Antimicrobial applications of treatments using light pulses emitted from light emitting diodes (LED)

by

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Abstract

Low water activity (a_w) foods have been associated with several foodborne outbreaks and recalls. Eradication of foodborne microorganisms like *Salmonella* and *Escherichia coli* from low a_w foods is challenging, as they are resistant to conventional decontamination practices. High intensity light pulses emitted from the Light Emitting Diode (LED) is an emerging decontamination technology, which is based on photodynamic inactivation. The overall objective of this research was to evaluate the inactivation efficacy of light pulses with selected wavelengths emitted from LEDs against foodborne pathogens at low and high a_w conditions and their biofilms and to understand their antimicrobial mechanisms.

First, the effects of the selected process and product parameters on the inactivation efficacy of LEDs emitting light pulses of 365 and 395 nm wavelengths against *S.* Typhimurium and *E. coli* in low a_w conditions were studied. The 365 nm LED treatments were more effective at low and high a_w conditions than the 395 nm LED treatments with the same dose. The second study focussed on evaluating the antibacterial efficacy of 455 nm LED treatment against *Salmonella* in dry powdered form and in low a_w pet foods. *S.* Typhimurium in dry powdered form was more resistant to the 455 nm LED treatment than in 0.75 a_w pet foods. Pre-treatment of *Salmonella* inoculated 0.75 a_w pet foods with 275 nm LED improved the inactivation efficacy of 455 nm LED treatment. Significant weight loss, surface temperature increase, and a_w reduction were observed in LED treated samples, indicating the drying potential of the 365, 395 and 455 nm LED light pulses. Also, significant effects of treatment time (or dose), strains used, sample type, power level, and illumination conditions were observed on the inactivation efficacy of the LED treatments.

The third study focussed on understanding the antibacterial mechanisms of the 365, 395 and 455 nm LED treatments against *S.* Typhimurium at low a_w conditions. A significant increase in the intracellular reactive oxygen species production and membrane lipid oxidation, after 365, 395 and 455 nm LED treatments were observed. The 395 nm LED treatments produced more membrane lipid oxidation in *S.* Typhimurium cells than the 365 nm LED treatment with the same dose.

Foodborne microorganisms can form biofilms on surfaces. Therefore, the fourth study focussed on understanding the efficacy of 275 and 455 nm LED treatment against single and mixed species biofilms of *S.* Typhimurium and *Aeromonas australiensis* on stainless steel surface. Both the LED treatments showed promising inactivation efficacy against *S.* Typhimurium and *A. australiensis* in single and mixed species biofilms. The sensitivity of *A. australiensis* towards 455 nm LED treatment was influenced by the presence of *S.* Typhimurium in the mixed species biofilms. Significant cell membrane damage of mixed species biofilms was observed due to the LED treatments.

This research shows the drying potential and the inactivation efficacy of the LED technology against foodborne pathogens in low a_w conditions and their biofilms. The knowledge gained in this research would help in further research and in the development of LED technology as an alternate decontamination technology.

Preface

This thesis is an original work done by Amritha Jaya Prasad at the Food Safety and Sustainability Engineering Lab at the University of Alberta under the supervision of Dr. Roopesh Mohandas Syamaladevi.

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I completed the experiments independently, analyzed the data, and wrote the manuscript. Dr. M. S. Roopesh guided the overall study and provided comments and suggestions for troubleshooting and manuscript editing. Dr. Gänzle provided comments and suggestions to troubleshooting and manuscript editing. A technical abstract related to the results of Chapter 3 was peer-reviewed and the poster presentation was delivered at Canadian Society for Bioengineering Annual General Meeting and Technical Conference joint with CIGR VI Technical Symposium, held at Winnipeg, MB, Canada in August 2017. Another technical abstract related to the results in Chapter 3 was peer-reviewed and an e-poster presentation was delivered at the Institute of Food Technologists annual meeting, held at Chicago, IL, USA from July 15-18, 2018.

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I completed the experiments independently, analyzed data, and wrote the manuscript. Dr. M. S. Roopesh guided the overall study and provided comments and suggestions on troubleshooting and manuscript editing. Dr. Gänzle provided comments and suggestions to troubleshooting and manuscript editing. A technical abstract related to the results in Chapter 4 was peer-reviewed and a poster presentation was delivered at the Institute of Food Technologists annual meeting, held at Chicago, IL, USA from July 15-18, 2018.

Following manuscripts are in preparation:

1) Amritha Prasad, Michael Gänzle and M. S. Roopesh, "Understanding the antibacterial mechanisms of 365, 395 and 455 nm light pulses emitted from light emitting diodes" (Based on Chapter 5).

2) Amritha Prasad and M.S. Roopesh, "Bacterial biofilm reduction by 275 nm and 455 nm light pulses emitted from LEDs" (Based on Chapter 6)

Dedication

I would like to dedicate this thesis to my beloved parents, Mr. KK Jayarama Krishna Prasad and Mrs. Rema Prasad. Thank you for your support and blessings.

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First and foremost, I would like to express my deepest gratitude to Dr. Roopesh Mohandas Syamaladevi for giving me the opportunity to pursue my Ph.D. program under his supervision. During the five years of my Ph.D. program, he has been patient and supportive of me, and has given me valuable guidance to improve my research work. He has always helped and encouraged me to become a better researcher for which I would be forever thankful. Without his efforts and valuable suggestions, it would have been impossible to complete my research and this thesis.

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List of Abbreviations

a_w	Water activity
CDC	Centre for Disease Control
CFU	Colony forming unit
CLSM	Confocal Laser Scanning Microscopy
CPD	Cyclobutane Pyrimidine dimers
CV	Crystal Violet
DNA	Deoxyribonucleic acid
EPSs	Extracellular Polymeric Substances
FDA	Food and Drug Administration
GAB	Guggenheim-Anderson-de Boer
LB-NS	Lennox broth with no salt agar plates
LED	Light Emitting Diode
LP	Low Pressure
MDA	Malondialdehyde
NIR	Near Infrared
NUV-Vis	Near Ultraviolet- Visible
PBS	Phosphate Buffered Saline

PDI	Photodynamic Inactivation
PI	Propidium iodide
PLA	Polylactic acid
RH	Relative Humidity
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SS	Stainless steel
TBA	Thiobarbituric acid
TEM	Transmission electron microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
VSA	Vapor Sorption Analyzer
YE	Yeast Extract

Chapter 1: General introduction and thesis objectives

1.1 Introduction

Low water activity (a_w) foods are comprised of food products with a_w less than 0.85, in which most of the water is bound by the solutes present in them, making it unavailable for the growth of foodborne microorganisms (Gurtler, Doyle, & Kornacki, 2014). However, many cases of foodborne outbreaks and recalls have been reported in low a_w foods including pet foods, wheat flour, powdered spices, breakfast cereals and dried coconut (Beuchat et al., 2011; CDC, 2018; FDA, 2019). These foodborne outbreaks in low a_w foods are mostly associated with *Salmonella enterica* and Shiga-toxin producing *Escherichia coli*.

