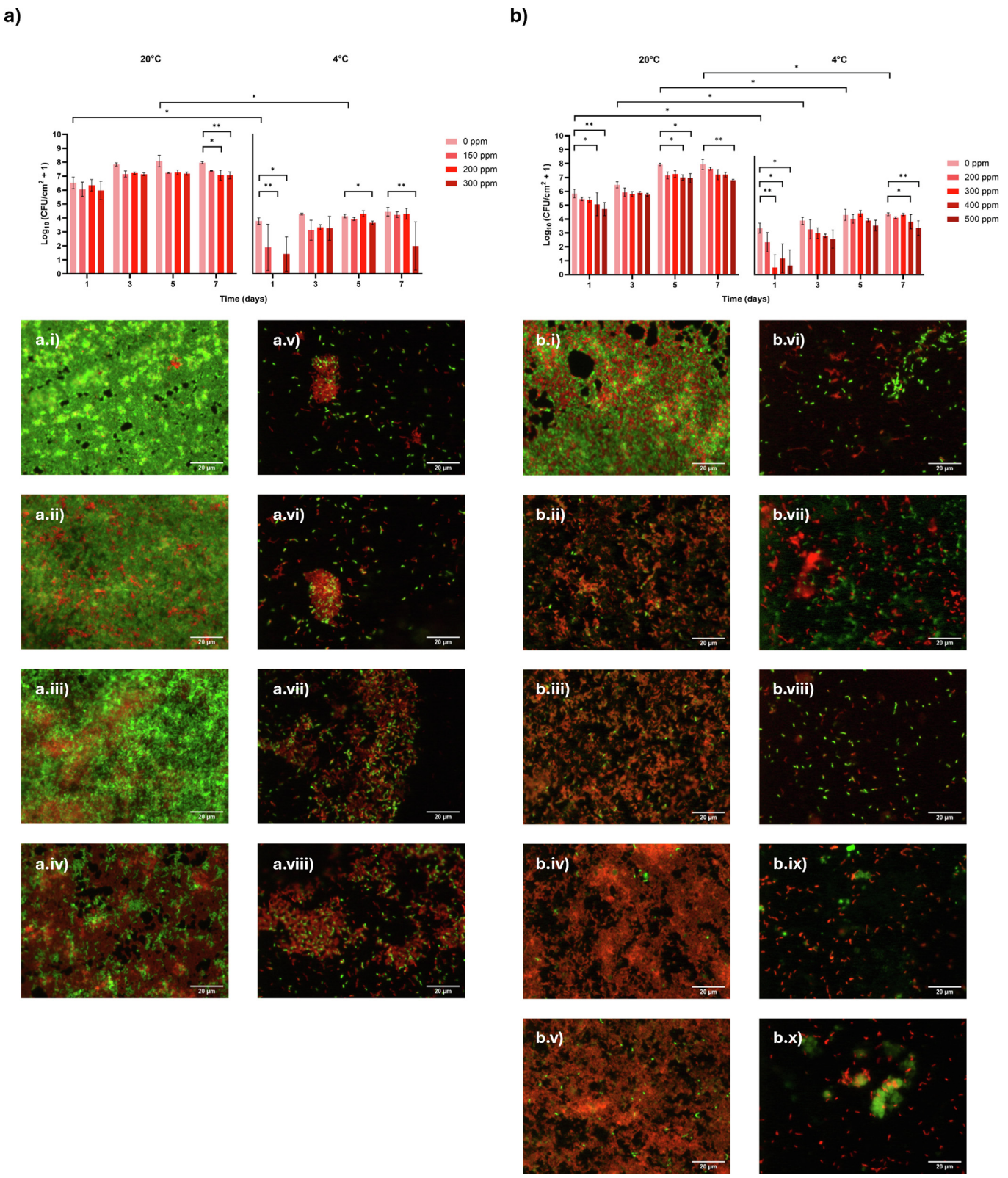

Figure 3. Culturable cell counts and EDIC microscopy of treated L. innocua NCTC 12210 biofilms. Biofilms were grown at 20 °C or 4 °C, on 1 cm2 stainless steel coupons for 7 days, and treated with either a) chlorine or b) peracetic acid on days 1, 3, 5, or 7. Culturability was enumerated by measuring colony-forming units (CFUs) after treatment. Error bars show ±SD for $n = 3$, and significance has been annotated with an asterisk (* = $p \leq 0.05$; ** = $p \leq 0.01$). The LIVE/DEAD® Bacterial Viability Kit (BacLightTM) has been used to distinguish between viable (green) and dead (red) cells and imaged using EDIC microscopy. (a.i–a.viii) Micrographs of chlorine-treated biofilms. (a.i–a.iv) Grown at 20 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively; (a.v–a.vii) Grown at 4 °C and treated with: 0 ppm, 150 ppm, 200 ppm, and 300 ppm Cl_2, respectively. (b.i–b.x) Micrographs of peracetic acid-treated biofilms. (b.i–b.v) grown at 20 °C and treated with: 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively; (b.vi–b.x) Grown at 4 °C and treated with 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm PAA, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

