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Bacterial biofilm reduction by 275 and 455 nm light pulses emitted from light emitting diodes

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Abstract

Eradication of biofilms from the food contact surfaces is a challenging task, owing to their increased resistance to sanitizers and regular cleaning practices. The treatment with the light pulses emitted from the light emitting diode (LED) is an emerging surface decontamination technology, that can produce the antibiofilm effect by photodynamic inactivation. The objective of this study was to understand the antibiofilm efficacy of the 275 (Ultraviolet-C [UV-C]) and 455 nm (Blue) light pulses emitted from the LEDs against single and dual-species biofilms of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 on stainless steel (SS) coupons formed at different time. The biofilm formation by *S.* Typhimurium was improved when grown with *A. australiensis* in dual-species culture. Both 275 and 455 nm light pulses showed significant antibiofilm activity against *S.* Typhimurium and *A. australiensis* in single and dual-species biofilms. For instance, the 275 nm LED treatment of surfaces of SS coupons with 1.8 J/cm^2 dose on each surface, produced reductions of 4.24 and 3.9 log (CFU/cm^2) in single (cell attachment) and dual-species biofilms of *S.* Typhimurium, and reductions of 4.45 and 4.99 log (CFU/cm^2) in single and dual-species biofilms of *A. australiensis*. However, the susceptibility of *A. australiensis* toward 455 nm LED treatments was influenced by the presence of *S.* Typhimurium in the dual-species biofilm. The confocal laser scanning microscopy images revealed significant cell membrane damage in the dual-species biofilms by the LED treatments with 275 and 455 nm light pulses. Overall, several factors like surface temperature increase, strains used, treatment dose, treatment time, and incubation period of biofilms influenced the inactivation efficacy of the 275 and 455 nm LED treatments against the biofilms formed on SS coupons. This study provides an insight into the inactivation efficacy of LED light pulses against bacterial biofilms on food grade SS surfaces.

1 | INTRODUCTION

Biofilm formation by foodborne pathogens like *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* is a major concern. These microorganisms form the biofilms as a defense mechanism in response to stress conditions in food products like vegetables, meat, poultry, seafoods, and on food contact surfaces such as, stainless steel (SS) equipment, plastics, and conveyor belts (Kumar & Anand, 1998). *Salmonella* can attach to

different surfaces and form biofilms and can thrive in the presence of biofilms formed by other strong biofilm forming bacteria (Joseph, Otta, Karunasagar, & Karunasagar, 2001; Sinde & Carballo, 2000; Stepanović, Ćirković, Ranin, & Svabić-Vlahović, 2004; Visvalingam, Zhang, Ells, & Yang, 2019). For example, *Aeromonas australiensis* 03-09 (isolated from beef processing plant) showed synergistic effect in multi species biofilm formation with *S*. Typhimurium (Visvalingam, Zhang, et al., 2019; Wang, He, & Yang, 2018). The secretion of extracellular polymeric substances that include polysaccharides, proteins, lipids, extracellular DNA in the biofilms create a complex matrix encasing the bacterial cells, which protects them from sanitizers and regular cleaning procedures in the food processing plants (Flemming et al., 2016; Visvalingam, Zhang, et al., 2019), thus, increasing the risk of cross contamination of foods. This necessitates the need for exploring novel technologies for eradication of biofilms from food contact surfaces. Several studies focused on understanding the antibiofilm activity of light based technologies such as UV light, pulsed light and light emitting diode (LED) technology (Argyraki, Markvart, Bjørndal, Bjarnsholt, & Petersen, 2017; Bumah, Masson-Meyers, & Enwemeka, 2020; Li, Kim, Bang, & Yuk, 2018; Silva-Espinoza et al., 2020).

LEDs have several advantages like absence of warm-up time, monochromatic light emission, compact size, which makes it easy to incorporate into the existing processing lines (D'Souza, Yuk, Khoo, & Zhou, 2015; Kebbi et al., 2020). The specific color and wavelength of light emitted by the LEDs involve doping of the semiconductor materials with impurities (Held, 2009; Prasad et al., 2020). LED technology produces antimicrobial effect by means of photodynamic inactivation (PDI). This involves generation of reactive oxygen species (ROS) in the presence of oxygen, when light absorbing molecules like porphyrin compounds in the bacteria absorb the light, which further leads to cytotoxic responses like, DNA oxidation, lipid oxidation, and inhibition of cell replication, eventually causing cell death (Luksiene & Zukauskas, 2009; Plavskii et al., 2018).

Previous studies have focused on understanding the antimicrobial efficacy of the LED system in high and low water activity foods (Du, Prasad, Gänzle, & Roopesh, 2020; Ghate et al., 2017; Ghate, Kumar, Zhou, & Yuk, 2016; Prasad, Gänzle, & Roopesh, 2021; Subedi, Du, Prasad, Yadav, & Roopesh, 2020). LEDs emitting light of wavelengths 275 nm (Ultraviolet-C [UV-C]) and ~460 nm (Blue) have shown promising antibacterial effect in high water activity foods and in wastewater disinfection (Ghate et al., 2016; Ghate et al., 2017; Josewin, Ghate, Kim, & Yuk, 2018; Kim, Kim, & Kang, 2016; Vilhunen, Särkkä, & Sillanpää, 2009). Also, UV-C and Blue LED treatments are being widely studied for biofilm inactivation in medical applications and has shown promising results (Angarano et al., 2020; Bak, Ladefoged, Tvede, Begovic, & Gregersen, 2010; Esper et al., 2019). There have also been studies exploring the inactivation efficacy of the LED technology emitting lights of wavelengths 405, 255, 265, 266, 285, and 295 nm against bacterial biofilms formed by *L. monocytogenes* and *Pseudomonas aeruginosa* (Argyraki et al., 2017; Gora, Rauch, Ontiveros, Stoddart, & Gagnon, 2019; Li et al., 2018). However, there have been limited to no studies focusing on the antibiofilm efficacy of LEDs emitting light pulses of wavelengths 275 and 455 (Blue) nm against single and dual-species biofilms of *S*. Typhimurium on SS surfaces.

LED treatment can be used in food industries to inactivate pathogen biofilms attached to food contact surfaces such as SS. Hence, understanding the antibiofilm effect of LEDs emitting light pulses of wavelengths 275 and 455 nm during the incubation period of biofilm formation on SS coupons is important. Moreover, information on their underlying mode of action against biofilms would help in developing LED treatment as an alternate surface sanitation technology for food contact surfaces. The objective of this study was to evaluate the potential of 275 and 455 nm LEDs for their antibiofilm efficacy against *S*. Typhimurium and *A. australiensis* and to understand more details about their antibiofilm mechanism.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium ATCC 13311 and *A. australiensis* 03-09 (meat plant isolate; [Visvalingam, Zhang, et al., 2019]) were used in this study. Tryptic soy agar (TSA; Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.6% yeast extract (YE; Fischer Bioreagents, Geel, Belgium) and Lennox agar with no salt agar plates (LA-NS) consisting of 10 g/L tryptone, 5 g/L yeast extract and 15 g/L granulated agar, were used to restore *S*. Typhimurium and *A. australiensis* by streaking them on their respective agar plates from their frozen stock cultures, respectively. This was followed by two consecutive transfers in Tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.6% yeast extract or Lennox broth no salt (LB-NS) and incubation at 37°C for 18–24 hr, respectively.

