Enhanced inactivation of *Escherichia coli* and *Listeria monocytogenes* by exposure to 405 nm light under sub-lethal temperature, salt and acid stress conditions

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Abstract

The antimicrobial effects of 405 nm light have generated interest in its use as an emerging disinfection technology with potential food-related applications. The aim of this study was to assess the bactericidal efficacy of 405 nm light for inactivation of *Escherichia coli* and *Listeria monocytogenes* under sub-lethally stressed environmental conditions. Bacteria were exposed to 405 nm light from a light emitting diode (LED) array under various temperature, salt (NaCl) and acid conditions to determine if bacterial susceptibility to 405 nm light inactivation is affected when exposed under these conditions. Non-stressed bacterial populations (10^5 CFU/mL$^-$) were exposed to increasing doses of 405 nm light (~70 mW/cm^2) and the inactivation results were compared with those generated under stress conditions. Bacteria were held at various temperatures (4°C, 22°Cand 45°C), acid concentrations (pH3 and 3.5 and 7) and salt concentrations (0%,0.8%,10% and 15% NaCl), and simultaneously exposed to 405 nm light. Enhanced inactivation of both *E. coli* and *L. monocytogenes* was achieved when light exposure was combined with each of the sub-lethal stresses, with significantly increased inactivation rates compared to non-stressed populations (P≤0.05). One exception was with *L. monocytogenes* when light-exposed in the presence of 15% salt, as this combination reduced bacterial inactivation. The greatest enhancement of 405 nm light inactivation for both bacterial species was achieved when light exposure was combined with sub-lethal acid stress conditions at pH3. This was demonstrated by a 5-log$_{10}$ reduction of *E. coli* following a 405 nm light dose of 84 J/cm^2 compared to 378 J/cm^2 for non-stressed populations (77% reduction in dose) and by a 5-log$_{10}$ reduction of *L. monocytogenes* achieved with a dose of 42 J/cm^2 which corresponded to 50% of the dose required for the equivalent reduction of non-stressed populations. This acid-enhanced 405 nm light

inactivation effect was demonstrated with *E. coli* and *L. monocytogenes* when dispersed in liquid suspension and when deposited on a test surface. Overall, results from this study have shown that sub-lethally stressed bacteria have increased susceptibility to 405 nm light inactivation, thereby providing a synergistic inactivation effect, findings which increase the potential of this new light-based decontamination technology for food related applications.

Keywords

405 nm Visible light; Decontamination; Food; Bacteria; Environmental stress

1. Introduction

Effective microbial decontamination of the food manufacturing and processing environment remains a key concern, and a wide range of both traditional and newer technologies are being employed to this end (Lo). The use of light for environmental and food surface decontamination has generated much interest over many years. Ultraviolet (UV) light applications, although not largely utilised, have been demonstrated for decontamination of equipment in bakeries, and cheese and meat plants (Koutchma 2008). However, despite the efficacy of this technology, due to the safety issues associated with UV light, its ability to degrade plastics and its limited transmissibility through various materials, overall applications have been limited.

Visible light has provided a more recent alternative, specifically 405 nm light, which has displayed extensive bactericidal properties without the detrimental effects associated with UV light. The inactivation mechanism of 405 nm light is accredited to the excitation of intracellular photosensitive porphyrin molecules, which results in the production of reactive oxygen species, inducing oxidative damage and consequently cell death (Maclean et al., 2008; Hamblin and Hassan, 2004, Dai et al., 2012).

A number of studies have demonstrated the bactericidal effects of 405 nm light against a range of pathogenic food-related organisms, both in vegetative and in spore form (Maclean et al., 2013; Maclean et al., 2009). Recent data (Murdoch et al., 2012; Endarko et al., 2012) have demonstrated significant reduction of *E. coli, S. enterica*, *S. sonnei* and *L. monocytogenes* populations when exposed to 405 nm light on nutritious surfaces. Further data from Murdoch et al. (2012) also highlights the efficacy of 405 nm light for inactivation of both *L. monocytogenes* and *S. enterica* on inert materials, with significant reductions

observed for both bacteria on acrylic surfaces (Murdoch et al., 2012). Inactivation of bacterial biofilms has also been shown with results demonstrating between 5-8 $\log_{10}$ reductions of *E. coli* biofilm on acrylic and glass surfaces following 405 nm light exposure (McKenzie et al., 2013).

Within foods and food residues and when dispersed as surface contaminants in the food processing environment microorganisms are exposed to a vast array of physical and chemical stresses including various temperatures, pH and osmotic conditions (Dykes and Withers., 1999; Lou and Yousef., 1997). Surviving bacteria may be in a stressed sub-lethally injured state and it is important to understand how these bacteria, as well as non-stressed bacteria, will respond to 405 nm light treatment. In the current study, *Escherichia coli* and *Listeria monocytogenes* are used as test species, to investigate, for the first time, the inactivating effects of 405 nm light on bacterial cells that are held under environmental stress conditions. The findings demonstrate that a synergistic bactericidal effect results from the combination of light and stress conditions employed. The potential application of 405 nm light technology for use in the food processing environment is also briefly discussed.

2. Materials and methods

2.1 Bacterial preparation

Escherichia coli NCTC 9001 was obtained from the National Collection of Type Cultures, Collindale, United Kingdom. *Listeria monocytogenes* LMG 19944 was obtained from the Laboratorium voor Microbiologie, Unversiteit Gent, Belguim. For experimental use, *E. coli* and *L. monocytogenes* were inoculated in 100 mL nutrient broth and tryptone soya broth (Oxoid Ltd, UK), respectively. Broths were cultivated for 18-24 hours at 37°C in a rotary incubator (120 rpm) and then centrifuged at 3939×g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 100 ml volume of phosphate buffer saline (PBS (BR0014G); Oxoid Ltd, UK) and serially diluted to give the required starting populations.

2.2 405 nm-light sources

The light sources used in this study were 405 nm light emitting diode LED arrays (Opto Diode Corp., USA/ PhotonStar Technologies, UK) with a bandwidth of approximately 14 nm. Arrays were attached to a heat sink and cooling fan, to dissipate excess heat and minimise heat transfer to test samples. Arrays were powered by a DC power supply. Irradiance of the LED sources was measured using a radiant power meter and photodiode detector (LOT Oriel USA). Temperature of the light-exposed bacterial suspensions was monitored using a thermocouple (Kane May KM340). A maximum temperature increase of 2°C occurred in samples that received the greatest light dose thereby verifying that inactivation was a direct result of 405nm light exposure and not due to a heating effect from the light source.

2.3 405 nm light exposure of bacterial suspensions

A 3 ml volume of bacterial suspension was transferred into a central well of a 12 well micro-plate. The LED array was mounted onto PVC housing designed to fit over the micro-plate, with the LED array sited directly above the sample well, at an approximate distance of 2 cm, providing an irradiance of approximately 70 mW/cm^2 across the sample surface. At the bacterial densities used no significant light attenuation occurred through the bacterial test volumes (Maclean et al., 2009) so that light exposure conditions in test wells were uniform. Test samples were exposed to increasing doses of 405 nm light, with dose calculated as the product of irradiance × exposure time. Exposure times were up to 20 minutes (84 J/cm^2) for *L. monocytogenes* and 90 minutes (378 J/cm^2) for *E. coli*. These results provided base-line inactivation kinetics for non-stressed bacteria, i.e. bacteria exposed whilst suspended in PBS (0.8% salt; pH 7) at room temperature (22°C). Control samples were set up simultaneously under identical environmental conditions but were without 405 nm light illumination. Post-exposure, *E. coli* samples were plated onto nutrient agar (NA), and *L. monocytogenes* samples onto tryptone soya agar (TSA) plates and incubated and enumerated as detailed in Section 2.7.