Foodborne microorganisms develop certain defense mechanisms under stress conditions, which makes their inactivation a challenging task. These mechanisms include maintaining the turgor pressure of the cell by the accumulation of compatible solutes like trehalose, as an immediate response to drying conditions or osmotic stress (Csonka, 1989; Csonka, & Hanson, 1991). Under desiccation stress, an upregulation of the genes for fatty acid catabolism and increase in rRNA degradation in bacteria were observed to make up for the energy required in the accumulation of trehalose in the cell (Deng, Li, & Zhang, 2012; Deutscher, 2006; Li et al., 2012). Low a_w conditions might also induce filamentation in *Salmonella* (Stackhouse et al., 2012). They can also enter into viable but non-culturable state as part of their response to the stress conditions (Oliver, 2010). *Salmonella* can survive in low a_w environment for more than a year and is infectious if present in small numbers (Gurtler et al., 2014; Lambertini et al., 2016). Therefore, these foodborne pathogens become resistant to the traditional decontamination methods like heat treatments (70 to 120°C) or oxidizing chemicals like propylene oxide in low a_w foods, which

necessitates the requirement for thermal treatments with high temperatures that can negatively affect the quality and nutritional composition of food products (Bari et al., 2009; Hasani et al., 2020). There is a need for exploring alternate decontamination methods for low a_w foods. Previously, non-thermal technologies like irradiation, high pressure processing, cold plasma and light-based technologies like ultraviolet (UV) light and pulsed light treatments have shown promising antimicrobial efficacy in low a_w foods (Calvo, Muguerza, & Cienfuegos-Jovellanos, 2007; Cheon et al., 2015; Hierro et al., 2009; Jeong et al., 2012; Niemira, 2012).

Light Emitting Diode (LED) technology is an emerging alternative decontamination method that can be studied for their antimicrobial efficacy in low a_w foods. LEDs consist of semiconductor material doped with certain impurities, which is responsible for the generation of light of specific color and wavelength upon application of electric field (Held, 2009; section 2.2, Chapter 2). The LED is a safer light source as compared to mercury lamps employed in UV light treatments (Song, Mohseni, & Taghipour, 2016) and requires no warm up time as opposed to long warm up times required in flash lamps used for pulsed light treatments (Elmnasser et al., 2007). Other advantages of LED include its cost-effectiveness, generation of uniform irradiation, compact size, ease of incorporation into the existing processing lines and long lifetimes (Elmnasser et al., 2007).

The inactivation efficacy of the LED technology has been extensively reported in wastewater treatment and high a_w foods like fresh-cut fruits, ready-to-eat fresh salmon, sliced camembert cheese and so on (Ghate et al., 2017; Hamamoto et al., 2007; Kim, Bang, & Yuk, 2017a; Kim, Kim, & Kang, 2016; Li et al., 2018a). LED technology produces antibacterial effect by means of photodynamic inactivation (PDI), which involves generation of reactive oxygen species (ROS) by photosensitization of endogenous light absorbing molecules like porphyrin

compounds, with an absorption spectrum of 400-500 nm wavelength (Plavskii et al., 2018). ROS further triggers cytotoxic actions like cell membrane damage, oxidation of cellular components like DNA, RNA, protein, etc., eventually leading to cell death (Luksiene, & Zukauskas, 2009). LEDs emitting light in the blue spectrum (~460 nm) induces oxidative stress when combined with exogenous photosensitizers like curcumin (Bhavya, & Hebbar, 2019). The antibacterial mode of action of the LEDs emitting light with different wavelengths might vary. For instance, UV-C light induces formation of cyclobutane pyrimidine dimers (CPD) in the DNA of the bacterial cell, that inhibits the cell replication (Diffey, 1991; Hamamoto et al., 2007; Sánchez-Maldonado, Lee, & Farber, 2018). The LEDs emitting light of wavelength 365 nm (UV-A) shows antibacterial effect by oxidizing the guanine bases of DNA (Hamamoto et al., 2007), while 395 nm (Near UV-Visible; NUV-Vis) light produces photo-oxidation in bacterial cells by the generation of singlet oxygen as an ROS (Birmpa et al., 2014; Feuerstein et al., 2005). Thus, indicating the promising antibacterial effect of 275 (UV-C), 365 (UV-A), 395 (Near Ultraviolet-Visible; NUV-Vis) and 455 (Blue) nm light wavelengths in high a_w systems. However, the studies focussing on understanding the antibacterial effect of LED technology and their underlying inactivation mechanism in low a_w conditions are limited.

The 405 nm LED treatments increased the surface temperature, which showed influence of illumination temperature on its antibacterial efficacy at high a_w conditions (Kim et al., 2017b; Kim, Bang, & Yuk, 2017a). Moreover, 395 nm (NUV-Vis) light showed reduction of weight of the treated chicken skin (Haughton et al., 2012). These studies show the potential drying effect of the LED treatments i.e., the high intensity light pulses emitted from LEDs can be used for drying of food products in addition to microbial inactivation. However, limited studies reported the effect of process and product parameters on the antimicrobial and drying efficacies of LED treatments

using light energy with different wavelengths. It is necessary to understand the influence of process parameters like treatment dose, treatment time, irradiance, environment conditions (illumination temperature and relative humidity), wavelength of the light pulses used; and product parameters like a_w of the sample and surface temperature, on the inactivation effect of the LED treatment at low a_w conditions.

The foodborne pathogens like *Salmonella*, *E. coli*, *Listeria monocytogenes*, form biofilms in food products like meat, poultry and on food contact surfaces like conveyor belts, stainless steel equipment and plastics as a defence mechanism under stress conditions. Biofilms consist of a complex matrix of extracellular polymeric substances (EPSs) (Flemming et al., 2016; Kumar, & Anand, 1998). This EPS matrix acts as a barrier to the regular cleaning practices and disinfectants used for cleaning the food contact surfaces in the food industry, increasing the risk of cross contamination of the food products in the post-processing stage (Flemming et al., 2016; Visvalingam et al., 2019a). LEDs can be installed in food processing facilities or LED treatment systems can be developed to decontaminate food contact surfaces such as conveyor belt and equipment surfaces. LEDs emitting light of wavelength 405 nm was shown to have promising inactivation effect against *L. monocytogenes* biofilms in ready-to-eat fresh salmon (Li et al., 2018a). However, limited studies reported the biofilm inactivation effectiveness of LED treatments using light with different wavelengths, for instance UV-C LED treatment.

1.2 Hypotheses

The LEDs emitting light pulses can reduce foodborne pathogens such as *Salmonella* in low a_w conditions, depending on the wavelength and dose. This inactivation efficacy is influenced by certain product and process parameters and is caused by the generation of ROS. Since the LED

treatments can increase the temperature because of the high intensity light pulses, the resulting heating and drying play a major role in the inactivation efficacy of the LED technology. This antimicrobial efficacy of the LEDs can be improved when the low a_w foods are treated by a combination of light pulses emitted at different wavelengths. Being a surface decontamination technology, the LEDs emitting light of specific wavelengths can be used for the disinfection of food contact surfaces. The biofilm reduction efficacy will be influenced by the wavelength of light pulses emitted from LEDs.

1.3 Objectives

The overall objective of this research was to develop an advanced decontamination process based on the LED technology for low a_w food systems and food contact surfaces for biofilm inactivation. The specific objectives of this research are:

1) To evaluate the antibacterial efficacy of treatments using light pulses emitted from 365 and 395 nm LEDs at low a_w conditions and to understand the effect of selected product (e.g., water activity, sample type) and process (e.g., wavelength, sample type) parameters (Chapter 3).