2.2 | Preparation of *S*. Typhimurium biofilms on stainless steel coupons

The SS coupons (food grade, Type 304) of 2.4 cm × 2.4 cm were used in this study. The SS coupons were washed thoroughly and were sterilized in 70% ethanol with a Bunsen burner followed by autoclaving at 121°C for 15 min to remove any residual bacteria after use. Overnight culture of *S*. Typhimurium ATCC13311 was diluted to 10^8 CFU/mL in TSBYE. The SS coupons were inoculated with this bacterial suspension in a 6-well microplate (Costar, Corning, NY) followed by static incubation at 37°C for 3 days. The inoculated SS coupons were washed thrice with 1 mL of 0.1% peptone water to remove the loosely attached cells from the coupons on Days 1, 2, and 3. To extract *S*. Typhimurium biofilms for enumeration, the washed coupons were placed in a 50 mL falcon tube containing ~3 g glass beads (4 mm diameter, DWK Life Sciences Kimble, Mainz, Germany) and 5 mL 0.1% peptone water. This tube was then vortexed at the highest speed for 2 min (Visvalingam, Wang, Ells, & Yang, 2019) and the resultant bacterial suspension was then serially diluted in 0.1% peptone water and enumerated by spread

plating on TSAYE plates followed by incubation at 37°C for 24 hr. The final cell counts were recorded in CFU/cm^2.

2.3 | Preparation of *A. australiensis* and dual-species biofilms on stainless steel coupons

Development of dual-species biofilms included both *S.* Typhimurium and *A. australiensis*. For the preparation of single species biofilm of *A. australiensis*, the overnight culture in LB-NS broth (~10^9 CFU/mL) was diluted 100-fold to get a final concentration of ~10^7 CFU/mL in the same broth. For the preparation of the dual-species biofilms, the overnight cultures of both *S.* Typhimurium (~10^9 CFU/mL) and *A. australiensis* prepared in LB-NS broth were mixed in equal volume and diluted 100-fold in LB-NS broth to get a final cell concentration of ~10^7 CFU/mL. The SS coupons were inoculated with the diluted single and dual-species bacterial suspension in a 6-well microplate and incubated at room temperature (~23°C) statically for 6 days. Cell counts were taken every day to check the growth of biofilms. The SS coupons were washed thrice with 1 mL 0.1% peptone water to remove the loosely attached bacterial cells. The biofilms were extracted from the SS coupons by vortexing in 5 mL of 0.1% peptone water and ~3 g glass beads as mentioned in Section 2.2. For the enumeration of single species biofilm of *A. australiensis*, the bacterial suspension was serially diluted in 0.1% peptone water and spread plated on LB-NS agar plates followed by incubation at 37°C for 24 hr. In the case of dual-species biofilm, the *S.* Typhimurium and *A. australiensis* cells were differentially enumerated based on their colony morphology and growth temperatures. Therefore, the spread plated LB-NS agar plates were incubated at: (a) 37°C for 24 hrs to get the cell counts for dual-species biofilms, (b) 42°C for 24 hrs to obtain the cell counts for *S.* Typhimurium cells in the dual-species biofilms (absence of *A. australiensis* colonies in 24 hr), and (c) 18°C for 48–65 hrs to obtain the cell counts of *A. australiensis* in the dual-species biofilms (*S.* Typhimurium colonies needed longer incubation period to appear). All the cell counts were reported in CFU/cm^2.

2.4 | Crystal violet staining assay

Single and dual-species cultures prepared as mentioned in Section 2.3 in LB-NS were used for the crystal violet (CV) staining assay. Two hundred microliters of single and dual-species cultures were inoculated in a 96-well microplate in triplicates (Costar, Corning, NY) and incubated at room temperature (~23°C) for 6 days. Similarly, 200 µL of sterile LB-NS was incubated for 6 days and was used as control. The assay was performed on Days 1, 2, 4, and 6 of the incubation period. For the CV staining, the supernatant was removed, and each well was washed twice with 200 µL of phosphate buffer saline of pH 7.4 (PBS; Gibco, life technologies, Waltham, MA) and air-dried in the biosafety cabinet for 30 min. Each well was stained with 100 µL of 0.1% CV stain and incubated at room temperature for 45 min in the dark. After incubation, the stain was removed, and each well was washed thrice with 200 µL of PBS. Two hundred microliters of 95% ethanol was added in each well to dissolve the remaining CV stain and incubated at 4°C for 30 min in the dark. The amount of dissolved CV stain was determined as optical density at 595 nm using a spectrophotometer (Variskon flash, Thermo Electron Corporation, Nepean, Ontario, Canada). The readings obtained for uninoculated LB-NS broth served as the background value and was subtracted from the sample readings. The CV samples were diluted two folds, when the OD_{595} value >1, and the reading was repeated again (Visvalingam, Zhang, et al., 2019).

2.5 | The light emitting diode system

The LED system comprised of a controller unit (CF3000, Clearstone Technologies, Inc., Hopkins, MN) and the LED heads of JL3 series (111 × 70 × 128 mm^3) emitting light pulses of wavelengths 275 and 455 nm. The LED system was used at 60% power level which signifies that the light pulses are produced with an ON time of 6 ms and OFF time of 4 ms during the treatment time at a frequency of 100 Hz (Prasad et al., 2021). Hence, an average value of the light intensity was calculated considering the ON time of 6 ms and OFF time of 4 ms. More details about the pulsed LED intensity, light frequency and duty cycle are provided in Subedi et al. (2020). The irradiance of the 455 nm LED was measured using a laser energy meter (7Z01580, Starbright, Ophir Photonics, Har Hotzvim, JRS, Israel), connected to a photodiode irradiance and dose sensor (PD300RM-8 W, Ophir Photonics, A Newport Corporation Brand, Har Hotzvim, JRS, Israel) at 4 cm from the LED head. Similarly, the irradiance of the 275 nm LED was measured using a radiometer (ILT2400, International Light Technologies, MA) attached to an intensity sensor at 4 cm from the LED head. The irradiance of the 275 and 455 nm LEDs at 60% power level at 4 cm distance was obtained as 0.006 and 0.291 W/cm^2, respectively. The dose of the light pulses was calculated by using the equation (Prasad et al., 2020) as follows:

$$E = I \times t \quad (1)$$

where E is the energy dose (J/cm^2), I is the irradiance of the emitted light of particular wavelength (W/cm^2), and t corresponds to the treatment time (in s).

2.6 | Light emitting diode treatment of single and dual-species biofilms on stainless steel coupons

The single and dual-species biofilms formed on the SS coupons were treated with 275 and 455 nm LED at 60% power level. The washed SS coupons were kept on a small petri dish with an area of ~28.3 cm^2 and at 4 cm from the LED heads for the treatments with the light pulses emitted from the LEDs and the biofilms formed on both the surfaces of the SS coupons were treated with the LEDs. This included treatment of a surface (Surface 1) of the SS coupons with the light pulses of selected wavelength and dose, immediately

followed by turning the same SS coupon and exposing the second surface (Surface 2) to the LED treatments with the same wavelength and treatment dose. The single species biofilms of *S.* Typhimurium on the SS coupons were treated with the 275 nm light pulses for 2 (0.72 J/cm^2 dose) and 5 (1.80 J/cm^2 dose) min and with 455 nm light pulses for 5 (87.30 J/cm^2 dose) and 10 (174.6 J/cm^2 dose) min on each surface of the coupons. The LED treatments were performed each day, during the 3 days incubation period of *S.* Typhimurium biofilm formation at 37°C. The inoculated SS coupons without any LED treatments on Days 1, 2, and 3 were considered as control.