2.4 Sub-lethal stressing of bacterial populations

Sub-lethal stressing of organisms was achieved by holding the bacterial populations under different stress conditions:

Temperature stress: 10^5 CFU/mL populations in PBS were held at 4°C (refrigerator), and 45°C (incubator).

Acid Stress: 10^5 CFU/mL populations in PBS were acidified to pH 3 and pH 3.5 using 1% citric acid (Sigma Aldrich, UK). The pH was measured using a pH meter and probe (Hanna Instruments pH210 microprocessor pH metre, USA).

Osmotic Stress: 10^5 CFU/mL populations were held in 10% and 15% salt suspension (Sodium chloride, Sigma Aldrich, UK).

In order to demonstrate that the stress conditions employed caused sub-lethal damage, the temperature, salt and acid treated *E. coli* and *L. monocytogenes* samples were plated onto both non-selective and selective agar. Bacteria that were sub-lethally damaged/injured were more likely to be unable to grow on selective media than on non-selective media, and this was used to verify that bacterial populations were sub-lethally damaged. Violet red bile agar (VRBA; Oxoid UK) and *Listeria* selective agar (LSA; Oxoid UK) were chosen as the selective media for *E. coli* and *L. monocytogenes*, respectively (Dykes and Withers 1998; Robinson and McKillip 2010; Smith et al., 2013). Samples were plated, incubated and enumerated as described in Section 2.7, and evidence of sub-lethal injury was ascertained from the difference in counts obtained on the non-selective and selective media.

2.5 405 nm light inactivation of bacteria under sub-lethal stress

Stressed bacterial suspensions were prepared as described above (Section 2.4) and were then exposed to 405 nm light under the stress conditions as detailed in Section 2.3. For 4°C and 45°C temperature stress, the exposure system and control samples were held at temperature and results compared to the inactivation curves for organisms exposed at room temperature (~22°C). For acid stress, bacterial suspensions at pH 3 and 3.5 were

exposed to increasing doses of 405 nm light and results compared to the inactivation curves for organisms exposed at pH 7 (i.e. in PBS). In the case of osmotic stress, bacterial suspensions in 10% and 15% salt solution were exposed to increasing doses of 405 nm light, and results compared to the inactivation curves for organisms exposed at 0.8% salt concentration (i.e. in PBS). Bacteria were also exposed to 405nm light in sterile distilled water in order to provide data for bacteria exposed at 0% salt concentrations. Non-exposed control samples were set up in all cases. Post-exposure, samples were plated onto NA (*E. coli*) or TSA (*L. monocytogenes*) as detailed in section 2.7.

2.6 Bacterial Inactivation on surfaces

In order to demonstrate bacterial inactivation on surfaces, bacteria were acid-stressed as previously described in Section 2.4. To attain a suitable population for enumeration (approx. 150-200 CFU/surface), bacteria were diluted in PBS to 10^1 CFU/ mL and 10 mL volumes of which were filtered onto nitro cellulose membranes which were then washed with a further 10mL sterile PBS, prior to 405nm light exposure. Nitro cellulose surfaces were placed on NA and TSA plates (*E. coli* and *L. monocytogenes* respectively) and exposed to 405 nm light at an approximate distance of 5cm, with an average irradiance of 60mW/cm^2 over the 12.6cm^2 surface area. Non-light exposed control samples were set-up for comparison.

2.7 Enumeration and Statistical Analysis

E. coli and *L. monocytogenes* suspension samples were plated onto agar plates in duplicate using a WASP 2 spiral plater (Don Whitley Scientific), or if bacterial populations were

expected to be low, larger sample volumes were plated manually onto agar plates. For surface exposure tests, nitro cellulose surfaces were incubated directly on NA/TSA plates. All plates were incubated at 37°C for 18 hours and then enumerated to calculate the number of surviving colony-forming units per millilitre (CFU/mL) (for suspension exposure tests), or the number of surviving CFU per surface (for surface exposure tests). Data points represent average results taken from a minimum of triplicate independent experiments, with a minimum of two samples plated for each experiment (n=≥6). Significant differences were calculated using one-way ANOVA (MINITAB 16 statistical software), with results found to be significant when P≤0.05.

3. Results

3.1 405 nm light inactivation of non-stressed bacteria:

In order to establish the inactivation characteristics of non-stressed bacteria, bacterial populations were exposed to 70 mW/cm^2 405 nm light whilst suspended in PBS (pH 7.2 and 0.8% salt concentration) at room temperature (22-24°C). The inactivation data for *E. coli* and *L. monocytogenes* under these "non-stress" conditions are incorporated into Figures 2, 3 and 4. In the case of *E. coli,* exposure to increased doses of 405 nm light initiated significant inactivation with 0.5-log$_{10}$, increasing to ~2-log$_{10}$, reductions after 189 and 252 J/cm^2, respectively and complete inactivation (5-log$_{10}$ reduction) of non-stressed *E. coli* was achieved upon exposure to a dose of 378 J/cm^2. Inactivation of *L. monocytogenes* occurred using much lower doses demonstrating the increased susceptibility of *L. monocytogenes* to 405 nm light. For *L. monocytogenes*, an approximate 0.5-log$_{10}$ reduction occurred after 63

J/cm^2 and complete inactivation of the 10^5 CFU/mL population was achieved following a dose of 84 J/cm^2. These 405 nm light inactivation kinetics for non-stressed cells form the baseline inactivation curves for comparing the effects of 405 nm light exposure of temperature, salt and acid stressed bacteria.

3.2 Assessment of sub-lethal damage in stressed bacterial populations

During preliminary studies, temperature, salt and acidity conditions were determined that would be likely to result in formation of sub-lethally stressed populations of *E. coli* and *L. monocytogenes*. These were temperatures of 4°C and 45°C, salt levels of 10% and 15% and acidity levels of pH 3.0 and 3.5. *E. coli* cells were exposed to these stress conditions for 90 minutes and *L. monocytogenes* cells were exposed to these conditions for 20 minutes. These exposure time periods corresponded to the time that would be required to achieve complete inactivation of non-stressed populations of each species when exposed to the 70 mW/cm^2 405 nm light treatment, as described in Section 3.1. In order to demonstrate and confirm that the temperature, salt and acid conditions caused sub-lethal stress in the test bacteria, samples were plated onto non-selective and selective media at intervals during the exposure period. Confirmation of sub-lethal damage was evidenced by the reduced counts shown by the stressed population when plated on the selective medium (VRBA for *E. coli* and LSA for *L. monocytogenes*).

The results of these tests are shown in Figure 1a for *E. coli* and Figure 1b for *L. monocytogenes.* Significant differences were observed between counts on selective and non-selective media by the end of the exposure periods, demonstrating that the

temperature, salt and acid conditions employed resulted in non-lethal stress of the exposed bacteria. The only exception to this pattern of results was with *L. monocytogenes* exposed to 15% salt stress, which demonstrated no significant difference between selective and non-selective counts over the 20 minutes exposure period.

<FIGURE 1>

3.2 405 nm light inactivation of bacteria under temperature stress conditions

Tests were carried out on *E. coli* (Figure 2a) and *L. monocytogenes* (Figure 2b) to determine if cells held under temperature stress conditions were more susceptible to 405 nm light inactivation than non-stressed cells. With both species, inactivation rates were significantly greater at the 4°C and 45°C stress temperatures than at the non-stress temperature of 22°C. Whilst the pattern of results was similar for both species, the 405 nm light dose levels required for inactivation of *E. coli* were substantially greater at all temperatures than those required for inactivation of *L. monocytogenes*. Complete inactivation of *E. coli* populations required 250 – 380 J/cm^2 whereas the equivalent values for *L. monocytogenes* were of the order of 40 – 80 J/cm^2.