2) To evaluate the simultaneous decontamination and drying potential of treatments using 455 nm light pulses emitted from LEDs on low a_w pet food pellets (Chapter 4).

3) To understand the antibacterial mechanisms of treatments using light pulses emitted from LEDs at low a_w conditions (Chapter 5).

5) To evaluate the biofilm inactivation efficacy of treatments using light pulses emitted from LEDs on food grade stainless steel surfaces (Chapter 6).

Salmonella enterica and *E. coli* are involved in several foodborne outbreaks and recalls. Highly resistant food and wastewater isolates of *Salmonella enterica* and *E. coli* were used in this research to evaluate the survival of these microorganisms at low a_w conditions after the LED treatments (Chapter 3). Pet food pellets (a_w ~0.54) were used in this study as salmonellosis (illness caused by *Salmonella*) can be transmitted through pets and several cases of recalls have been reported related to the pet foods (Chapters 3 and 4). *Aeromonas australiensis* was used in the biofilm study as it is a strong biofilm former and is isolated from a beef processing plant (Visvalingam et al., 2019b) (Chapter 6).

Chapter 2: Applications of Light Emitting Diodes (LEDS) in food processing and water treatment

2.1 Introduction

Artificial light treatments using light energy with different wavelengths have been used in agriculture and the food industry to disinfect water and food and to improve plant health and growth (Lian et al., 2010; Koutchma, & Orlowska, 2012; Song, Taghipour, & Mohseni, 2018). Conventional approaches, such as UV light emitted by mercury vapor lamps or pulsed light produced in xenon lamps, have been used to inactivate microorganisms such as bacteria, yeasts, viruses, and fungi. Disadvantages of these treatments include the possibility of contamination by mercury residues, and a short life span of equipment. Light emitting diodes (LED) made of semiconductor materials and producing monochromatic light have been used in agriculture and the food industry, as they have several advantages over conventional sources. For example, harmful microorganisms in food and water can be eliminated by light with specific wavelengths and pulsed or continuous modes of operation, making LEDs effective. LEDs are nonhazardous (no mercury), and their compact size makes them easy to incorporate into existing food processing applications. LEDs offer high performance, robustness, a long lifetime (> 10,000 h), low power use, and cost effectiveness, making them a promising option for effective disinfection and for plant growth applications (Song, Taghipour, & Mohseni, 2018). This review explains the fundamentals of LED applications to microbial inactivation in different food products and water. It describes the potential quality changes in recipients of LED treatment, the mechanisms of microbial inactivation during treatments using light of different wavelengths, and the challenges and future opportunities for LED technology in the food processing sector.

2.2 LED fundamentals

An LED is a semiconductor that emits light when electricity passes through it. LEDs work on the principle of electroluminescence, that is, they produce light upon application of an electric or a magnetic field. In an electric or a magnetic field, excited electrons reach lower energy states by emitting light and releasing energy in the form of electromagnetic radiation. An LED is a semiconductor material doped with impurities that create a boundary or interface (known as a p-n junction) between two types of semiconductor materials, one type (the positive or p-type) having an excess of holes and the other type (the negative or n-type) having an excess of electrons. The color and the wavelength of the light emitted depends on the semiconductors and the impurities used in the LED formation (Table 2.1). LEDs are similar to conventional diodes, with the p-side called the anode and the n-side called the cathode. Additionally, the diode consists of a nonconducting region between the p and n sides, known as the depletion region (Held, 2009).

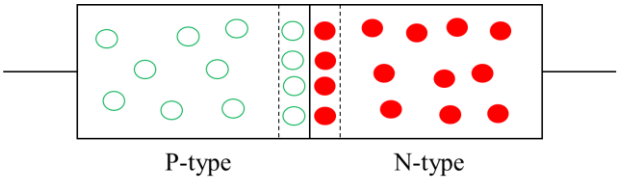
LEDs consist of a chip of semiconductor material doped with certain impurities that enable it to emit light of a particular color and wavelength. A p-type semiconductor can be formed by impregnating a group II element such as magnesium (Mg) into a group III element substrate to provide extra holes in the substrate. An n-type semiconductor is formed by doping a group IV element into a group III element substrate to provide extra free electrons in the substrate. The extra holes on the p-side and the free electrons on the n-side fuse together at the p-n junction to form a nonconducting, depletion region (Chen, Loeb, & Kim, 2017). A radiative recombination of free electrons and holes is an important event in the LED system.

Table 2.1: The semiconductors and applications of LEDs emitting light of different wavelengths (Held, 2009).

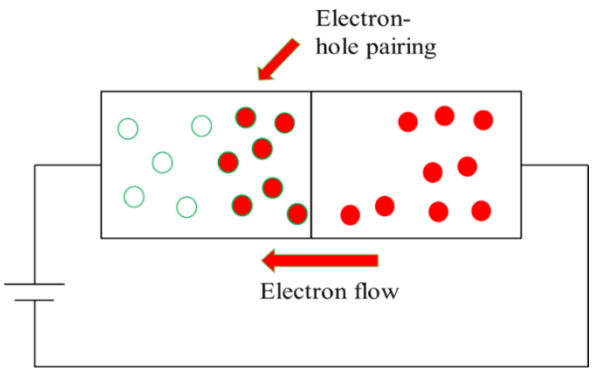
Semiconductor	**Voltage drop (ΔV)**	**Wavelength (nm)**	**Color**	**Applications**
Gallium arsenide (GaAs), Aluminium gallium arsenide (AlGaAs)	<1.9	>760	Infrared	Home-entertainment remotes, night-vision cameras, security systems, wound healing
Aluminium gallium arsenide (AlGaAs), Gallium arsenide phosphide (GaAsP), Aluminium gallium indium phosphide (AlGaInP), Gallium phosphide (GaP)	1.6-2.0	610-760	Red	Traffic light systems, wound healing, dental implants, algaculture
Gallium arsenide phosphide (GaAsP), Aluminium gallium indium phosphide (AlGaInP), Gallium phosphide (GaP)	2-2.1	590-610	Orange/ amber	Cell phone screens
Gallium arsenide phosphide (GaAsP), Aluminium gallium indium phosphide (AlGaInP), Gallium phosphide (GaP)	2.1-2.2	570-590	Yellow	Traffic light systems
Gallium phosphide (GaP), Aluminium gallium indium phosphide (AlGaInP), Aluminium gallium phosphide (AlGaP)	1.9-4.0	500-570	Green	Traffic light systems, lipid production in microalgae, wound healing, dental whitening
Indium gallium nitride (InGaN), Silicon carbide (SiC)	2.5-3.7	450-500	Blue	Telecommunications, message boards, traffic control devices, algaculture, wound healing, dental care
Indium gallium nitride (InGaN)	2.8-4.0	400-450	Violet	Adhesive curing, tooth bleaching
Aluminium nitride (AlN), Aluminium gallium nitride (AlGaN), Aluminium gallium indium nitride (AlGaInN), Diamond (C)	3.1-4.4	<400	Ultraviolet	Sterilization and air disinfection system, adhesive curing, 3D-printing

On the passage of electric current through the LED, the higher energy electrons in the conduction band (the n-side) combine with the holes in the p-side forming electron-hole pairs and move to the valence band of the p-type semiconductor where the energy is lower (compared to the n band with its free electrons) (Figure 2.1). This bandgap can either be direct, where momentum is conserved, or indirect, where momentum is not conserved, and the transition is lower.