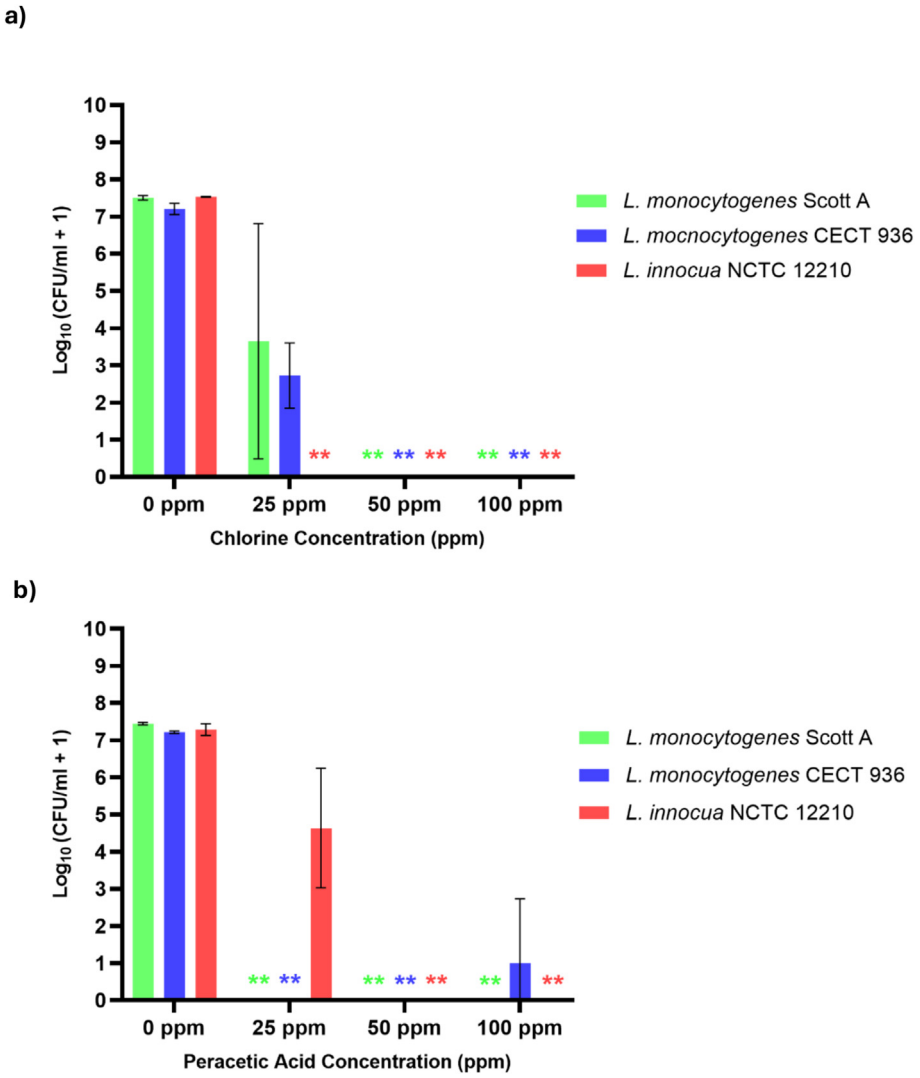

Figure 4. Planktonic cultures of Listeria treated with (a) chlorine or (b) peracetic acid. Overnight cultures were diluted to ~107 CFU/mL and then treated with 0 ppm, 25 ppm, 50 ppm, and 100 ppm of Cl_2 or PAA. Culturability was enumerated by measuring colony-forming units (CFUs) after treatment. Error bars show ±SD for $n = 3$, and significance has been annotated with an asterisk ($^{**}p = \leq 0.001$).

and maintaining structural integrity (Flemming et al., 2007). This explains why there are large amounts of red fluorescence in the room temperature biofilms, where there is a large amount of EPS. This shows that although SYTO 9 and PI costaining may be appropriate for viability staining of planktonic cultures (Rosenberg et al., 2019), it is not suitable for complex biofilms where eDNA is abundant. Gião and Keevil, (2014) reported similar findings when growing *L. monocytogenes* biofilms, where older biofilms appeared completely red when costained with SYTO 9 and PI due to the presence of eDNA. Additionally, plasmid DNA has been shown to remain intact when treated with both chlorine and PAA, despite the bacterium being killed (C. Zhang, Brown, & Hu, 2019), which again could facilitate binding of PI. The survival of plasmid DNA after treatment is a considerable concern as these could be beneficially acquired by other microorganisms via horizontal gene transfer (Rossi et al., 2014).