The temperature enhanced light inactivation results were particularly striking with *L. monocytogenes* (Figure 2b). Under temperature stress conditions not only were inactivation rates significantly increased, but also dose levels resulting in complete population inactivation were significantly reduced. For example, complete inactivation at 45°C required approximately 50% less dose than that required for complete inactivation at 22°C (approximately 40 J/cm^2 as compared with a dose of approximately 80 J/cm^2).

<FIGURE 2>

Population densities of non-exposed control samples held at various sub lethal stress temperatures demonstrated no significant reduction, when plated onto non-selective media, over the duration of the experiment for both *E. coli* and *L .monocytogenes* (P=0.394, 0.321 and 0.166, 0.23 for each bacteria respectively, at 4°C and 45°C), indicating inactivation was a direct result 405 nm light exposure.

3.3 405 nm light inactivation of bacteria under acid stress conditions

The results in Figure 3 demonstrate the enhanced inactivation of both *E. coli* and *L. monocytogenes* when exposed to 405 nm light under sub-lethal acid conditions. Although the pattern of results was similar for both organisms, as referred to previously, much higher dose levels of light were required for inactivation of *E. coli* than *L. monocytogenes*.

Results for *E. coli* (Figure 3a) show that not only were inactivation rates greatly increased but also complete inactivation of *E. coli* at pH 3.5 was achieved with approximately 50% of the dose necessary for complete inactivation of non-acid stressed bacteria. When acidity was increased to pH 3, the susceptibility of *E. coli* to 405 nm light was further increased, with complete inactivation achieved after 84 J/cm^2; 50% of the dose required for complete inactivation at pH 3.5, and 25% of that necessary for complete inactivation of non-acid stressed *E. coli*.

The results for *L. monocytogenes* (Figure 3b) demonstrate similar acid enhanced light inactivation effects as was observed with *E. coli* albeit at considerably reduced light dose levels. Significantly greater inactivation of *L. monocytogenes* cells occurred at pH 3.5 compared to non-acid stressed cells at pH 7. When acidity was further increased to pH 3,

inactivation rates were further enhanced, with complete inactivation achieved following exposure to 42 J/cm^2, which was significantly less than the dose required for complete inactivation of both non-stressed cells and cells exposed at pH 3.5 (P=0). The results demonstrate that non-stressed *L. monocytogenes* required 50-100% greater applied dose of 405 nm light to achieve complete inactivation than when cells were exposed to the light at pH 3.5 and 3 respectively.

Non-light exposed control samples that had been sub-lethally stressed with acid demonstrated no significant reduction in the absence of 405 nm light over the duration of the experiment, when plated onto non-selective media. P values were noted as 0.209 and 0.408 for *E. coli,* and 0.713 and 0.502 for *L. monocytogenes*, at pH 3 and pH3.5, respectively.

<FIGURE 3>

3.4 405 nm light inactivation of bacteria under salt stressed conditions

As shown in Figure 4a, 405 nm light inactivation of *E. coli* is enhanced when cells are exposed to sub-lethal salt concentrations of 10 and 15%. It was also found that the light inactivation curves of *E. coli* at 0.8% salt concentration (i.e. non-stressed in PBS) and in 0% salt followed similar trends, albeit with significantly greater inactivation when exposed in PBS.

When salt concentration was increased to 10%, the *E. coli* inactivation rate increased significantly and complete inactivation (5-log$_{10}$) was achieved with exposure to a dose of 252 J/cm^2: significantly greater than the 1.2 and 2-log$_{10}$ reductions observed with 0% and 0.8% salt suspensions respectively (P≤0.05).

Increasing the salt concentration to 15%, further enhanced *E. coli* inactivation with inactivation rates significantly greater at all applied doses when compared with 0%, 0.8% and 10% salt concentrations (P=0). Complete inactivation was achieved with a dose of 189 J/cm^2, giving a 5-log$_{10}$ reduction compared to the 3-log$_{10}$ reduction achieved when at 10% salt, and only 0.5 log$_{10}$ for 0.8% salt concentrations.

Figure 3b illustrates the 405 nm light inactivation curves of *L. monocytogenes* when held at varying salt concentrations (0%, 0.8% 10% and 15%). Bacterial inactivation at light dose levels of 21 J/cm^2 and 42 J/cm^2 was negligible at all salt concentrations tested. At dose levels above 40 J/cm^2 the inactivation trends were approximately similar for cells held at salt concentrations of 0, 0.8 and 10%, with complete inactivation achieved following exposure to 84 J/cm^2 of 405 nm light. The inactivation rate for *L. monocytogenes* held at 15% salt was similar to that of 0.8% salt (PBS) until 63 J/cm^2, after which the inactivation rate decreased relative to other salt concentrations and complete inactivation at 15% salt required a 50% greater dose (126 J/cm^2) than for cells at all other salt concentrations. Again control data of non-light exposed samples plated onto non-selective agar, demonstrated no significant reduction in bacterial population when exposed to various osmotic concentrations over the entire duration of the experiment. P values were shown to be 0.704, 0.81, 0.702 (*E. coli*) and 0.726, 0.448, 0.576 (*L. monocytogenes*) for 0%, 10% and 15% salt concentrations, respectively.

<FIGURE 4>

3.5 405 nm light inactivation of acid stressed bacteria on an exposed surface

It was considered important to determine if the key finding of the current study, namely that environmental stress factors enhance the bactericidal effectiveness of 405 nm light, applied to bacteria, not only when treated in liquid suspension, but also when treated on an exposed surface. For this purpose, a comparison was made of the inactivation rates of acid stressed and non-stressed bacteria seeded onto a test nitro cellulose membrane surface. These experiments differed in one respect from the liquid suspension tests in that bacteria were acid stressed prior to filtration and deposition on the membrane surface but acid stress was not maintained during the light exposure. Data shown in Table 1, highlights the enhanced 405 nm light inactivation of acid stressed *E. coli* and *L. monocytogenes* on surfaces compared to non-stressed populations. Results for *E. coli* demonstrate a significant 95% reduction (P=0.000) in stressed bacteria was achieved when exposed to 36 J/cm^2 of 405 nm light, compared to a 26% reduction for non-stressed bacteria. Similarly inactivation of prior stressed *L. monocytogenes* was enhanced when exposed to 405nm light on the test surface. Following an applied dose of 36 J/cm^2, non-stressed *L. monocytogenes* demonstrated a 13% reduction in bacterial count compared to a significant 99% reduction (P=0.00) when bacteria were sub lethally stressed with acid, prior to light exposure.

<TABLE 1>

4. Discussion

The results of experiments comparing inactivation rates of stressed and non-stressed *E. coli* and *L. monocytogenes* cells clearly demonstrated that the bactericidal effects of 405 nm light are greatly enhanced when applied in combination with environmental conditions that exert physiological stress on the exposed microbial populations. Manipulation of temperature, salt and acidity are amongst the most important parameters that can be used to control microbial populations in various environments. The precise temperature, salt concentrations and acidity conditions chosen for use in the current study were those that were experimentally established to induce sub-lethal stress on one or both of the test organisms *E. coli* and *L. monocytogenes.* Cell suspensions of the test bacteria were held under the defined sub-lethal stress conditions and then subjected to 405 nm light treatment in order to investigate bactericidal effects resulting from combined stress and light. For experimental purposes a relatively high irradiance level of approximately 70 mW/cm^2 of 405 nm light was used to treat bacterial samples. The experimental set up ensured that no significant temperature increase occurred in the sample and that inactivation was caused by 405 nm light and not by a heating effect. Also under the test conditions and bacterial densities employed, no light attenuation occurred through the sample thereby ensuring that light conditions were uniform throughout the sample (Maclean et al., 2009). Under these carefully controlled test conditions enhanced inactivation between 405 nm light and the tested stress parameters was demonstrated. It is reasonable to speculate from the results obtained that this enhanced inactivation effect will not be restricted to the precise temperature, salt and acidity conditions tested but will represent a more general phenomenon whereby the bactericidal properties of 405 nm light will be enhanced when

target cells are simultaneously subjected to a variety of environmental stress conditions as will be the case, for example, with bacterial contaminants within the food processing environment.