(a)



(b)



(c)

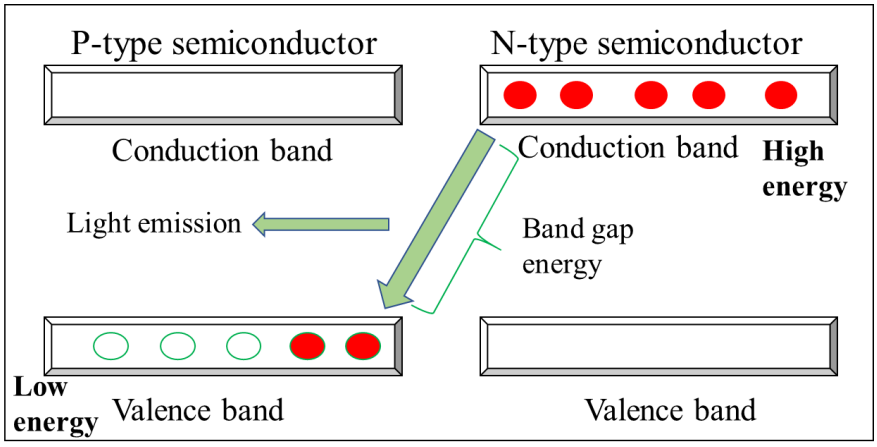


Figure 2.1: The electrons on the n-type semiconductor and holes in the p-type semiconductor forms a depletion region at the p-n junction without external current (a), Forward-biased diode resulting in the recombination of electrons and holes (b) Light emission with energy equivalent to the band gap energy (c) (Held, 2009).

The transition can be improved by the addition of isoelectronic traps (DenBaars, 1993). The difference between the energy of the free electrons and the energy of the electron-hole pairs (i.e., the band gap energy) is emitted as photons, carriers of electromagnetic radiation of certain color and wavelength (Figure 2.1). Highly efficient LEDs are based on group III-V semiconductors and are formed by direct band gap alloys. Varying the chemical compositions of these alloys can vary the band gap energy and hence the wavelength of the light emitted. On applying the law of conservation of energy with an assumption that the thermal energy produced is much less than photon energy produced, the energy produced in the form of light will be equal to the bandgap of the diodes (Schubert, 2006):

$$E_c - E_v \approx E_g \quad (2.1)$$

where, E_c is the energy of electrons in the conduction band, E_v is the energy of the holes in the valence band, and E_g is the energy in the bandgap of diodes, and signifies the total energy generated during the electroluminescence.

According to the conservation of energy, if the thermal energy is much less than E_g,

$$hv = E_c - E_v \approx E_g \quad (2.2)$$

where, hv describes the energy of a photon emitted, h is Planck's constant, and v is the frequency of the photon of light and is inversely proportional to the wavelength of the light. Equations 2.1 and 2.2 clearly satisfy the fact that a material with the higher conduction band is needed to emit a smaller wavelength of light and vice-versa. For instance, AlGaN has a larger bandgap than GaN and InGaN, so it is preferred over the others to produce deep UV light (wavelengths shorter than 365 nm) or near UV light (320-400 nm) (Chang et al., 2002; Bao et al., 2015).

Packaging of a LED chip can affect the efficiency of a LED system. For example, if the packaging film absorbs most of the light emitted by a LED source due to total internal reflection,

the amount of light perceived by the human eye is affected, thus varying the overall luminous efficiency. Overall luminous efficiency provides the efficiency of a light source to convert the electrical energy into the optical power perceived by a human eye under standard conditions. The voltage and current requirements of an LED varies based on the semiconductor material used in the diode and the wavelength of the light emitted; usually the voltage ranges from 1.5-3 V and the current ranges from 10-30 mA (Held, 2009). Regulation of the electric current and the duty ratio (proportion of time operated) helps to regulate the light intensity and the spectral output of the LED.

The irradiance (I) of the LED is an important parameter determining the process effectiveness. The irradiance is the radiant power exposed to unit surface area of the sample. Radiometers can be used for the measurement of irradiance of LEDs emitting light of different wavelengths at specific distance from the source and checked periodically during the lifespan of the source to monitor the source power (Kim, & Yuk, 2017; Du et al. 2020; Ghate et al., 2017; Subedi et al., 2020). The radiant energy exposure (the energy dose) of a sample to light at a constant height and exposure time, is equal to the product of the irradiance and the exposure time, as expressed in equation (2.3)

$$E = I \times t \tag{2.3}$$

where, E represents the energy dose of the LED light per unit area (mJ/cm^2), I is the irradiance of the LED light (mW/cm^2) and t represents the duration of the exposure of the LED light (in s) (Ghate et al., 2017). The irradiance values are dependent on the power level (or pulse width) of the light pulses emitted from the LEDs. For example, for the LEDs operating at the frequency of

100 Hz, a power level of 60% would indicate the ON and OFF times of the light pulses to be 6 and 4 ms, respectively.

Another important parameter is the photon fluence, which is the quotient of dN by dA.

$$\emptyset = \frac{dN}{dA} \tag{2.4}$$

where, $\emptyset$ is the photon fluence (cm^{-2}), dN is the number of photons incident on an imaginary sphere and dA is the cross-sectional area of this imaginary sphere (Seuntjens et al., 2005; Podgorsak, 2005). The dN can be determined as the ratio of total photon energy incident on the surface to the energy of one photon ($\frac{hc}{\lambda}$), where h is the Plank's constant (6.626×10^{-34} J.s), c is the speed of light (3×10^{8} m/s), λ is the wavelength of light (m).

2.3 LED technology for antimicrobial applications

LEDs have been applied to air disinfection, water treatment, surface decontamination, and curing (Koutchma, & Orlowska, 2012). Light with wavelengths in the range of 200-280 nm (UV-C), 280-320 nm (UV-B), 320-400 nm (UV-A and near UV-visible, NUV-Vis), and 400-470 nm (blue light) have been studied to understand their antimicrobial efficacy. Longer wavelengths, i.e., infrared and red (630-1000 nm) are used for applications such as phototherapy, dying and curing of coatings, and ink curing (Lim, 2011). The antimicrobial effectiveness of light energy emitted by lamps, especially in the UV-C range, has been well documented (Rowan et al., 1999; Wekhof, Trompeter, & Franken, 2001; Uslu, Demirci, & Regan, 2015). Most LED studies have focused on the application of UV-C LEDs for water disinfection (Chatterley, & Linden, 2010; Rtele et al., 2011; Song et al., 2016). However, the application of LEDs that emit light at wavelengths such as

365 nm, 395 nm, and 455 nm is emerging in food processing (Haughton et al., 2012; Josewin et al., 2018) and water treatment.