For the LED treatment of the SS coupons with *A. australiensis* biofilms with 275 and 455 nm light pulses; treatment times chosen were 5 min of 275 nm light pulses corresponding to an energy dose of 1.80 J/cm^2, and 2 and 5 min of 455 nm light pulses corresponding to dose of 34.92 and 87.30 J/cm^2, respectively on each surface at 60% power level and 4 cm distance from the LED head. Similarly, the SS coupons with dual-species biofilm comprising of *S.* Typhimurium and *A. australiensis*, were treated for 5 min with 275 nm light pulses and for 2 and 5 min for 455 nm light pulses at 60% power level for each surface at 4 cm from the LED heads. The LED treatments of the single (*A. australiensis*) and dual-species biofilms were performed at 23°C on Days 2, 4, and 6 of the incubation period of 6 days. The inoculated SS coupons without any LED treatments on Days 2, 4, and 6 were used as control.

The cell counts were obtained by extraction of the attached biofilms from the SS coupons, followed by serial dilution and spread plating as mentioned above and were expressed as CFU/cm^2. The surface temperatures of the LED treated SS coupons were determined by using a thermocouple attached to a digital thermometer (1507726, Fischer Scientific, Hampton, NH) during the LED treatments on both the surfaces. Here, the temperature increase on the first surface (indicated as Surface 1) was monitored, immediately followed by recording the temperature on the second surface (indicated as Surface 2) by turning the same SS coupon. The surface temperatures of the SS coupons before LED treatment of each surface were considered as control.

2.7 | Confocal laser scanning microscopy imaging

The biofilms on the SS coupons inoculated with dual-species of *S.* Typhimurium and *A. australiensis* were analyzed with the confocal laser scanning microscopy (CLSM) microscope. For imaging, the dual-species biofilms on SS coupons were washed thrice in 1 mL of 0.1% peptone water in a small petri dish (~28.3 cm^2) and treated with 275 (5 min) and 455 (2 and 5 min) nm light pulses at 60% power level on the surface to be analyzed at 4 cm distance from the LED head on Day 2 of the incubation period. LIVE/DEAD BacLight viability kit (L7012, Molecular Probes, Inc., Eugene, OR) consisting of propidium iodide (PI) and SYTO9 dyes was used for the analysis of dual-species biofilms using the CLSM. Here, PI stains the damaged bacterial cells and SYTO9 stains both intact and damaged bacterial cells. For staining, equal volumes of both the dyes were mixed thoroughly and 3 µL of the dye mixture was added to 1 mL PBS, which was loaded onto the SS coupons surface and incubated in the dark for 15 min for staining the biofilms. After the staining, the SS coupons were washed thrice with PBS to remove the excess stain from the coupons. These steel coupons were then imaged with CLSM (Zeiss LSM 710, Jena, Germany) by using the 488 (green) and 594 (red) nm lasers. The z-stack images were taken in triplicates to facilitate the quantification of the bacterial cells stained with PI (red) and SYTO9 (green) using Fiji (ImageJ 1.53f51) software. The untreated stained dual-species biofilms on SS coupons were considered as control.

2.8 | Statistical analysis

The experiments were done in triplicates ($n = 3$). The statistical analysis was done using SAS University edition (SAS studio 9.4) and the significant differences between the means was performed by two-way ANOVA by Tukey's LSD test ($p < .05$).

3 | RESULTS

3.1 | Determination of single and dual-species biofilm formation by using crystal violet assay

Crystal violet assay was used to analyze the biofilm forming capability of *S.* Typhimurium and *A. australiensis* in single as well as in dual-species culture in 96-well microtiter plate. CV stains the biomass

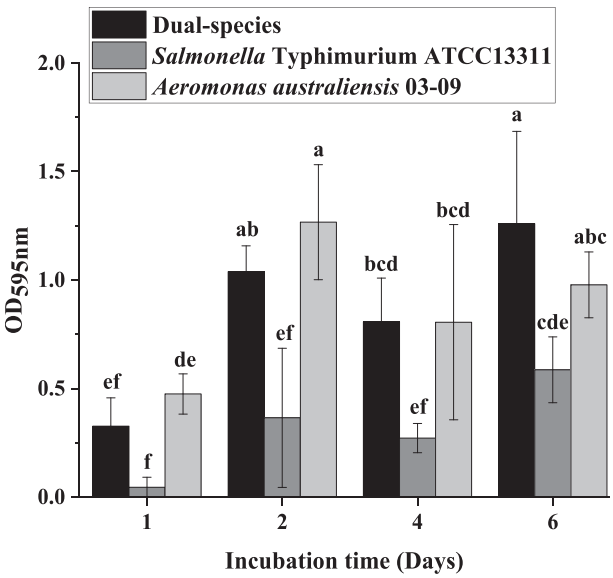

FIGURE 1 Biofilm formation by single and dual-species of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 on 96 well microtiter plate, incubated at ~23°C for 6 days. The quantification of the crystal violet (CV) stained biomass was done by their absorbance measurement at 595 nm on Days 1, 2, 4, and 6 of the incubation period. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p < .05$).

produced by the cells on the surface, which can be attributed to the biofilm forming capacity of the bacteria. Here, the biomass produced by *S.* Typhimurium alone was significantly lower than the biomass produced by both *A. australiensis* and dual-species cultures during the entire incubation period of 6 days ($p < .0001$) (Figure 1). However, no difference in biomass production between *A. australiensis* and dual-species cultures were observed. The effect of incubation period on the biofilm formation by single and dual-species culture was also analyzed on Days 1, 2, 4, and 6. A significant effect of incubation period on the biomass production by both the single and dual-species cultures was observed ($p < .0001$). Biofilm production was observed to be more pronounced on Days 2 and 6. For example, the OD_{595nm} values for *A. australiensis* and dual-species culture were 1.267, 1.038, and 0.977, 1.259 on Days 2 and 6, respectively (Figure 1). The highest OD_{595nm} value for *S.* Typhimurium was 0.586 on Day 6 of the incubation period. Also, the lowest OD_{595nm} values were obtained on Day 1 of the incubation period for both single and dual-species cultures. Incubation period influenced the biofilm forming capacity of the single and dual-species cultures.