Sub-lethal injury of bacteria implies a degree of damage to cell structure and/or function, without inducing cell death (Wesche et al., 2008). In order to demonstrate the sub-lethal effect of each of the chosen stress conditions, bacterial populations were plated onto selective and non-selective media, prior to light exposure to confirm the presence of sub-lethally damaged cells. The differences in selective and non-selective CFU counts were indicative of the extent of sub-lethally damaged bacterial populations, as cells that had been structurally/ metabolically damaged were not able to grow on selective media (Dykes and Withers 1998; Smith et al., 2013).

405 nm light exposure of optimally-grown *E. coli* and *L. monocytogenes* suspended in PBS (0.8% salt; pH 7) at room temperature (22°C) established the inactivation results of non-stressed populations. These results demonstrated that complete inactivation of 5-$\log_{10}$ populations could be achieved by exposure to a 405 nm light dose of 84 J/cm^2 (70 mW/cm^2 for 20 min) in the case of *L. monocytogenes*, and 378 J/cm^2 (70 mW/cm^2 for 90 min) for *E. coli*. These findings are consistent with the results of previous studies which have demonstrated the higher sensitivity of *L. monocytogenes* to 405 nm light compared to *E. coli* (Endarko et al., 2012; Maclean et al., 2009). Differences in inactivation susceptibility to 405 nm light between bacterial species have been attributed to variance in cellular structure and endogenous photosensitizing molecules (Maclean et al., 2009; Guffey and Wilborne 2006; Demidova et al., 2005).

Enhanced cellular inactivation upon combination of environmental stress and 405nm light may be a direct result of multi-target inactivation, supporting the principle of hurdle technology, whereby the bacteria are targeted at multiple sites, by multiple stresses, enhancing the rate of inactivation. In the current study, the major factor involved in the bacterial inactivation was exposure to 405 nm light with lethality probably resulting from photochemically induced oxidative damage (Hamblin and Hassan., 2004; Maclean et al., 2008), but the temperature, acid stress and salt (in the case of *E. coli*) conditions employed significantly amplified the bactericidal efficacy of the 405 nm light. Regarding the role played by the environmental stress conditions, it is likely that some form of structural and/or metabolic stress is induced whilst the bacteria are exposed to sub-lethal environmental stresses, that alone is not enough to induce cellular inactivation, but is sufficient to increase susceptibility to oxidative damage during 405nm light exposure. The combined effect observed in these studies can be described as synergistic inactivation as the net inactivation result is considerably greater than the sum of the component effects.

Regarding the mechanism of 405 nm light induced damage, previous studies investigating photodynamic inactivation have identified that oxidative damage resulting from visible light exposure is non-specific to cellular components (Donelly et al., 2008; Gourmelon et al., 1994), however a recent study has suggested the production of reactive oxygen species (ROS) may directly affect the cell membrane (Wasson et al., 2012). It is worth noting that exposure to high osmotic concentrations, low pH and stress temperatures, can directly affect cellular structure and induce changes in the cellular membrane (Beales 2003) and it is therefore possible that bacteria exposed to a sub-lethal stress may have wea kened or

damaged membranes, making them increasingly susceptible to oxidative damage when exposed to 405nm light.

Of the three environmental stress conditions tested, low acidity interacted with 405 nm light exposure to produce the greatest enhancement of light induced inactivation. Inactivation under the most acidic conditions (pH 3), demonstrated the greatest enhanced susceptibility in both bacteria with a 77% and 50% reduction in inactivation dose required for *E. coli* and *L. monocytogenes*, respectively, when compared to non-stressed populations exposed solely to 405nm light. Results also indicated that *E. coli* may have increased light susceptibility under both acid and increased salt (osmotic) conditions, when compared to *L. monocytogenes*. Figure 4 shows a 50% reduction in the dose required for inactivation of *E. coli* during osmotic stress (at 15% salt concentration), but a 50% increase in dose for *L. monocytogenes*, when compared to tests conducted at lower salt concentrations. The latter result was the only instance where an apparent stress parameter decreased sensitivity to 405 nm light inactivation but this result was understandable in view of the finding that exposure of *L. monocytogenes* to 15% salt concentration in fact caused no-sub-lethal damage prior to 405 nm light exposure. These results are consistent with the known osmotolerance characteristics of *L. monocytogenes*.

The fact that for *L. monocytogenes*, sub-lethal damage and susceptibility to 405nm light was decreased with increased osmotic concentration suggests that an adaptive cellular protective mechanism is involved. Previous studies have suggested that stress reactions may be non-specific, thereby a response to a particular stress may provide “cross protection” to other applied stresses (McMahon Leistner 2000, Koutsoumanis et al., 2003). This phenomenon is known as stress hardening and previous studies highlighting stress

hardening have shown microbial adaptations resulting from continued exposure to lethal and sub-lethal environmental conditions allowing for partial or complete resistance against particular stress factors (Koutsourmanis et al., 2003; Lou and Yousef 1997). Stress hardening is a major concern in the food industry, whereby bacterial ability to generate increased tolerance or resistance to multiple environmental challenges may compromise food safety standards.

Although this study focussed largely on determining the inactivation characteristics of sub-lethally stressed bacteria in suspension, tests were also conducted to investigate the enhanced susceptibility of stressed bacteria to 405 nm light whilst exposed on an inert surface. For these tests, acid stressed (pH 3) bacterial populations were seeded onto a nitro cellulose membrane surface. The results demonstrate that significantly enhanced reduction of both *E. coli* and *L. monocytogenes* was achieved on the test surface when cells were sub-lethally acid-stressed prior to 405 nm light exposure. This finding demonstrates that the enhanced susceptibility of sub-lethally stressed bacteria to 405 nm light, observed when bacteria were present in liquid suspension, also applies to bacteria when treated on exposed surfaces. These surface inactivation results reinforce the previously published data by Murdoch et al (2012) and McKenzie et al (2013) that have demonstrated the application of 405 nm light for inactivation of bacteria on inert surfaces and in surface-associated biofilms.

Although the studies reported here were conducted with only two species, *E. coli* and *L. monocytogenes,* it is anticipated that the key findings of the study will apply to a wide range of pathogenic and problematic bacteria as the broad spectrum efficacy of 405 nm light against a wide range of Gram positive and Gram negative bacteria has already been

established (Dai et al., 2012; Murdoch et al., 2012; Endarko et al., 2012; Maclean et al., 2009). Given this wide spectrum bactericidal activity which applies when bacteria are distributed either in liquid suspension or on exposed surfaces, and with effectiveness further enhanced under environmental stress conditions, then these findings indicate that 405 nm light could have application as an environmental decontamination technology in the food processing environment. There is for example a need for additional methods and approaches to tackle the problem of food contamination by *L. monocytogenes* which can arise from diverse sources within the food processing environment (Campdepardros et al., 2012; Carpentier and Ceff., 2011).