2.3.1 LED treatment of solid foods

The efficacy of LED treatments of solid foods depends on the type and nature of the food products and components, the water activity (a_w), and the food surface morphology. Parameters such as light wavelength, treatment duration, dose, illumination temperature, relative humidity, and microbiological parameters are also important. Ready-to-eat fresh cut fruits have a high market demand. These products are stored in refrigerators, but they are susceptible to resistant microorganisms, although the growth of such organisms is limited at low temperatures. LEDs have shown promising antibacterial effectiveness in such products, although their antibacterial efficacy is affected by many product and process parameters, including type of product, composition, treatment temperature, and environmental conditions. LEDs emitting light at 405 nm induced a reduction of 1-1.2 log CFU/cm^2 (colony forming units per cm^2) in fresh-cut papaya inoculated with *Salmonella*. The papaya was treated with a total dose of 1.7 kJ/cm^2 at a set temperature of 4°C for 48 h (Kim, Bang, & Yuk, 2017a). The antibacterial effectiveness of 405 nm LEDs was supported by another study on fresh-cut mango by Kim et al. (2017c), where the cell counts in a three strain cocktail of *E. coli* O157:H7, three serotypes of *L. monocytogenes*, and five serotypes of *Salmonella* spp. were reduced to less than 1.6 log CFU/cm^2 with a total dose of 2.6-3.5 kJ/cm^2 for 36-48 h. The *E. coli* O157:H7 and *Salmonella* in the cocktail culture were reduced to below the detection limit with 36 h of treatment at 4°C and at 10°C, indicating the antibacterial efficacy of the LED is dependent on the type of bacteria. The sterilization effects of visible light LED treatment on fresh-cut fruits has also been studied. Ghate et al. (2017) tested the antibacterial effects of a 460 nm LED at different illumination temperatures and irradiances on fresh-cut

pineapples infected with a cocktail of *S. enterica*. A maximum reduction of 1.72 log CFU/g was achieved with 7950 J/cm^2 (92 mW/cm^2 irradiance) at 16°C illumination temperature. Varying the irradiances had no effect on the inactivation. High energy doses used for long times with small reductions in target pathogens may limit the practical applications of LED treatment unless the antimicrobial efficacy is improved.

Seafoods like molluscs and crabs are rich sources of protein and other nutritional components and are prone to microbial contamination by many sources, either due to pollution or by pre- or post-processing sources. LEDs, an emerging non-thermal antibacterial technology, have been tested on contaminated seafoods. In a study by Josewin et al. (2018), the efficacy of a blue LED (460 nm) with a riboflavin photosensitizer was studied on smoked salmon inoculated with a 4-strain cocktail of *L. monocytogenes.* The synergistic effects of the LED treatment with 2400 J/cm^2 dose (15 mW/cm^2 irradiance) and riboflavin (100 μM) produced reductions of 1.2 and 1.1 log CFU/cm^2 at surrounding temperatures of 4°C and 12°C, respectively. The LED treatment of seafoods might render it susceptible to a subsequent acidic condition. This was reported in a study of ready-to-eat salmon inoculated with an *L. monocytogenes* and *Salmonella* spp. cocktail. A 405 nm LED treatment for 8 h with a total dose of 460.8 J/cm^2, produced a reduction of 0.4 and 0.3 log CFU/cm^2 in cell counts of *L. monocytogenes* and a 0.5 log reduction of *Salmonella* spp. at 4°C and at 12°C. Although the inactivation was low, both bacteria had reduced D-values (time required to reduce 90% of the population in simulated gastric fluid) compared to untreated samples, and the treated samples were more sensitive to simulated gastric fluid. However, this effect varied for both strains, as *Salmonella* spp. (gram-negative) showed more susceptibility than *L. monocytogenes* (gram-positive), indicating that the treatment inactivated gram-positive and gram-negative bacteria differentially (Li, Kim, & Yuk, 2018b).

Owing to a high water content, ready-to-eat meat products are highly susceptible to contamination by foodborne pathogens. Kim et al. (2017b) measured the effect of the treatment of cooked chicken inoculated with *S.* Enteritidis with light pulses of wavelength 405 nm emitted from the LEDs. A total dose of 3.8 kJ/cm^2 at 4°C produced a reduction of 0.8-0.9 log CFU/cm^2. A similar experiment at room temperature produced a smaller reduction in *S.* Enteritidis. LED systems can be designed to produce either continuous or pulsed treatments, according to the objective requirements, but treatment efficiencies can vary based on the design. This aspect was reported in recent research conducted on white mushrooms and commercial ready-to-eat sausages. The treatment with light pulses emitted from UV-C LED with 20 Hz frequency and a duty ratio of 50% showed better antibacterial efficacy than continuous UV-C LED treatment against three-strain cocktails containing *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes.* Continuous treatment resulted in 2, 1.5, and 2 log reductions, whereas light pulses emitted from the LED at a 5 J/cm^2 dose resulted in 3, 4, and 4 log reductions in *E. coli*, *Salmonella*, and *Listeria*, respectively, in ready-to-eat sausage. In white mushrooms, continuous irradiation resulted in 2, 1, and 1 log reductions and light pulses emitted from the LED produced 2, 1.5, and 1.8 log reductions, in *E. coli*, *Salmonella*, and *Listeria*, respectively (Kim, & Kang, 2018a). LEDs emitting light in the visible spectrum need further evaluation.

There have been many reported cases of illness in North America caused by the bacterial contamination of cheese. The presence of high moisture in cheese products supports the growth and survival of foodborne pathogens. Treatments with the light pulses emitted from the LEDs have the potential to decontaminate these products. In a recent study conducted on sliced camembert cheese, a UV-C LED emitting light of wavelength 266 nm produced 4.88, 4.72, and 3.52 log reductions in camembert cheese containing *E. coli* O157:H7, *S.* Typhimurium, and *L.*

monocytogenes, respectively. Also, higher wavelength UV-C LED treatments (266-279 nm) showed 4-5 log reductions in *E. coli* O157:H7 and *Salmonella* spp., while a 3-4 log reduction in *Listeria* spp. in sliced camembert cheese was achieved with a treatment of 3 mJ/cm^2 (Kim et al., 2016a).

Contamination of low water activity (a_w) foods such as dry nuts, cereals, and pet foods (a_w < 0.85) is a global concern, as thriving microorganisms eventually develop resistance to decontamination efforts. Foodborne pathogens can survive for long periods in a dormant state and become active on exposure to a favourable environment. There have been limited studies on the antibacterial efficacy of LED treatments in low a_w foods, but the studies conducted have shown promising results. Lacombe et al. (2016) treated shelled almonds with 405 nm LED and achieved maximum reductions of 2.44, 0.96, 1.86, and 0.7 log CFU/g in *E. coli* O157:H7, *S.* Typhimurium, *E. coli* K12, and *S.* Enteritidis, respectively. Further research is needed to improve the antimicrobial efficacy of LED treatments of foods with low a_w using different wavelengths (275, 365, 395, and 455 nm) of light energy. Results of LED treatments of solid foods are listed in Table 2.2.

Surface characteristics of food influence the inactivation efficacy of LED treatment. The variable effects of UV-C LED on white mushrooms and sausages were likely due to the limited penetration of light into the food matrix (Kim, & Kang, 2018a). However, it is unclear why the elimination of gram-positive bacteria required a higher LED dose than the elimination of gram-negative bacteria. The bacterial inactivation improved with an increase in the duty ratio as well (Kim, & Kang, 2018a). In the visible range, a 461 nm LED deployed better bacterial inactivation efficacy than 521 nm and 642 nm LEDs (Ghate et al., 2013). The illumination temperature of the

Table 2.2: The antimicrobial efficacy of LED in solid foods.