3.2 | Biofilm formation of single and dual-species on stainless steel coupons

The biofilm formation of single and dual-species of *S.* Typhimurium and *A. australiensis* on food grade SS coupons were monitored by microbial enumeration over the incubation period of 3 or 6 days. For single species biofilm study for *S.* Typhimurium, an inoculum of ~8 log (CFU/mL) and a shorter incubation period of 3 days was used in comparison to ~7 log (CFU/mL) inoculum and 6 days incubation period used for *A. australiensis*. *A. australiensis* showed higher biofilm formation than *S.* Typhimurium on SS coupons ($p < .0001$) (Figure 2a). For example, the highest cell attachment obtained in case of *S.* Typhimurium was 4.35 log (CFU/cm^2) on Day 1 (with no effect of the incubation period) and 6.78 log (CFU/cm^2) for *A. australiensis* on Day 2 with the inoculum of 8 and 7 log (CFU/mL), respectively (Figure 2a). Since, the cell attachment of single species of *S.* Typhimurium on SS coupons is low, the *S.* Typhimurium cell counts obtained on SS coupons can be considered as surface attached cells. A significant interaction of bacteria and incubation period was also observed ($p < .0001$).

For dual-species biofilm study on SS coupons, the cell counts of *S.* Typhimurium and *A. australiensis* were obtained by incubating the dual-species culture at different incubation temperatures ideal for the faster growth of these individual bacteria. The dual-species inoculum was 7.27 log (CFU/mL), from which the inoculum counts of *S.* Typhimurium and *A. australiensis* were 7.12 and 7.01 log (CFU/mL), respectively. Overall, the biofilm formation by dual-species culture ($p < .0001$) and *A. australiensis* ($p = .0004$) were significantly higher than *S.* Typhimurium (Figure 2b). Incubation period also produced a significant effect on the dual-species biofilm formation on SS coupons. A significant decrease in the biofilm formation was observed from Day 4 till Day 6 (Figure 2b). Also, a significant interaction of bacteria and incubation period was observed in the dual-species biofilm study ($p < .0001$).

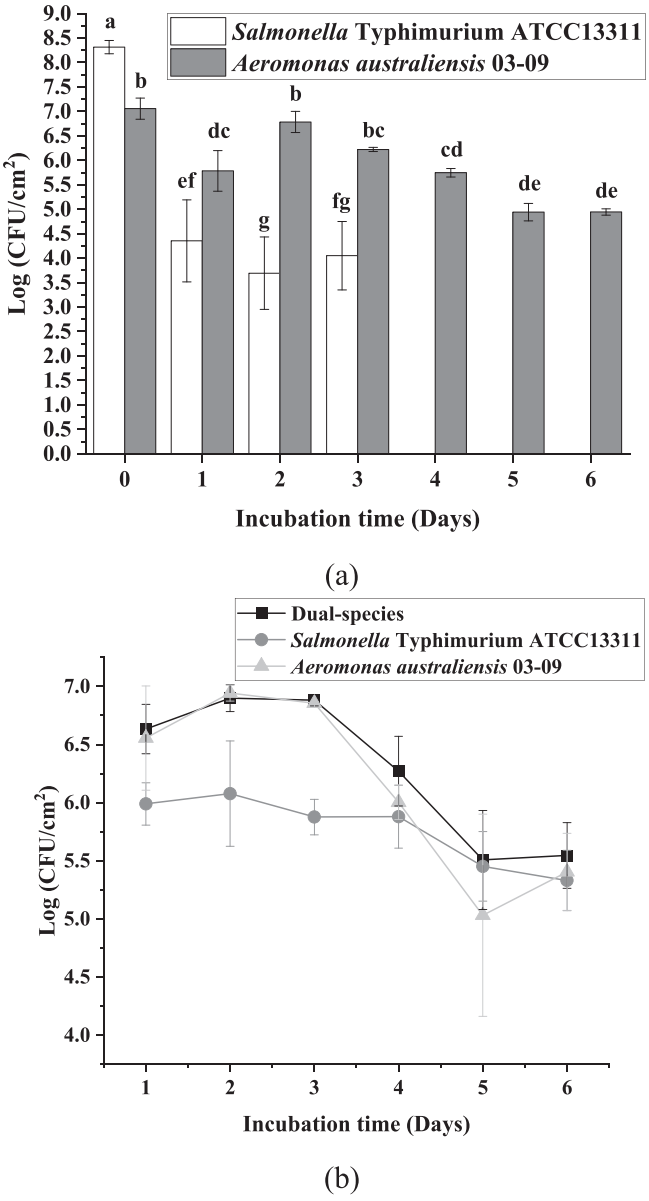

FIGURE 2 Biofilm formation by single species of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 on stainless steel coupons, incubated at 37°C and 23°C for 3 and 6 days, respectively (a). Values for Day 0 corresponds to log (CFU/mL) of the inoculum used. Biofilm formation by *S.* Typhimurium and *A. australiensis* in dual-species cultures incubated at 23°C for 6 days (b). Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p < .05$).

3.3 | Effect of 275 and 455 nm light pulses against single and dual-species biofilms

The efficacy of the 275 and 455 nm LED light pulses were tested against single and dual-species biofilms on SS coupons by treating both surfaces of the coupons at 4 cm distance from the LED head at 60% power level. The single species *S.* Typhimurium cell attachment and *A. australiensis* biofilms showed significant sensitivity to both