The practical application of 405 nm light technology for environmental decontamination has previously been demonstrated in the clinical setting with the use of 405nm lights, arranged as overhead lighting luminaires for environmental decontamination of hospital isolation rooms, with the light providing continuous disinfection of occupied patient environments (Maclean et al., 2010; Bache et al., 2011). The results of the current study support the idea that 405 nm light technology could potentially have similar decontamination applications for the food processing environment. However for commercial food-related applications, larger scale studies are required involving a variety of environmental and food contact surface materials that should be tested under realistic operating conditions in the industrial environment. This scale of study was out with the scope of the current investigation which focussed on establishing and characterising the synergistic inactivation effect of 405 nm light inactivation when combined with environmental stress conditions.

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References

Abram, F., Starr, E., Karatzas ,A.G., Matlawska-Wasowska, K., Boyd, A., Wiedmann, M., Boor K.J, Connally D, O'Byrne C.P. 2008. Identification of componenets of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. Journal of Applied and Environmental Microbiology 74, 6848-6858

Bache, SE., Maclean, M., MacGregor, SJ., Anderson, JG., Gettinby ,G., Coia, JE., Taggart, I., 2011. Clinical studies of High-Intensity-Narrow-Spectrum environmental decontamination system (HINS-light EDS), for continuous disinfection in the burn unit inpatient and outpatient settings. Burns 1, 69-76

Beales, N. 2003. Adaptations of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Comprehensive Reviews in Food Safety and Food Science 3, 1-20

Beumer, RR., Giffel, MC., Cox,LJ., Rombouts, FM., Abee, T ., 1994. Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. Applied and Environmental Microbiology 60, 1359–1363

Buchovec, I., Paskeviciute, E., Luksiene, Z., 2010. Photodynamic inactivation of food pathogen *Listeria monocytogenes*. Food Technology and Biotechnology 48, 207-213

Campdepardros, M., Stchigel AM., Romeu M., Quilez J and Sola R., 2012. Effectiveness of two sanitation procedures for decreasing the microbial contamination levels (including L. moncytogenes) on food contact and non-food contact surfaces in a dessert processing factory. Food Control 23, 26-31

Carpentier, B and Cerf, O., 2011. Review- Persistence of Listeria monocytogenes in food industry equipment and premises. International Journal of Food Microbiology 145,1-8

Centre for Disease Control and Prevention. 2011. CDC Estimates of Foodborne Illness in the United States. *(http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html;* 10 May 2013*)*

Chaturongakul, S., Boor, K.J. 2006. σ^B activation under environmental and energy stress conditions in *Listeria monocytogenes*. Journal of Applied and Environmental Microbiology 72, 5197-5203

Cole, M.B., Jones, M.V., Holyoak, C., 1989. The effect of PH, salt concentration and temperature on survival and growth of *Listeria monocytogenes.* Journal of Applied Bacteriology 69, 63-72

Cotter, PD., Gahan, CG., Hill, C., 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Molecular Microbiology 40, 465-475

Dai, T., Asheesh, G., Huang,Y., Yin, R., Murray, C.K., Vrahas, M.S., Sherwood, M., Tegos, G.P., Hamblin, M.R. 2012. Blue light rescues mice from potentially fatal Pseudomonas aeruginosa infection: Efficacy, safety and mechanism of action. Antimicrobial Agents and Chemotherapy 57, 1238-1245

Demidova, T.N., Hamblin, MR., 2005. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. Antimicrobial Agents and Chemotherapy 49, 2329-2335.

Dykes, G.A, Withers, K.M. 1998. Sub lethal damage of Listeria monocytogenes after long term chilled storage. Letters in Applied Microbiology 28, 45-48.

Endarko, E., Maclean, M., Timoshkin, I.V., MacGregor, S.J., Anderson, J.A., 2012. High intensity 405 nm light inactivation of *Listeria monocytogenes*. Journal of Photochemistry and Photobiology 40, 734-737.

Ferreira, A., O'Byrne, CP., Boor, KJ., 2001. Role of Sigma (B)in heat, ethanol, acid and oxidative stress resistance and during carbon starvation in Listeria monocytogenes. Applied and Environmental Microbiology 10, 4454-4457.

Gardan, R., Cossart, P., 2003. The European Listeria Genome Constorium and Labadie J. Identification of *Listeria monocytogenes* genes involved in salt and alkaline tolerance. Journal of Applied and Environmental Microbiology 69, 3137-3143.

Gourmelon M., Cillard J., Pommepuy M., 1994. Visible light damage to Escherichia coli in seawater: oxidative stress hypothesis. Journal of Applied Bacteriology 77, 105-112

Guffey, J.S., Wilborn, J., 2006. In vitro bactericidal effects of 405 nm and 470nm blue light. Photomedicine and Laser Surgery 24, 684-688.

Hamblin, M.R., Hassan, T., 2004. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochemistry and Photobiology 5, 436-450

Herbert, R.A. 1989. Microbial growth at low temperatures. Mechanisms of action of food preservation procedures. London. Elsevier Applied Science. P71-96

Hill, C., Cotter, P.D., Sleator, R.D., Gahan, C.G.M., 2002. Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. International Dairy Journal 12, 273-283

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J., Wiedmann, M., 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. Journal of Bacteriology 185, 5722-5734

Koutsoumanis, K.P., Kendall, P.A., Sofos, J.N., 2003. Effect of food processing related stresses on acid tolerance of *Listeria monocytogenes*. Journal of Applied and Environmental Microbiology 69, 7514-7516

Lado, B.H., Yousef, A.E., 2002. Alternative food preservation technologies: efficacy and mechanisms. Microbes and Infection 4, 433-440

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology 55, 181-186

Loretz, M., Stephan, R., Zweifel, C. 2010. Antimicrobial activity of decontamination treatments for poultry carcasses: A literature survey. Journal of Food Control 21, 791-804

Lou, L., Yousef, A.E., 1997. Adaptation to sublethal environmental stresses protects Listeria monocytogenes against lethal preservation effects. Journal of Applied and Environmental Microbiology 63, 1252-1255

Lucht, J.M., Bremer, E., 1994. Adaptation of Escherichia coli to high osmolarity environments: Osmoregulation of high affinity glycine betaine transport system ProU. FEMS Microbiology reviews 14, 3-20

Maclean, M., MacGregor, S.J., Anderson, J.G., Woolsey, G.A., 2008. The role of oxygen in visible light inactivation and wavelength sensitivity of *Staphylococcus aureus*. Journal of Photochemistry and Photobiology 92, 180-184

Maclean M., MacGregor S.J., Anderson J.G and Woolsey G.A. 2009. Inactivation of bacterial pathogens following exposure to light from a 405nm light emitting diode array. Applied and Environmental Microbiology 75, 1932-1937

Maclean, M., MacGregor, S.J., Anderson, J.G., Woolsey, G.A., Coia, J.E., Hamilton, K., Taggart, I., Watson, S.B., Thakker, B., Gettinby, B., 2010. Environmental decontamination of a hospital room using high intensity narrow spectrum light. Journal of Hospital Infection 76, 247-251

Maclean, M., Murdoch, L.E., MacGregor, S.J., Anderson, J.A., 2013. Sporicidal effects of 405 nm visible light on endospore forming bacteria. Journal of Photochemistry and Photobiology 89, 120-126.

McMahon, M., Xu, J., Moore J., 2007. Environmental stress and antibiotic resistance in food pathogens. Journal of Applied and Environmental Microbiology 73 211-217

McKenzie, K., Maclean, M., Endarko, E., Timoshkin, I.V., MacGregor, S.J., Anderson J.G.,2013. Inactivation of bacteria attached to glass and acrylic surfaces by 405nm light: Potential application for biofilm decontamination. Photochemistry and Photobiology, *In Press*, DOI: 10.1111/php. 12077

Meury, J., 1989. Gylcine betaine reverses the effects of osmotic stresson DNA replication and cellular division in Escherichia coli. Archives of Microbiology 149, 232-239

Murdoch, L.E., Maclean, M., MacGregor, S.J., Anderson, J.G., 2010. Inactivation of *Campylobacter jejuni* by exposure to high intensity 405 nm visible light. Foodborne Pathogens and Diseases 7, 1211- 1216.