Tested food product	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Sliced camembert cheese	UV emitting peak wavelengths 266, 270, 275 and 279 nm; dose - 1, 2 and 3 mJ/cm^2, respectively; radiation intensity -about 4 W/cm^2	*E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*		The 3 mJ/cm^2 dose resulted in 4 to 5 log reduction in *E. coli*, *S.* Typhimurium and *L. monocytogenes*	Kim, Kim, & Kang (2016)
Shelled almonds	405 nm MBL LED lights; working distance - 7 cm; treatment time - 0, 1, 2, 4, 6, 8 and 10 min	Pathogenic *E. coli* O157:H7, non-pathogenic *E. coli* K12, pathogenic *S.* Enteritidis (PT30, Stanley and Anatum) and non-pathogenic *S.* Typhimurium strain Chi3985 with 8 or 5 CFU/g inoculum levels		Following log reductions for higher and lower inoculum levels, respectively: *E. coli* O157:H7- 2.44 and 1.44 log CFU/g *E. coli* K12- 1.85 and 1.63 log CFU/g *S.* Enteritidis- 0.7 and 0.55 log CFU/g *S.* Typhimurium- 0.54 and 0.97 log CFU/g	Lacombe et al. (2016)
Fresh cut papaya	405±5 nm LED treatment with 0.9-1.7 kJ/cm^2 (24-28 h), 10±1 mW/cm^2; working distance 4.5 cm in the temperature- controlled incubator (4, 10 or 20°C)	*S.* Agona, *S.* Newport, *S.* Saintpaul and *S.* Typhimurium	No cellular lipid oxidation, significant DNA oxidation, no color change and antioxidant capacity, 1.5-1.9 times higher total flavonoid content, no significant change in the ascorbic acid, β-carotene and lycopene content	0.3-1.3 log CFU/cm^2 reduction in *Salmonella* cells at 1.3-1.7 kJ/cm^2 dose	Kim, Bang, & Yuk (2017a)

Table 2.2 (continued)

Tested food product	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Fresh cut mango	405±5 nm LED; irradiance- 20±2 mW/cm^2; at 4, 10, or 20°C illumination temperature; treatment times - 24-48 h; total dose - 1.7–3.5 kJ/cm^2 in a temperature-controlled incubator	Three strains of *E. coli* O157:H7, 3 serotypes of *L. monocytogenes* and 5 serotypes of *Salmonella*	No significant changes in the color, antioxidant capacity, total flavonoid content, ascorbic acid content and β-carotene of the LED treated mangoes	2.6-3.5 kJ/cm^2 of LED light resulted in 1-1.6 log CFU/cm^2 at 4 and 10°C in all the bacterial species and; 1.2 log CFU/cm^2 reduction in *Salmonella* at 20°C with 1.7 kJ/cm^2 dose	Kim et al. (2017c)
Fresh cut pineapples	460 nm LED with irradiance of 92.0, 147.7 or 254.7 mW/cm^2, corresponding to the working distances (4.5, 3.5 and 2.5 cm, respectively); Illumination times (24, 13.91 and 8.66 h) to produce the same dose of 7950 J/cm^2 to the slices.	A cocktail of five of *Salmonella enterica serovars* Gaminara, Montevideo, Newport, Saintpaul and Typhimurium	Discolouration of treated pineapple slices	Significant effect of illumination temperature on the antibacterial efficacy; inactivation ranged from 0.61 to 1.72 log CFU/g	Ghate et al. (2017)
Satsuma mandarin fruits	465 nm Blue LEDs with low (8 µmole/m^2/s) and high (80 µmol/m^2/s) fluency	*Penicillium italicum*		Radial growth of sporulation zone was found to be 0.3 and 3 mm/day for high and low fluency, respectively. LED-80 produced suppression of sporulation till day 6 after inoculation	Yamaga et al. (2015)

Table 2.2 (continued)

Tested food product	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Blueberries	Green, red, blue and white LEDs; working distance-30 cm	*Bacillus amyloliquefaciens* and *Lactobacillus brevis* for fermentation and *Propionibacterium acnes* and *Staphylococcus epidermis* for antimicrobial study	Green and white LED increased the total phenolic content and total flavonoid content in fermented blueberry extract	White and green LED effective in improved fermentation for 72 h and improved antibacterial activity	Jeong et al. (2018)
White mushrooms and commercial ready-to-eat (fully cooked) sausages	Three UV-C LEDs (280 nm) were combined; working distance-3 cm; varied duty cycle; irradiance- 1 to 5 J/cm^2, continuous and light pulses irradiation	*E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *Salmonella enterica serovar* Typhimurium (ATCC 19585, ATCC 43971 and DT 104), and *Listeria monocytogenes* (ATCC 19111, ATCC 19115 and ATCC 15313)	LED treatment reduced formazan formation levels in *E. coli* and *Listeria*; light pulses from LEDs produced more ROS and membrane lipid peroxidation compared to continuous irradiation; no significant effect on membrane damage but effect on membrane potential of *E. coli* and *Listeria*	Continuous and pulsed irradiation resulted in the following inactivation, respectively: **White mushrooms**: *E. coli*- 2 and 2 log CFU/g; *Salmonella*- 1 and 1.5 log CFU/g; *Listeria*- 1 and 1.8 log CFU/g **RTE sausages**; *E. coli*- 2 and 3 log CFU/g; *Salmonella*- 1.5 and 4 log CFU/g; *Listeria*- 2 and 4 log CFU/g	Kim, & Kang (2018a)

Table 2.2 (continued)

Tested food product	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Cooked chicken	405±5 nm LED; working distance- 3 cm; surface intensity on cooked chicken 22.0 ± 1.1 mW/cm^2; dose- 1.58-3.80 kJ/cm^2; treatment times- 20-48 h; Illumination temperature- 4, 10, and 20°C	*Salmonella enterica* Enteritidis (124, 125 and 130)	*Salmonella* cells incapable of cellular repair at 4°C	0.8-0.9 log CFU/cm^2 reduction on cooked chicken in all *Salmonella* spp. with 3.8 kJ/cm^2 dose at 4°C; growth delays observed at 10 and 20°C	Kim et al. (2017b)
Smoked salmon	460 nm LEDs; working distance- 9 cm (intensity of 15 mW/cm^2) to 5.4 cm (intensity of 58 mW/cm^2); dose maintained at 2400 J/cm^2	Four serotypes of *Listeria monocytogenes*		0.7-1.2 log CFU/cm^2 reduction with LED treatment in combination with riboflavin (25, 50 and 100 mM)	Josewin et al. (2018)
Ready-to-eat fresh Salmon	405 nm LEDs; radiation intensity- 26 ± 2 mW/cm^2; working distance- 4.5 cm	*Listeria monocytogenes* inoculated on salmon exudates	LED treatments effective in reduction of *Listeria* during the biofilm formation	LED treatment produced 2-2.8 log reduction in planktonic cells on stainless steel or acrylic coupons in salmon exudates during 8h storage	Li et al. (2018a)
	405 nm LED; intensity- 16± 2 mW/cm^2; treatment time- 8h; illumination temperature- 4 and 12°C; working distance -7.9 cm	*Listeria monocytogenes* and *Salmonella enterica* serotype Enteritidis, Typhimurium and Newport	LED treatment produced no colour change; it improved the sensitivity of the bacteria to simulated gastric fluid	Reduction of 0.4 and 0.3 log CFU/cm^2 in *L. monocytogenes*; reduction of 0.5 and 0.4 log CFU/cm^2 in *Salmonella* at 4 and 12°C, respectively	Li, Kim, & Yuk (2018b)

treatment influenced the efficacy of the LED based on the wavelength of the LED used in the treatment (Ghate et al., 2017; Kumar et al., 2015).