Additionally, the residual organic matter within the biofilm could be quenching the oxidizing agent before it can penetrate into the lower cells of the biofilm. This is important to note, as throughout the fresh food supply of leafy greens, bacteria will be supplied with organic matter from wash water which not only acts as a carbohydrate source but will also reduce the efficacy of chlorine or peracetic acid disinfection (Domínguez Henao et al., 2018; Zhang et al., 2019, 2009). Therefore, several studies have suggested a minimum concentration of free chlorine or peracetic acid must be maintained in wash water (Banach et al., 2020; Gómez-López et al., 2014; Luo et al., 2011) to minimize the effect of changes in pH or organic load. Another possible explanation for the reduced susceptibility is that there is an upregulation of stress response genes in response to the sanitizer treatment. This was seen in a study using chlorine dioxide as a sanitizer (Pleitner et al., 2014), whereby there was an increase in the transcription of oxidative stress-related genes (thioredoxin, oxidoreductase, superoxide dismutase, catalase, and glutathione reductase), although gene expression responses to chlorine and peracetic acid treatment may differ.

The attachment of *Listeria* to stainless steel has been shown to be very good (Wilks et al., 2006), and this is confirmed by the CFU counts of one-day-old biofilms in this study. Fig. 5 depicts the microstructure of the stainless steel surface, showing how *Listeria* cells preferentially adhere to the cracks and dents of the surface. This highlights the difficulty in cleaning these surfaces due to the protection afforded to the pathogen by the topography of the steel. In some cases, factory surfaces are used for extended periods without replacement, which may contribute to long-term biofilm persistence, especially in hard-to-clean areas.