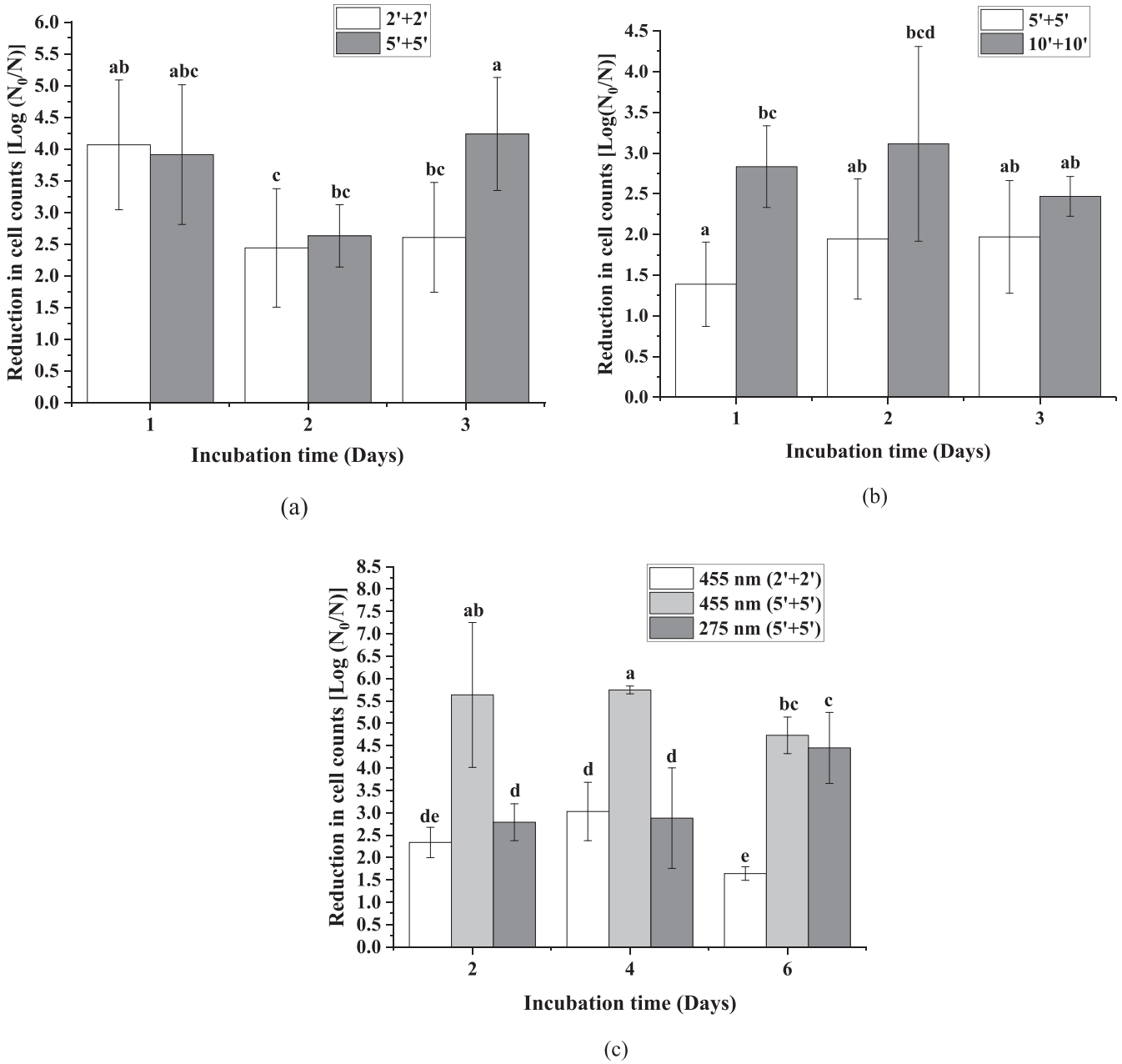

FIGURE 3 Reduction in cell counts (log (N_0/N) of single species biofilms of *Salmonella* Typhimurium ATCC13311 on stainless steel (SS) coupons formed at 37°C on Days 1, 2, and 3 after treatment of the coupon surfaces with 275 nm (a) and 455 nm LED (b). The treatment of biofilms of *S*. Typhimurium with 275 nm light pulses was performed for 2 (0.72 J/cm^2 dose) and 5 (1.80 J/cm^2 dose) min and with 455 nm light pulses was performed for 5 (87.30 J/cm^2 dose) and 10 (174.6 J/cm^2 dose) min. Reduction in cell counts of single species biofilms of *A. australiensis* 03-09 at 23°C on Days 2, 4, and 6 after treatment with 275 and 455 nm LEDs (c). The treatment of single species biofilms of *A. australiensis* with 275 nm light pulses was performed for 5 (1.80 J/cm^2 dose) min. and with 455 nm light pulses was performed for 2 (34.92 J/cm^2 dose) and 5 (87.30 J/cm^2 dose) min. The irradiance values for the 455 and 275 nm LEDs are 0.291 and 0.006 W/cm^2. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p < .05$).

275 and 455 nm LED treatments (Figure 3a–c). The incubation period produced a significant effect on the inactivation efficacy of the 275 nm LED treatment against *S*. Typhimurium ($p = .0078$) while there was no effect ($p = .065$) of increasing the 275 nm LED treatment time from 2 to 5 min on each surface on its inactivation efficacy. For instance, the 275 nm LED treatment for 2 and 5 min on each surface corresponding to dose of 0.72 and 1.80 J/cm^2 on Day 2, produced significantly lesser log (CFU/cm^2) reduction in *S*. Typhimurium cell counts as compared to that observed on Days 1 and 3, respectively (Figure 3a). Also, the incubation period produced a significant effect on the sensitivity of *A. australiensis* biofilms toward 275 nm LED treatment for 5 min (~1.80 J/cm^2 dose) on each surface. For example, the reduction in the cell counts of *A. australiensis* biofilms increased from 2.79 and 2.88 log

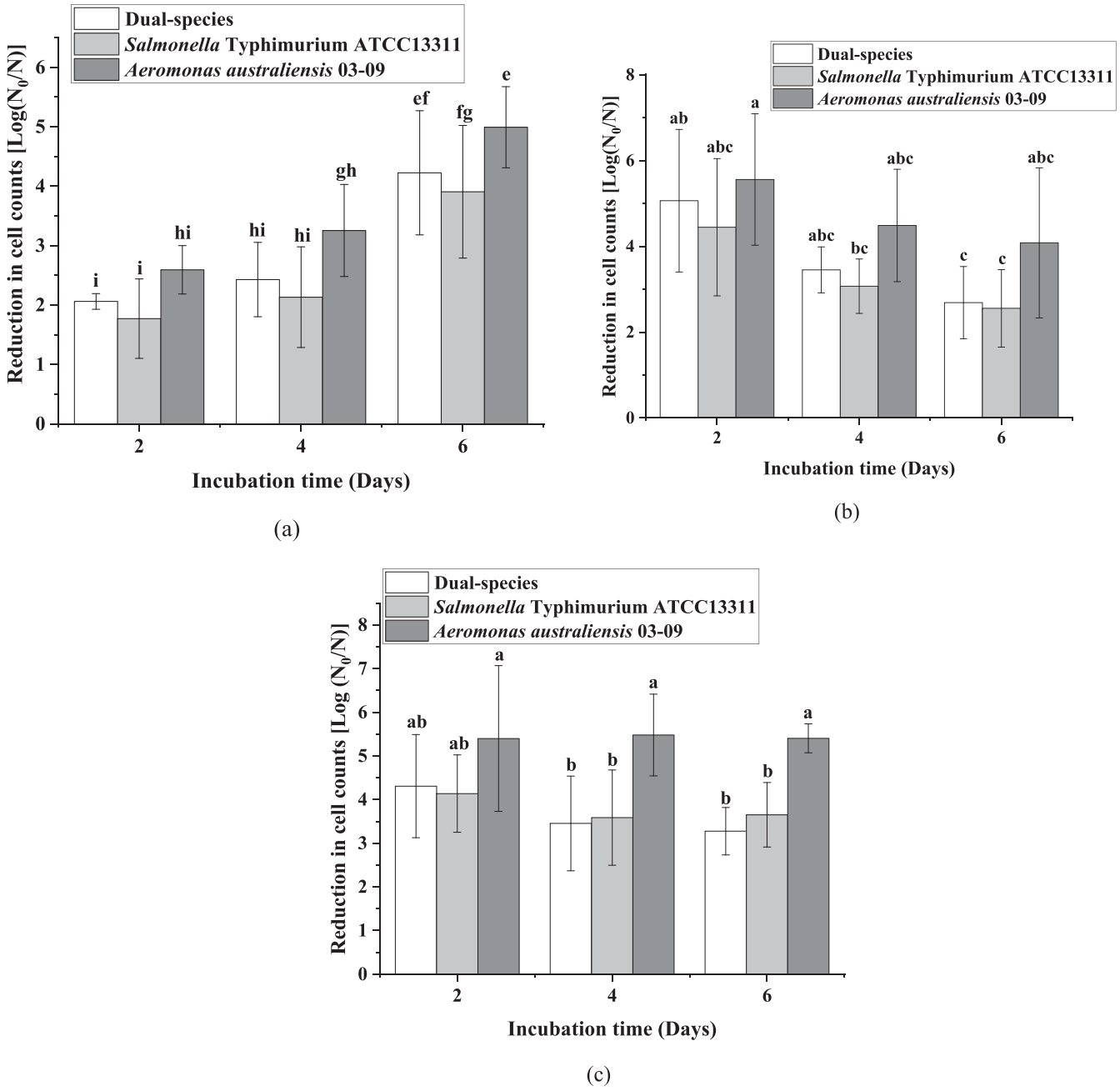

FIGURE 4 Reduction in cell counts [log (N_0/N)] of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 in mixed species biofilms formed on stainless steel (SS) coupons at 23°C on Days 2, 4, and 6 after treatment of SS coupon surfaces with 275 nm LED for 5 min (1.80 J/cm^2 dose) (a); and with 455 nm LED for 2 min (34.92 J/cm^2 dose) (b) and 5 min (87.30 J/cm^2 dose) (c). The irradiance values for the 455 and 275 nm LEDs are 0.291 and 0.006 W/cm^2. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other (p < .05).