2.3.2 LED treatment of liquid foods

Liquid foods such as beverages are vulnerable targets for pathogenic contamination because of their high a_w and carbohydrate composition. Commonly, chemical preservatives are added into liquid foods to extend their shelf life and reduce microbial growth. However, due to the growing demand for foods with no additives and consumers' increasing concerns about safe food ingredients, treatments such as ultraviolet light as a physical means to reduce pathogens have been extensively studied in liquid foods. The disinfection effects of UV treatment using a wide range of wavelengths produced from different sources (e.g., mercury lamps, excimer lamps, microwave lamps) on liquid foods, such as, apple cider, juices, beer, and milk have been studied (Koutchma, 2009). The studies have covered common foodborne pathogens, such as *E. coli*, *C. parvum oocyst*, *S. cerevisiae*, *L. innocua*, yeasts, and molds. LEDs can emit light in a broad wavelength range including visible, UV-A, UV-B, and UV-C; therefore, its antimicrobial activity has been applied on several liquid foods.

Studies of the antimicrobial effects in liquid foods of LED treatments have mainly focused on apple juice, orange juice, and milk. Compared to water, liquid foods are complex systems containing pigments, fibers, and insoluble particles, and the turbidity and color of liquid foods can affect the antimicrobial efficacy of LED treatments. Lian et al. (2010) used a UV-A LED to evaluate its disinfection activity in both colored solutions and orange juice inoculated with *E. coli* DH5α. Different food colors, carotenoids, the flavonoid carthamus yellow, and mixed food colorants of melon color-L, and grape color RCG were prepared at different concentrations from

0.001% to 0.1% with *E. coli* DH5α, and UV-A LED light of 126 J/cm^2 was used to treat the solutions (Lian et al., 2010). This amount of energy used was substantial; however, technically possible, especially with 365, 395 and 455 nm LEDs. The authors used UV-A LEDs with 70 mW/cm^2 (126 J/cm^2 dose) irradiance for 30 min. There are a number of studies reported, showing huge energy dose of UV-A and blue light pulses emitted from LEDs, used for microbial inactivation in various solid/liquid food matrices (Kim et al., 2017a, b & c; Li, Kim, & Yuk 2018b). However, the reported energy doses of UV-C LEDs were significantly lower as mentioned in this and previous sections, compared to other wavelengths. Lower antimicrobial activity after LED treatment was obtained at higher concentrations of colored solutions and the log reductions in cell counts in different colored solutions were diverse. A maximum log reduction of 1.75 log CFU/mL was achieved in the 0.001% β-carotene colored solution, which was far lower than the 2.5 log reduction in the control phosphate buffered saline (PBS) solution. Similar results were obtained in orange juice, in which the log reduction was much lower than that of the transparent control solution after treatment. Pigments and other suspended particles in liquid foods may reflect and scatter the light, reducing the LED efficiency of bacteria elimination. Since reactive oxygen species (ROS) induced by ultraviolet A (UV-A, 320-400 nm) light are central to the bactericidal effect, the antioxidant activity of food colors such as carotenoids in liquid foods can be reduced, resulting in oxidation and quality change.

LEDs emitting blue light (400-480 nm) were tested for their ability to destroy pathogens in orange juice and milk (Ghate et al., 2015; Srimagal, Ramesh, & Sahu, 2016). A 2 to 5 log reduction of *Salmonella* was observed in pasteurized orange juice inoculated with a cocktail of *Salmonella* and treated with a 460 nm LED at different irradiance and temperature combinations (Ghate et al., 2016). Conditions that produced the highest *Salmonella* inactivation were 92

mW/cm^2 with very long treatment time of 13.6 h at a huge energy dose of 4500 J/cm^2 at 12°C. Ghate et al. (2016) maintained the irradiance of 92, 147.7 and 254.7 mW/cm^2 by adjusting the distance of the sample from the 460 nm LED and used a total dose of 4500 J/cm^2 for the treatment by regulating the treatment times corresponding to 13.6, 8.46 and 4.91 h, respectively. The long treatment time and the enormous energy used on products during LED treatments need to be justified if this technology using UV-A and blue light pulses should be developed for commercial disinfection of food products. One approach would be exploring the use of this technology for other applications (e.g., heating or drying, as huge energy used will heat and remove water from products) along with microbial inactivation, simultaneously.

Srimagal et al. (2016) compared the inactivation of *E. coli* in milk using blue LEDs at 405, 433, and 460 nm at 5, 10, and 15°C and treatment times of 0 to 90 min. Microbial inactivation was highest at elevated temperatures and lower wavelengths, with a maximum of 5.27 log CFU/mL reduction of *E. coli* O157:H7 after 60 min irradiation at 405 nm. The 460 nm LED resulted in a 2 to 5 log reduction, with a stronger effect on bacterial inactivation at higher temperatures, similar to the findings reported by Ghate et al. (2015). Both these studies reported significant changes in food product colors (orange juice and milk) after exposure to blue LEDs, suggesting that the blue LED altered the quality of the liquid foods. LED lights in the blue range lower bacterial activity mainly through photodynamic inactivation (PDI) of the microorganisms. The photons produced with the LED light can be absorbed by endogenous photosensitizers (e.g., porphyrins, cytochromes, flavins) and NADH in bacteria, which are sensitized after being illuminated (Durantini, 2006; Luksiene, 2003) as described in section 2.2. Srimagal et al. (2016) reported an optimum condition (405 nm, 13.8°C, for 37.83 min) under which treated milk was pasteurized with no change in physicochemical properties in comparison to untreated milk. Also, when

refrigerated, the shelf-life of the treated milk increased significantly to almost twice of that of untreated milk.

A recent study published by Akgün, & Ünlütürk (2017) examined the *E. coli* K12 inactivation by UV-C LED at 254 (0.3 mW/cm^2 irradiance, 707.2 mJ/cm^2 dose) and 280 nm (0.3 mW/cm^2 irradiance, 771.6 mJ/cm^2 dose), and UV-C LED coupled with 365 (0.8 mW/cm^2) and 405 nm (0.4 mW/cm^2) (UV-A LED) in both cloudy and clear apple juice. The combinations of emission wavelengths included 280 nm/365 nm, 280 nm/405 nm, 254 nm/365 nm, 254 nm/405 nm, and 254 nm/280 nm/365 nm/405 nm. The highest antimicrobial activity was achieved when the cloudy apple juice was treated with 280 nm alone and a 280 nm/365 nm combination, with log reductions of 2.0 ± 0.1 and 2.0 ± 0.4 log CFU/mL, respectively on LED treatment of 40 min. A significantly greater inactivation was observed in the clear apple juice than in the cloudy apple juice. The highest log reduction was obtained at 4.4 log CFU/mL in the clear apple juice treated solely with 280 nm (771.6 mJ/cm^2, 40 min). The hybrid system treated with 280 and 365 nm UV-LEDs resulted in log reductions of 3.9 ± 0.2 log CFU/mL, similar to the 280 nm treatment of cloudy apple juice for the same treatment time (40 min). It was also demonstrated that these hybrid LED treatments showed better inactivation effects on polyphenol oxidase. Even though the fully pasteurized state (~5 log reductions) could not be accomplished in apple juice by the combined UV-A and UV-C LEDs, this study suggests that UV-A and UV-C LEDs have a synergistic potential for disinfection, with a potential to preserve food colors. An additional disinfection effect might be obtained by increasing the dose of the UV-A and UV-C LEDs. The higher efficiency of the UV LED combination and their low energy consumption make them more advantageous than traditional mercury lamps for polyphenol oxidase inactivation. Studies on the inactivation effect of LEDs on liquid systems are listed in Table 2.3.