Murdoch, L.E., Maclean, M., Endarko, E., MacGregor, S.J., Anderson, J.G., 2012. Bactericidal effects of 405 nm light exposure demonstrated by inactivation of *Escherichia, Salmonella, Shigella, Listeria* and Mycobacterium Species in liquid suspensions and on exposed surfaces. Scientific World Journal 54, 1-8.

Robinson, A.L, McKillip, J.L. 2010. Biology of Escherichia coli O157:H7 in human health and food safety with emphasis on sublethal injury and detection. Applied Microbiology, 1096-1105.

Roth, W.G., Leckie, M.P., Dietzler, D.N., 1985. Osmtic stress dramatically inhibits active transport of carbohydrates by Escherichia coli. Biochemistry and Biophysics Research Communications 126, 434-441

Schmitt, W., Gehrmann, M., Brunet, M., Multhoff., Garrido, C., 2007. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. Journal of Leukocyte Biology 81, 15-27

Smith, A.R., Ellison, A.L., Robinson, A.L, Drake, M, McDowell, S.A, Mitchell, J.K, Gerard, P.D, Heckler, R.A, McKillip, J.L. 2013. Enumeration of sublethally injured Escherichia coli 0157:H7 ATCC 43895 and Escherichia coli strain B 41560 using selective agar overlays versus commercial methods. Journal of Food Protection 76, 674-679.

U.S FDA/ Centre for Food Safety and Applied Nutrition. 2003. *Listeria monocytogenes* risk assessment question and answers.

(http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm208993.htm; 13May 2013)

Wasson, J.W., Zourelias, J.L., Aardsma, N.A., Eells, J.T., Ganger, MT., Schober, J.M., Skwor, T.A., 2012. Inhibitory effects of 405 nm irradiation on *Chlamydia trachomatis* growth and characterization of the ensuing inflammatory response in HeLa cells. British Medical Council Microbiology 12, 1471-2180

Wesche, A.M., Gurtler J.B., Marks B.P., Ryser E.T.,2009. Stress, sublethal injury, resuscitation and virulence of bacterial foodborne pathogens. Journal of food protection 72, 1121-1138

Table 1: *Inactivation of non-stressed and acid stressed (pH 3) E. coli and L. monocytogenes on nitro cellulose surfaces following exposure to 36 J/cm^2 405nm light. Results are expressed as CFU counts per surface.*

Bacterial Species	**Acid Stress (pH 3)**	**Bacterial Surface Contamination (CFU per surface (±SD))**		**% reduction**
		Non-exposed	**Light-exposed**	
E. coli	No	148 (±3.6)	110(±5.5)	26
	Yes	159(±13.1)	9(±8.7)	95*
L. monocytogenes	No	181(±21.2)	156(±16.5)	13
	Yes	205(±7.8)	3(±4.6)	99*

* Indicates where acid stressed sample reduction was statistically significant from non-acid stressed sample reduction (P≤0.05 calculated at a 95% confidence interval).

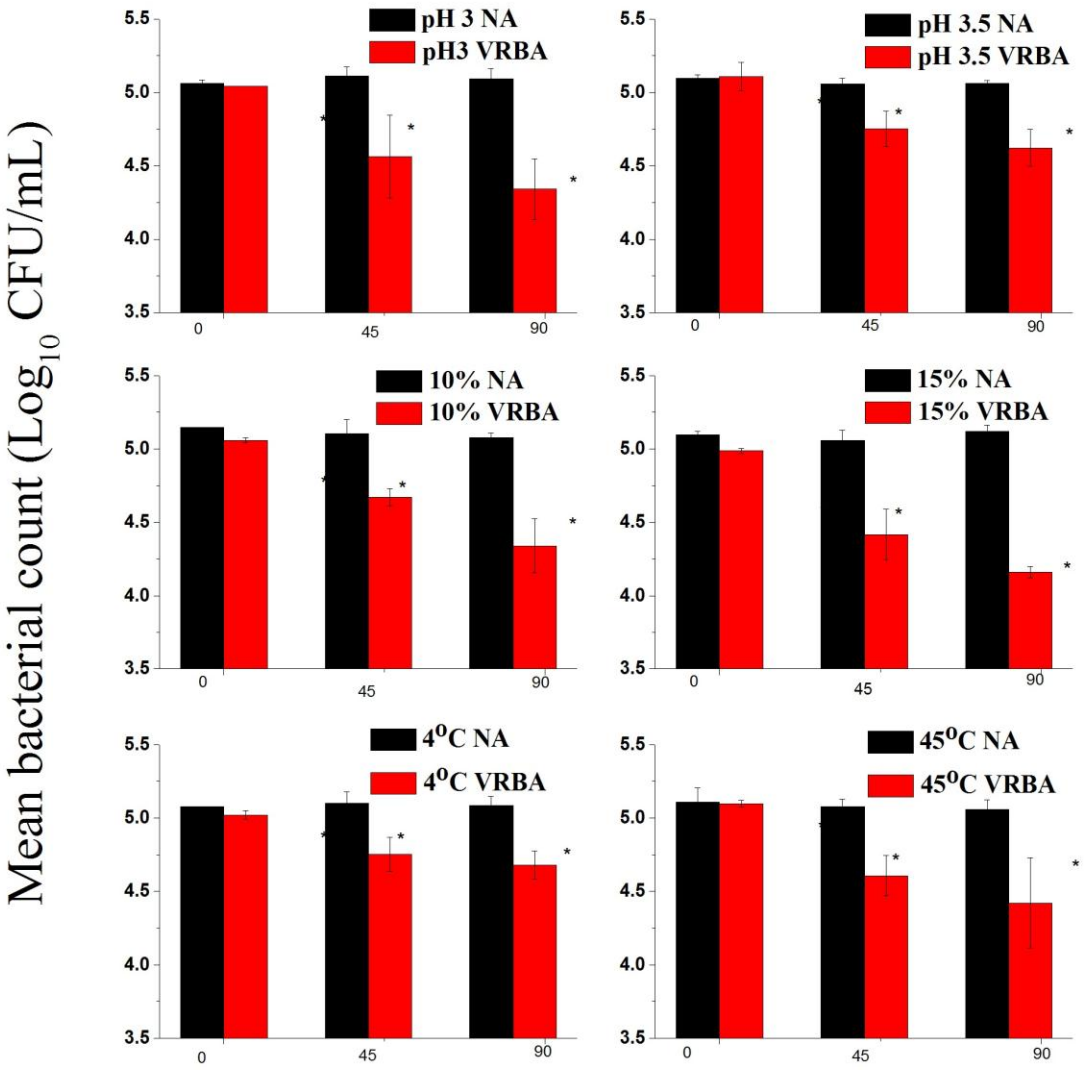
FIGURE CAPTIONS

Figure 1: *Quantification of sub -lethally damaged bacterial populations by comparing surviving bacterial counts on selective media (nutrient agar, NA/tryptone soya agar, TSA) and non-selective media (violet red bile agar,VRBA/listeria selective agar, LSA), for (a) E. coli and (b) L. monocytogenes, respectively . * represents statistically significant differences (P≤0.05) between bacterial populations on selective and non-selective media.*