(CFU/cm^2) on Days 2 and 4, respectively, to 4.45 log (CFU/cm^2) on Day 6 (Figure 3c).

The 455 nm LED treatment for 10 min (174.6 J/cm^2 dose) on each surface of the SS coupons produced significantly higher (p = .0089) reduction in cell counts of *S.* Typhimurium as compared to the 5 min (87.30 J/cm^2 dose) treatment on each surface on Day 1 (Figure 3b). However, the same trend was not observed on increasing the incubation period, indicating that increasing the treatment time (or dose) of the 455 nm LED treatment improved the inactivation efficacy of the LED against single species cell attachment of *S.* Typhimurium on only Day 1. The maximum log reduction of 3.11 log (CFU/cm^2) was recorded with the 455 nm LED treatment of 10 min (174.6 J/cm^2 dose) against *S.* Typhimurium on Day 2 (Figure 3b). On the contrary, the sensitivity of *A. australiensis* toward the 455 nm LED treatments improved significantly (p < .0001) on increasing the treatment time from 2 min (34.92 J/cm^2 dose) on each surface of the SS

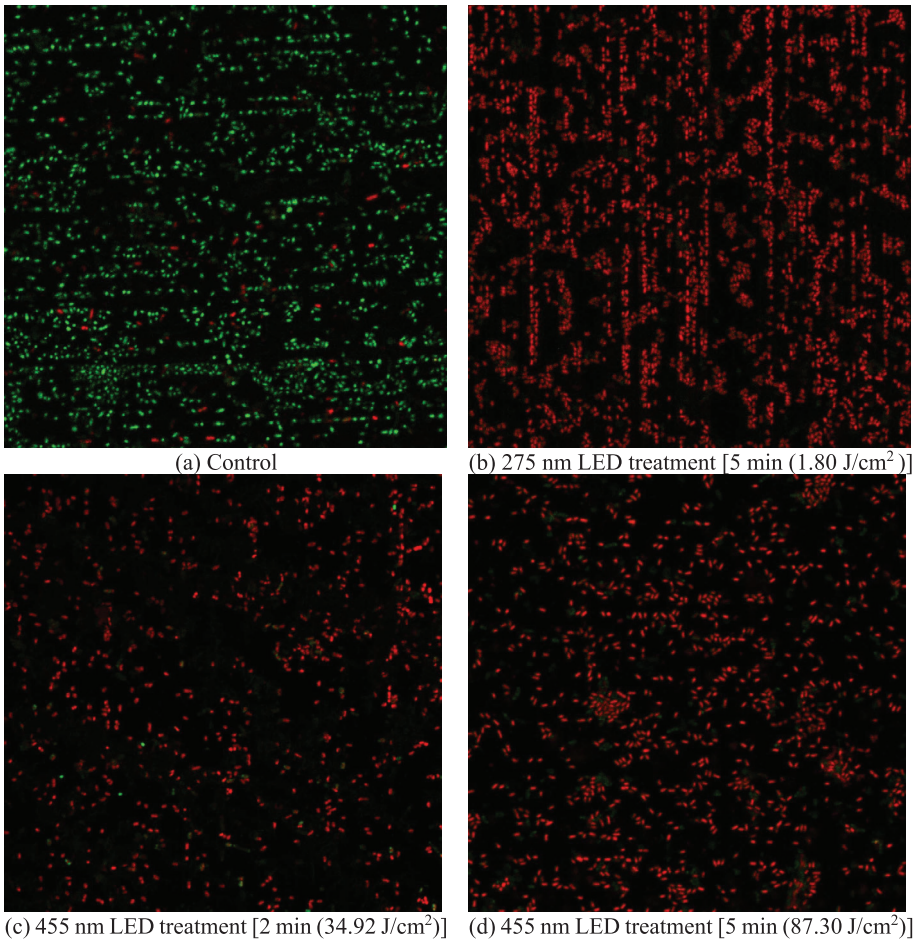

FIGURE 5 Confocal laser scanning microscopy images of dual-species biofilms of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 stained with SYTO9 and propidium iodide (PI). These images represent the cell membrane damage in the dual-species biofilms on stainless steel coupons due to the treatments with 275 and 455 nm light pulses on Day 2 of the incubation period. The treatments include (a) Control, (b) 275 nm LED treatment with 5 (1.80 J/cm^2 dose) min, (c) 455 nm LED treatment with 2 (34.92 J/cm^2 dose) min, and (d) 455 nm LED treatment with 5 (87.30 J/cm^2 dose) min. The irradiance values for the 455 and 275 nm LEDs are 0.291 and 0.006 W/cm^2.

coupons to 5 min (87.30 J/cm^2 dose) on Days 2, 4, and 6 of the incubation period (Figure 3c). Overall, the efficacy of the 455 nm LED decreased significantly on Day 6 compared to Day 4 of the incubation period.

For dual-species biofilm study, the 275 nm LED treatment for 5 min (1.80 J/cm^2 dose) on each surface of the SS coupons resulted in significantly ($p < .0001$) higher log reduction in Day 6 compared to Days 2 and 4, indicating the significant effect of increasing the incubation period on the inactivation efficacy of the 275 nm LED against dual-species biofilms (Figure 4a). However, there was no significant difference in the log reduction by the 275 nm LED treatment between Days 2 and 4. This trend was also observed in case of *S.* Typhimurium and *A. australiensis* individually in the dual-species biofilms (Figure 4a). A significant interaction between the 275 nm LED treatment and the incubation period was observed ($p < .0001$).

The 455 nm LED treatment showed significant inactivation efficacy against dual-species biofilms (also *S.* Typhimurium and *A. australiensis*) individually in the dual-species culture (Figure 4b,c). In this study, the 455 nm LED treatment for 5 min (87.30 J/cm^2 dose) on each surface of the coupons produced significantly higher reduction in *A. australiensis* cell counts as compared to *S.* Typhimurium ($p < .0001$) and dual-species ($p = .0015$) (Figure 4c) biofilm cell counts, indicating that the inactivation efficacy of the 455 nm LED treatment was strain dependent. Overall, there was no significant difference in the inactivation effect observed on increasing treatment time (or dose) of the 455 nm LED from 2 min (34.92 J/cm^2 dose) to 5 min (87.30 J/cm^2 dose). Also, there was no influence of the incubation period observed on the inactivation efficacy of the 455 nm LED against dual-species biofilms on SS coupons.