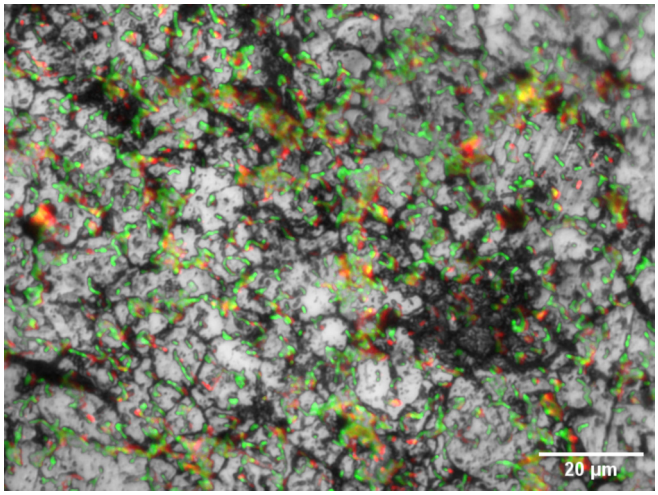

Figure 5. L. monocytogenes Scott A biofilm grown at 20 °C on stainless steel. The LIVE/DEAD® Bacterial Viability Kit (BacLightTM) has been used to distinguish between viable (green) and dead (red) cells in a biofilm and imaged using EDIC /EF microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This study focused on single-species *Listeria* biofilms; however, the biofilms in the fresh food supply chain are typically polymicrobial (Agustín & Brugnoni, 2018), which may affect the efficacy of sanitizers and is a limitation of this study. It has previously been observed that pathogens make up only a small proportion of the total microbial population on food samples (Bloomfield et al., 2023), so the contribution of this microflora to pathogen persistence should be considered in future studies. The nature of polymicrobial biofilms could raise further concerns for disinfection procedures, as horizontal gene transfer could enhance bacterial persistence and resistance (Rossi et al., 2014). Although avirulent, it was noted in this study that *L. innocua* produced thick biofilms with visible EPS (Fig. 3), which could contribute to its tolerance to chlorine and peracetic acid. When in a mixed-species biofilm, *L. innocua* may be able to protect pathogenic strains from disinfection. Similar findings were found in a salmon processing facility (Langsrud et al., 2016), whereby *Pseudomonas aeruginosa* promoted survival of *L. monocytogenes* in a polymicrobial biofilm.

L. monocytogenes Scott A (serotype 4b) is widely used in food-related biofilm research and is highly relevant to listeriosis outbreaks. Despite this, its status as a clinical strain means that it may not fully reflect the behavior of environmental strains that persist in food processing environments. Nevertheless, the findings of this study provide important and comparable data that can be complemented in the future by using environmental isolates – therefore increasing relevance to food safety.

A limitation of this study is that sanitizers were applied directly to the intact biofilm, without a prior cleaning step, which differs from standard food industry sanitization protocols. Therefore, it is possible that the results of this study do not represent the outcome when cleaning and sanitization steps are combined. However, this study was intended to model hard-to-reach factory areas such as machinery niches and pipework that may be missed during cleaning, allowing biofilm establishment and maturation. This “worst-case” approach investigated in this study provides valuable insights into the inherent reduced susceptibility of *Listeria* biofilms to sanitizers when a prior cleaning step is not carried out.

This study showed that when biofilms are established, sanitization becomes almost impossible, and single sanitizer treatment is ineffective. In this instance, a combination approach to cleaning may be necessary – Hua and Zhu (2024) found a >6-log reduction of seven-day-old *L. innocua* biofilms when using a combination of PAA and saturated steam treatments. An ideal decontamination regime should comprise mechanical and chemical components, conducted regularly to physically disrupt and remove biofilms from surfaces in addition to sanitizing them (Dawan et al., 2025). This study therefore provides important information on sanitizing efforts in the fresh food supply chain, concerning factory temperatures, surface material, and age of biofilm. This study determines that mature *Listeria* biofilms are tolerant to high levels of chlorine and PAA treatment, emphasizing the need for rigorous cleaning and sanitizing practices.

CRediT authorship contribution statement

Lucy Sutton: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Megan Humphreys:** Validation, Investigation. **Callum Highmore:** Supervision. **Sandra Wilks:** Supervision. **Charles William Keevil:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jfp.2025.100652.

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