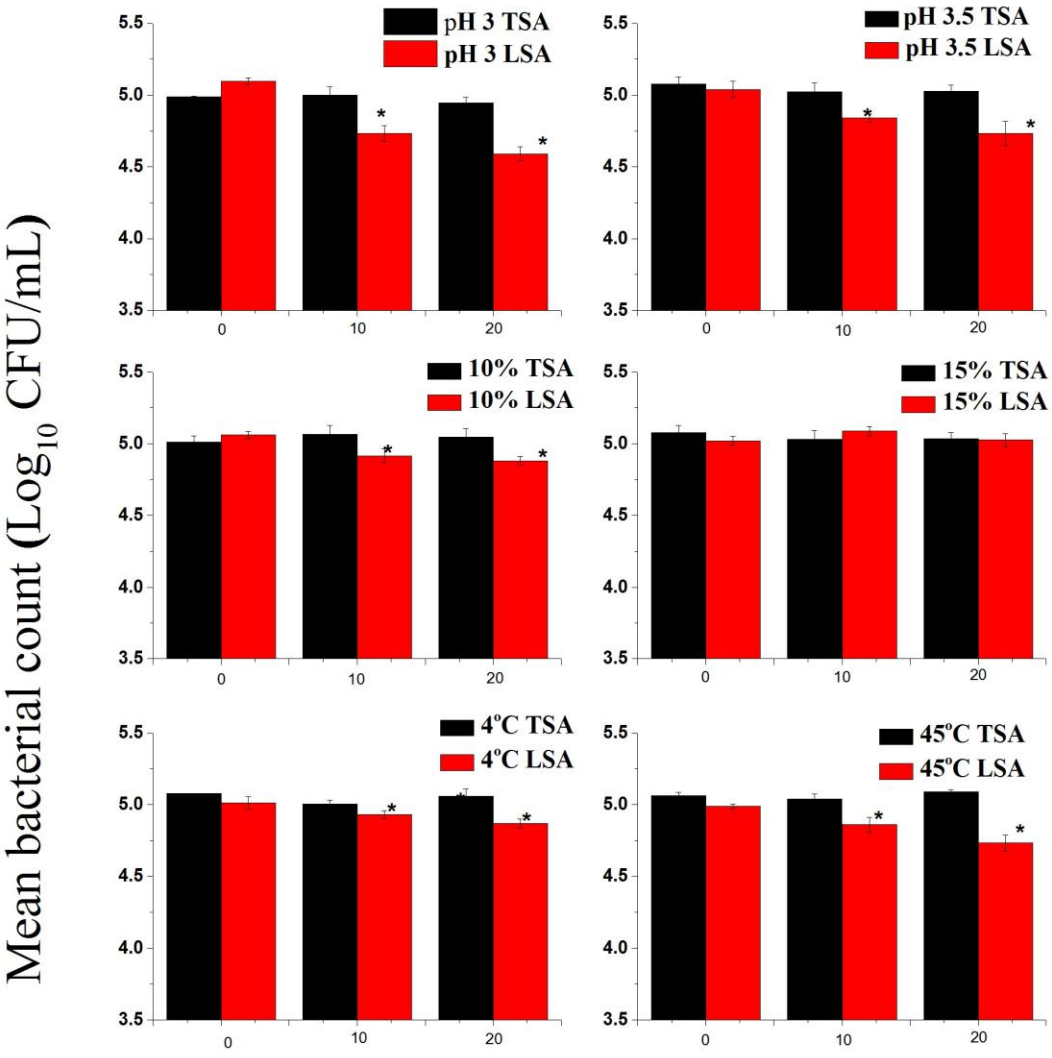
Figure 2: *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW/cm^2 irradiance) combined with temperature stress. Bacterial populations were suspended in PBS and exposed to 405 nm light at 4°C and 45°C. Inactivation kinetics for exposure at 22°C (room temperature) provided a non-stressed comparison. * represents the data points which have significantly increased bacterial inactivation (P≤0.05) compared to the non-stressed population (22°C). No significant changes were observed in non-light exposed control samples, data not shown.*

Figure 3: *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW/cm^2 irradiance) combined with acid stress. Bacterial populations were suspended in PBS at pH 3 and pH 3.5 and exposed to 405 nm light. Inactivation kinetics for exposure at pH 7 (PBS without acid) provide a non-stressed comparison. * represents the data points which have significantly increased bacterial inactivation (P≤0.05) compared to the non-stressed population (pH7). No significant changes were observed in non-light exposed control samples, data not shown.*

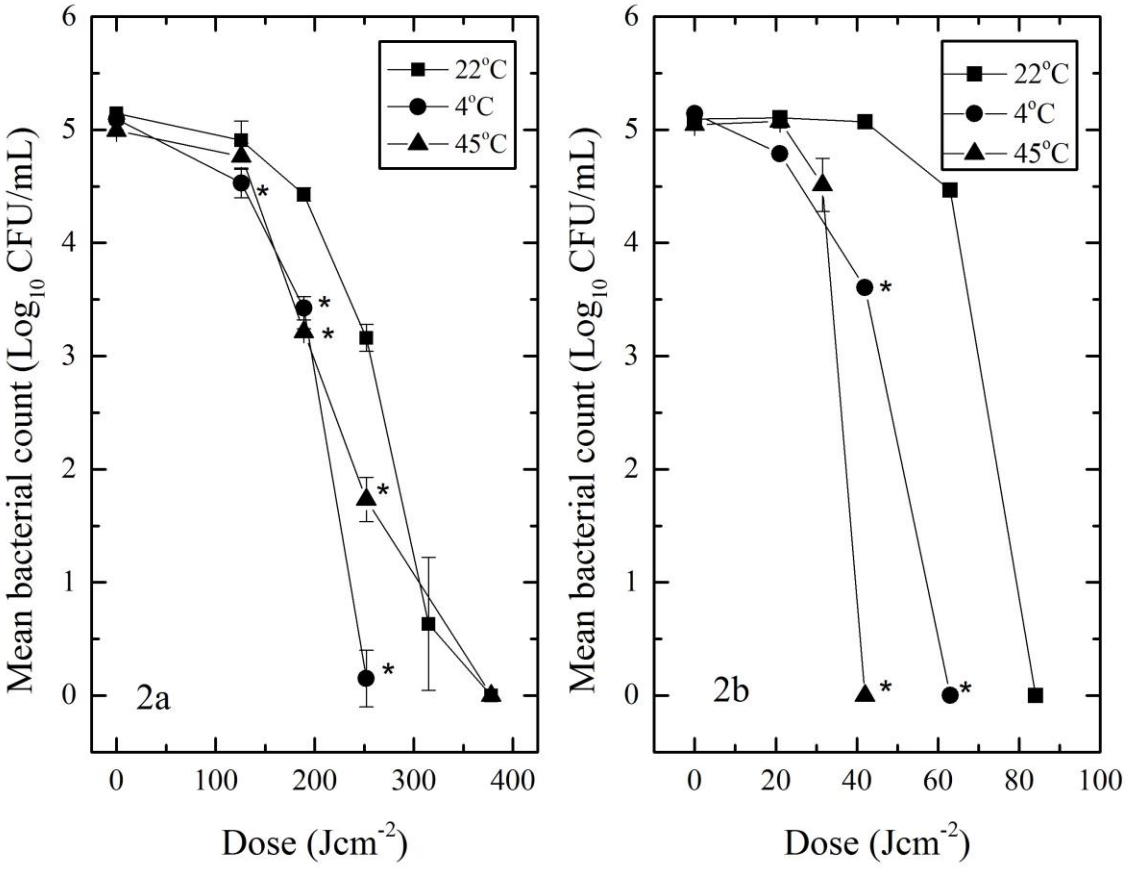
Figure 4: *Inactivation of (a) E. coli and (b) L. monocytogenes by exposure to 405 nm light (70 mW/cm^2 irradiance) combined with osmotic (salt) stress. Bacterial populations were suspended in PBS with a salt concentration of 10% and 15% and exposed to 405 nm light. Inactivation kinetics for exposure at 0% (water) and 0.8% (PBS) provide non-stressed comparisons. * represents the data points which have significantly increased bacterial inactivation (P≤0.05) compared to the non-salt stressed populations (0% and 0.8%). No significant changes were observed in non-light exposed control samples, data not shown.*