The antibiofilm efficacy of the 275 and 455 nm LED light pulses was influenced by the incubation period, which depends upon the strains used in the biofilm formation. For instance, the incubation period had a significant effect on the inactivation efficacy of the 455 nm LED treatments against single species biofilms, but this effect was not observed in the case of dual-species biofilms.

3.4 | Analysis of the images obtained by using confocal laser scanning microscope

LIVE/DEAD assay kit was used to understand the inactivation mechanism of the LED system emitting light of wavelengths 275 and 455 nm at 60% power level, by using CLSM. The SS coupons with dual-species biofilm were treated at 4 cm from the LED head. The LED treatment resulted in cell membrane damage as can be observed in Figure 5. The control has few damaged cells labeled with PI (Figure 5a) while the LED treatment with 275 and 455 nm wavelength

light pulses produced significant damage in the bacterial cell membrane (Figure 5b–d). In the case of 455 nm light pulses, the treatment for 2 min (~34.92 J/cm^2) produced significant cell membrane damage and there was no difference obtained by increasing the treatment time to 5 min (~87.30 J/cm^2). Since, light pulses had limited penetration, the z-stack images were quantified to understand its effect on the multilayer biofilms formed on the SS coupons. Figure 6 shows the percentage of cell population labeled with PI on one surface of the SS coupons. Here, the light pulses from LEDs produced significant damage in the bacterial cell membrane. However, there was no significant effect of the wavelength or treatment time on this effect.

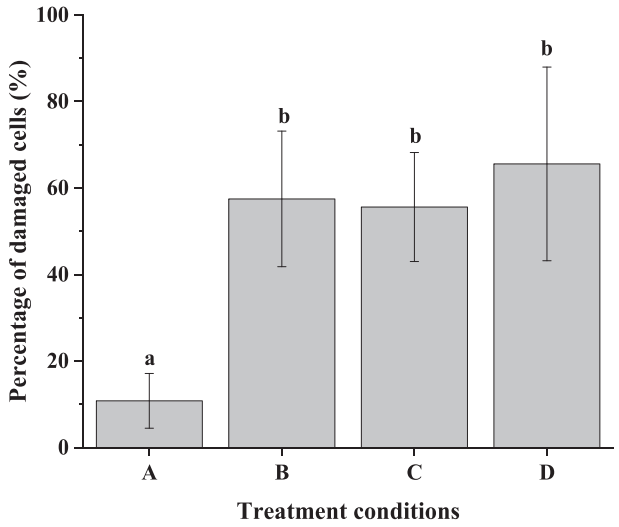

FIGURE 6 Percentage of damaged cells (%) in mixed species biofilms of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 stained with SYTO9 and propidium iodide (PI) on stainless steel coupon. The treatments were applied on biofilm formed at 23°C on Day 2 and include (a) Control, (b) 275 nm LED treatment for 5 min (1.80 J/cm^2 dose), (c) 455 nm LED treatment for 2 min (34.92 J/cm^2 dose), and (d) 455 nm LED treatment for 5 min (87.30 J/cm^2 dose). Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p < .05$).

3.5 | Temperature increase

LED treatment with 455 nm light pulses can produce surface temperature increase in foods like pet foods (Prasad et al., 2021). Therefore, the surface temperature of both surfaces of the SS coupons was monitored during the 275 and 455 nm LED treatments in this study (Table 1). The initial temperature of the second surface of SS coupons was significantly higher than the surface 1 due to the impact of the LED treatment on surface 1. The 275 nm LED treatments produced low but significantly higher surface temperature of SS coupons in comparison to the control (Table 1). For instance, the maximum temperature observed was 27°C with the 275 nm LED treatment with no effect of increasing the treatment time (or dose). However, significant increase in the surface temperature of the SS coupons were observed with the LED treatments using 455 nm light pulses, where the maximum temperature observed was 53°C after 10 min (174.6 J/cm^2 dose) treatment.

4 | DISCUSSION

The LED technology has shown promising antimicrobial activities and has been studied for the decontamination of high- and low-moisture foods (Du et al., 2020; Ghate et al., 2017; Hamamoto et al., 2007; Prasad et al., 2020; Prasad et al., 2021; Prasad, Gänzle, & Roopesh, 2019). This makes it an ideal technology to be tested for its antibiofilm activity to prevent cross contamination of the food products in food industry. Therefore, this study focused on understanding the inactivation efficacy of the LED technology, using light pulses of wavelengths 275 and 455 nm against biofilms formed by *S.* Typhimurium and *A. australiensis* in single and dual-species on SS coupons.

Differential biofilm forming capacity of *S.* Typhimurium and *A. australiensis* in 96-well microplate was observed in CV staining assay when grown in single species in this study. *S.* Typhimurium showed the lowest biomass production compared to *A. australiensis* alone and in the dual-species culture (Figure 1), indicating that *S.*

TABLE 1 Increase in the surface temperature of the stainless steel coupons treated with the LEDs emitting light pulses of wavelengths 455 and 275 nm at 60% power level and at 4 cm distance from the LED head.

Treatment time (min)	Dose (J/cm^2)	Surface 1 (°C)*	Surface 2 (°C)*
455 nm LED treatments			
0	0	24.33 ± 0.58^{f}	33.33 ± 0.58 ^{e}
2	34.92	46.33 ± 2.89^{d}	48.00 ± 1.00^{cd}
5	87.30	50.00 ± 1.00^{bc}	52.67 ± 0.58^{ab}
10	174.6	53.67 ± 1.53^{a}	53.00 ± 3.61^{ab}
275 nm LED treatments			
0	0	24.67 ± 0.58^{k}	26.00 ± 0.00^{j}
2	0.72	27.00 ± 1.00^{ij}	27.33 ± 0.58^{i}
5	1.80	27.33 ± 0.58^{i}	27.67 ± 0.58^{i}

Note: The irradiance values for the 455 and 275 nm LEDs are 0.291 and 0.006 W/cm^2. Values are shown as means ± standard deviation of three independent replicates.
*Values with same superscripts for the same LED treatments does not differ significantly ($p < .05$).

Typhimurium is a weak biofilm former and *A. australiensis* is a strong biofilm former in this study. This was supported by the viable cell counts obtained from the biofilms formed on SS coupons (Figure 2a,b). However, the CV staining can stain the biomass produced by both viable and dead cells (Merino, Procura, Trejo, Bueno, & Golowczyc, 2019; Pitts, Hamilton, Zelver, & Stewart, 2003), which might show contrasting observation with viable cell plating method. For example, in this study, the incubation period influenced the biomass formation of *A. australiensis* and dual-species culture in CV staining assay with highest values obtained at Days 2 and 6 (Figure 1), while the CFU enumeration showed that their biofilm formation reduced significantly after Day 3 (Figure 2a,b). Previously, a reduction in the biofilm formation by *Pseudomonas aeruginosa* was also observed with increasing incubation period (Pang & Yuk, 2018). This reduction in the cell numbers could be due to the detachment of the biofilms from the SS coupons upon maturation by secretion of certain enzymes by the bacteria (Nijland, Hall, & Burgess, 2010; Visvalingam, Zhang, et al., 2019).