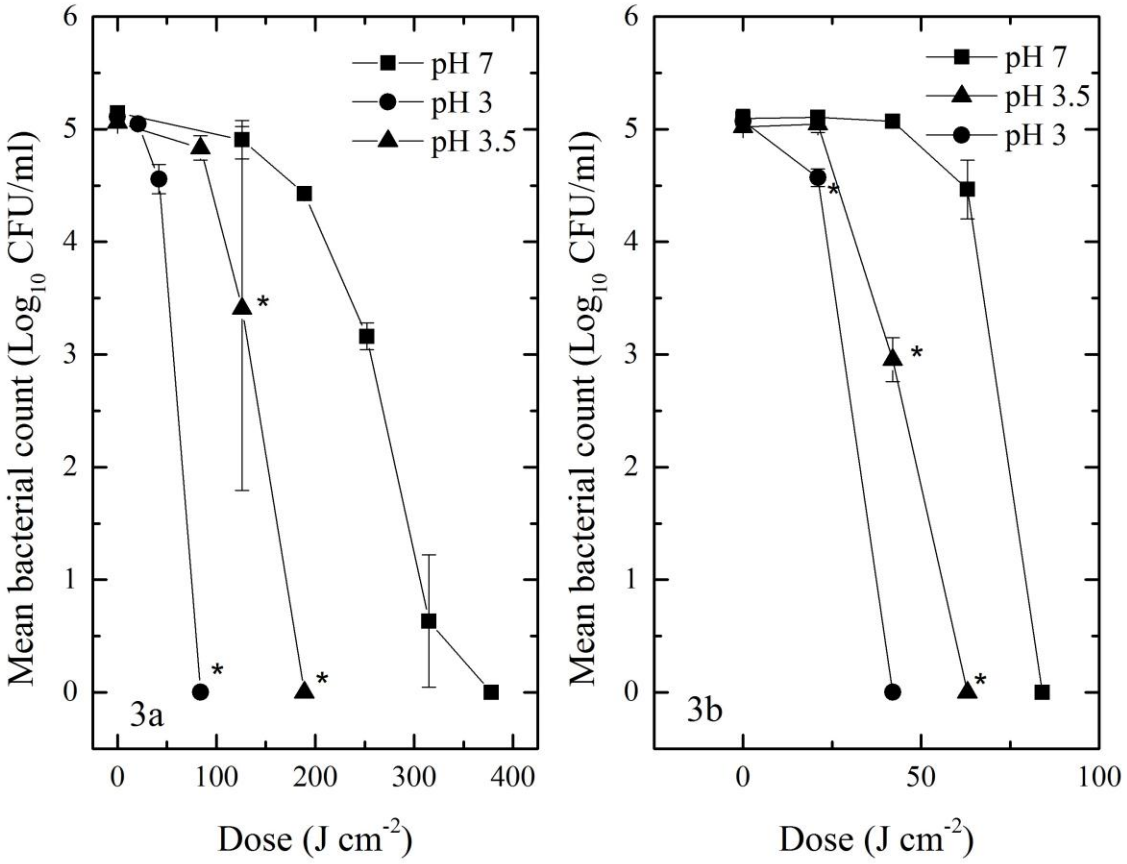


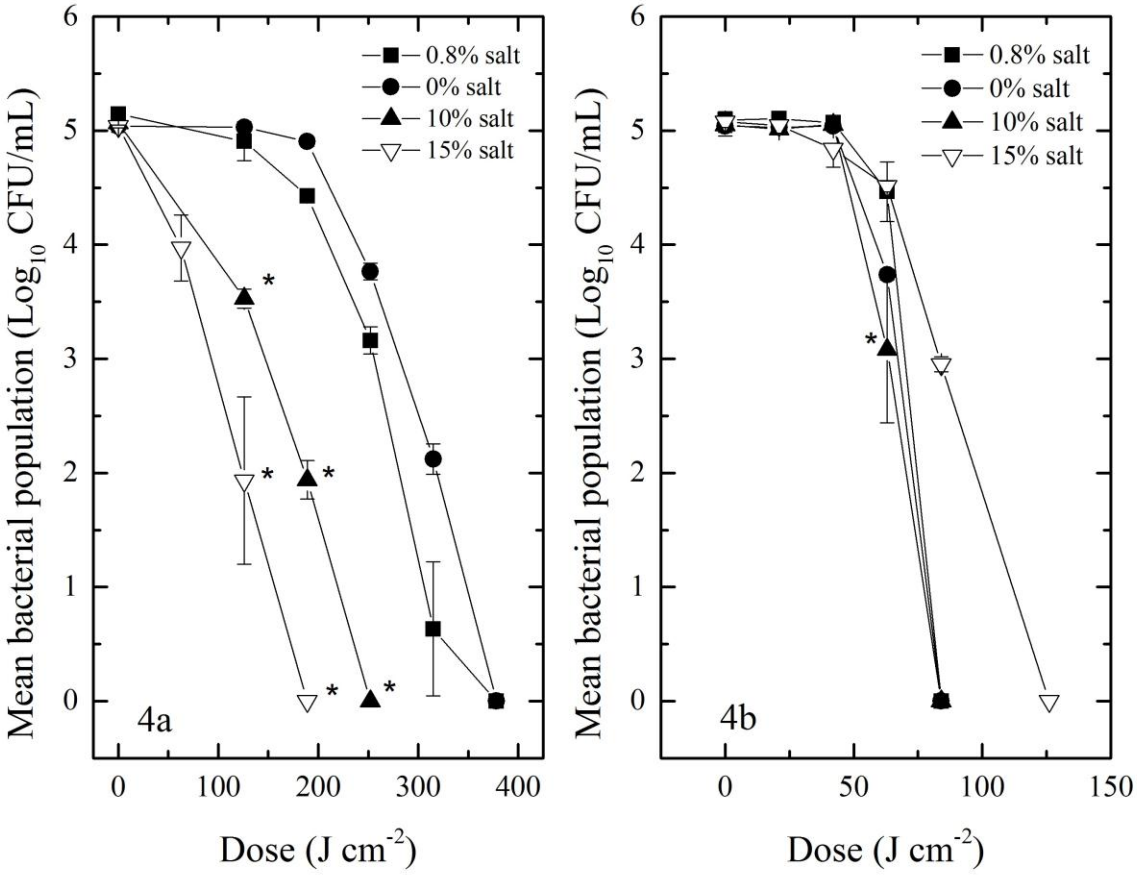
1a Exposure time to stress (minutes)


pH 3 TSA
pH 3 LSA
pH 3.5 TSA
pH 3.5 LSA
10% TSA
10% LSA
15% TSA
15% LSA
4°C TSA
4°C LSA
45°C TSA
45°C LSA
Mean bacterial count (Log10 CFU/mL)
Exposure Time to Stress (minutes)

1b


22°C
4°C
45°C
Mean bacterial count (Log10 CFU/mL)
Dose (Jcm-2)
2a
2b
*


pH 7
pH 3
pH 3.5
Mean bacterial count (Log10 CFU/ml)
Dose (J cm-2)
3a
3b
*


0.8% salt
0% salt
10% salt
15% salt
Mean bacterial population (Log10 CFU/mL)
Dose (J cm-2)
4a
4b
*