In contrast, *S.* Typhimurium in single and dual-species biofilm study showed no effect of incubation period on its growth based on CV staining and microbial cell enumeration, which is supported by a previous study by Wong et al. (2010). However, Pang and Yuk (2018), showed that cell counts of *S.* Enteritidis in single species biofilm in chicken juice increased with incubation period, indicating that the growth of biofilms is affected by factors like growth media and bacterial strain used. *S.* Typhimurium showed improved attachment on the SS coupons in dual-species biofilm with *A. australiensis* compared to single species biofilm, indicating that the *S.* Typhimurium cells might thrive in the presence of a strong biofilm former (*A. australiensis*) with no difference in the cell counts of *A. australiensis* among single and dual-species biofilms. This synergistic effect of bacteria in multi-species biofilms on the biofilm formation of *S.* Typhimurium has been previously reported by Visvalingam, Zhang, et al. (2019). In mixed species biofilms, interspecies cooperation might occur by means of intercellular communication (Flemming et al., 2016), which might aid in the synergistic effect in mixed species biofilms. Moreover, *A. australiensis* might have produced a favorable micro-environment that facilitated the biofilm formation of *S.* Typhimurium in dual-species biofilms (Visvalingam, Zhang, et al., 2019).

The biofilms formed by *S.* Typhimurium and *A. australiensis* in single and dual-species showed significant sensitivity toward treatments with LEDs emitting light pulses of wavelengths 275 (UV-C) and 455 (Blue) nm. Previously, *S.* Typhimurium ATCC14028 biofilms formed on SS coupons on Day 2 of the incubation period at 37°C showed a reduction of 2.9 log (CFU/cm^2) when treated with 0.62 J/cm^2 dose of UV-C light (Silva-Espinoza et al., 2020). Similarly, a reduction of 2.44 log (CFU/cm^2) in single species cell attachment of *S.* Typhimurium ATCC13311 was observed at Day 2 with 2 min (~0.72 J/cm^2 dose) treatment on each surface of the SS coupons with 275 nm (UV-C) light pulses in this study. However, the inactivation efficacy of the LED treatment is influenced by several factors like, strain used, cell attachment on the food contact surface, distance of the sample from the light source, sample type, power level, treatment time, treatment dose, illumination temperature, relative humidity, etc. (Prasad et al., 2019, 2021). We observed that the inactivation efficacy of the 275 nm LED against *A. australiensis* in single and dual-species biofilms was lesser on Days 2 and 4 and was maximum on Day 6 of the incubation period for treatment of 5 min (~1.80 J/cm^2) on each surface (Figures 3c and 4a). In contrast, the cell counts of biofilms formed by *A. australiensis* alone and dual-species were higher on Days 2 or 4 and it reduced significantly on Day 6 of the incubation period (Figure 2a,b), indicating the presence of more extracellular polymeric substances in biofilms on Days 2 or 4 compared to Day 6, which could act as a barrier toward the UV-C LED treatment (Flemming et al., 2016; Silva-Espinoza et al., 2020).

Higher treatment doses of 87.30 J/cm^2 (5 min) and 174.6 J/cm^2 (10 min) were required on each surface of the SS coupons in the case of 455 nm LED to produce comparable reduction in single species cell attachment of *S.* Typhimurium to 275 nm LED treatments (Figure 3b). The biofilm attachment of dual-species and *A. australiensis* in single and dual-species biofilms reduced after Day 3 of the incubation period (Figure 2a,b). Also, the inactivation efficacy of the 455 nm LED treatments reduced after Day 4 against the single species biofilms of *A. australiensis* (Figure 3c). The detachment of *A. australiensis* single species biofilms might have resulted in the attachment of only the highly resistant biofilms on the SS coupons, thus reducing their susceptibility to the high intensity 455 nm LED treatments (Leriche and Carpentier, 1995). However, this trend was not observed in the case of *A. australiensis* in dual-species biofilms, indicating that the presence of *S.* Typhimurium in the dual-species biofilm might have influenced the efficacy of the 455 nm LED treatments against *A. australiensis* in dual-species biofilm. Also, *A. australiensis* in single species biofilms was more resistant to the 455 nm LED treatment for 2 min (~34.92 J/cm^2 dose) on each surface than for the dual-species biofilms (Figures 3c and 4b). The presence of other bacteria in the mixed species biofilms can have antagonistic or synergistic effect on its susceptibility to the antibacterial treatments on other bacterial strains present in the biofilms (Pang & Yuk, 2018; Visvalingam, Zhang, et al., 2019).

This antibiofilm effect of the 455 nm LED treatment could be attributed to the generation of ROS in the presence of oxygen, which causes the cytotoxic responses in the bacterial cell, leading to cell death (Luksiene & Zukauskas, 2009). The 275 nm (UV-C) light might inactivate the microorganisms by the formation of thymine dimers in the DNA leading to the inhibition of the cell replication (Diffey, 1991; Sánchez-Maldonado, Lee, & Farber, 2018). The treatment of biofilms in mixed and single species has shown reduction in the intact cells and hence, decreased fluorescence of SYTO9 due to the treatments with light pulses of wavelengths of UV-C and blue regions of the electromagnetic spectrum (Bumah et al., 2020; Silva-Espinoza et al., 2020; Yang et al., 2017). Similarly, we observed that the green fluorescence corresponding to the SYTO9 labelling reduced with the LED treatment using 275 and 455 nm light pulses (Figure 5a–d). Also, the treated SS coupon surfaces showed majority of PI labeled red fluorescence (Figure 5b–d), indicating the cell membrane damage as one of the major antibacterial mechanisms of the 275 and 455 nm

light pulses. Most bacterial cells in the treated coupons were labeled entirely with PI, which can be correlated with the high log reductions obtained with the LED treatments of dual-species biofilms. In the case of 455 nm LED treatments, the surface temperature increase could have also contributed to the increased cell membrane damage and high log reductions (Prasad et al., 2021). Since, LED treatment is a surface decontamination technology, the confocal 2D images in this study, represents the surface of the biofilms treated. Therefore, to get a detailed understanding of the number of cells damaged by the LED treatments, the 3D images of the biofilms were quantified for cell counts, which showed an increase in the number of cells that suffered cell membrane damage after the LED treatments (Figure 6). There was no difference in the percentage of cells damaged by 275 and 455 nm light pulses (Figure 6) in this study, therefore, more studies focusing on understanding the inactivation mechanisms of LED technology emitting light pulses of different wavelengths against higher concentration of biofilms is important.

In conclusion, the 275 and 455 nm light pulses emitted from the LEDs showed significant inactivation efficacy against single and dual-species biofilms of *S*. Typhimurium and *A. australiensis* on SS coupons. This inactivation efficacy was influenced by the strain used and the incubation period of the biofilms. The inactivation of the *A. australiensis* by the 455 nm LED treatment was influenced by the presence of *S*. Typhimurium in the dual-species biofilms. A significant increase in the surface temperature of the SS coupons was observed due to the treatment with high intensity pulses of 275 and 455 nm, while this temperature increase was more pronounced in the case of 455 nm LED treatments. The treatment of the dual-species biofilms with LEDs emitting light pulses of wavelengths 275 and 455 nm showed significant increase in the cell membrane damage of the bacterial cells. Overall, this study provided an insight into the potential application of LED treatment using 275 and 455 nm light pulses for the inactivation of biofilms formed in food contact surfaces, like SS surface.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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