Table 2.3: The antimicrobial efficacy of LED in liquid systems.

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Suspension in suitable buffers	UV-A LED (365 nm); working distance- 20 mm	*E. coli* DH5α, Enteropathogenic *E. coli*, *Vibrio parahaemolyticus*, *Staphylococcus aureus* and *Salmonella enterica serovar* Enteritidis		3.9 log reduction in *E. coli* DH5α with 54 J/cm^2 dose; the inactivation was higher at the illumination temperature of 20°C and pH 8 and varied for different bacterial species	Mori et al. (2007)
Suspension in PBS	UV-A LED (365 nm); maximum current of one diode was 0.5 A, the voltage was 4.5 V; intensity was 70 mW/cm^2, working distance- 2 cm.	*E. coli* DH5α, Enteropathogenic *E. coli*, *Vibrio parahaemolyticus*, *Staphylococcus aureus* and *Salmonella enterica serovar* Enteritidis	Oxidative DNA damage observed (2.6 folds higher 8-OHdG formation); involvement of ROS like OH^- and H_2O_2 observed in the LED inactivation effect	*E. coli* DH5α, Enteropathogenic *E. coli*, *Vibrio parahaemolyticus*, *Staphylococcus aureus* were reduced by >5 log CFU/mL by 75 min treatment with 315 J/cm^2 dose; *Salmonella* was reduced by >4 log CFU/mL with 672 J/cm^2 dose for 160 min with UV-A LED	Hamamoto et al. (2007)
Ultrapure water, nutrient water and nutrient water with humic acids	UV LEDs (269 and 276 nm); sample volume 25 mL with stirring	*E. coli* K12		3 to 4 log CFU/cm^3 reduction observed; presence of humic acids and turbidity affected the UV irradiation and inactivation caused; 269 nm LED was more effective	Vilhunen, Särkkä, & Sillanpää (2009)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Bacterial suspension in PBS	265 nm LEDs; placed over a 6.5 mm wide aluminum channel 1 mm above the water surface; water depth- 7 mm; treatment dose- 0 to 20 mJ/cm^2	*E. coli* K12		>3 log CFU/mL reduction with 20 mJ/cm^2 dose of UV LED treatment	Chatterley, & Linden (2010)
Bacterial suspension in appropriate buffers	255, 280, 365 and 405 nm LEDs; pH tested- 6 and 8; treatment times- 60, 120 and 180 s	3 strains of *E. coli* and 2 strains of *E. faecalis*	pH did not show any significant effect	280/365 and 280/405 nm combination of LED treatment were most effective for bactericidal effect; 20 h after the UV irradiation all the tested samples showed 7 log reduction in all treated strains	Chevremont et al. (2012)
Bacterial suspension in deionized water	269 and 282 nm LEDs	*Bacillus subtilis*		269 nm LED produced better germicidal effect than 282 nm LED treatment	Rtele et al. (2011)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Water samples from tertiary effluent from the City of Regina wastewater treatment plant (WWTP) and bacterial suspension in suitable broth	260 nm UV LEDs	*E. coli* ATCC 25922		High turbidity of WWTP resulted in inconsistent effect; 1-2.5 log reduction obtained with 20 and 50 min treatment in a time dependent manner	Nelson et al. (2013)
Bacterial suspension in PBS	UV LEDs emitting wavelengths 265, 280 and 310 nm; 0.7, 1.3 and 1.1 mW output power, respectively; used for treatment individually and in combinations	*E. coli* K12		310 nm LED showed least antibacterial effect in batch system; 265 and 280 nm LEDs produced ~4 log reduction in both batch and flow-through system with dose of 10.8 and 13.8, and 16.4 and 25.5 mJ/cm^2, respectively	Oguma et al. (2013)
Bacterial suspension in 0.9% saline solution	UV-C LED (281.8 nm); Glass tube (quartz) and soda lime glass; 9 mL of bacterial suspension; treatment times- 10, 40 and 90 s; doses- 8.64, 34.59 and 77.82 mJ/cm^2	*Escherichia coli* DSM 498 *and Bacillus subtilis* DSM 402		Quartz glass had better transmittance of light; *B. subtilis* was reduced by 1.04 (soda lime glass) and 1.79 log CFU/mL (quartz glass) and, *E. coli* was reduced by 1.85 (soda lime glass) and 2.8 log CFU/mL (quartz glass) with 90s treatment; mixing of the samples improved the inactivation	Gross et al. (2015)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Bacterial suspension in appropriate buffer	260 nm (UV) LEDs and low pressure UV lamp	*Escherichia coli* B, a non-enveloped virus (MS-2), and a bacterial spore *Bacillus atrophaeus*		Comparable inactivation efficacy for *E. coli* B and MS-2; LED produced better inactivation for *Bacillus atrophaeus*; dose required for 4 log reductions for UV LEDs were as follows: *E. coli* B- 6.2 mJ/cm^2 MS-2- 58 mJ/cm^2 and *B. atrophaeus*- 18.7 mJ/cm^2	Sholtes et al. (2016)
Bacterial suspension in 0.05 M NaCl	Semi-commercial LED arrays (270–740 nm); treatment time- 6h	*Escherichia coli* K12 ATCC W3110 and *Enterococcus faecalis* ATCC 19433		270, 365, 385 and 405nm arrays produced >5 $\log_{10}$ reduction; 430 and 455 nm LED arrays resulted in ≈4.2 and 2.3-$\log_{10}$ reduction in *E. coli* and *E. faecalis* cell counts; 310 nm produced insufficient disinfection doe commercial application; 525, 590, 623, 660 and 740 nm arrays produced insignificant disinfection	Lui et al. (2016)
	Four UV-LED units emitting wavelengths 265, 280 nm, the combination of 265/280 (50%), and 265/280 (75%)	*E. coli*	Photoreactivation and dark repair decreased in case of 280 nm LED treatment	265 nm LED resulted in the maximum inactivation	Li et al. (2017)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Microbes in appropriate buffers	UV-C LED emitting 260 and 280 nm LED and 260/280 nm combination used for treatment	*Escherichia coli*, MS2 coliphage, human adenovirus type 2 (HAdV2), and *Bacillus pumilus* spores	DNA and RNA damage observed for individual LED treatments	Over 3 log reduction observed in *E. coli* with all UV LEDs; 260 nm LED was most effective in the inactivation of MS2 coliphage; A dose of 122, 89, and 105 mJ/cm^2 of 260, 280, and 260/280 nm LEDs required for 4-log reduction; 260 and 260/280 nm LED more effective for *B. pumilis* inactivation	Beck et al. (2017a)
Real wastewater samples and suspension in laboratory water	UV LED (265 nm); sample volume- 50 mL; frequency tested- 0.1, 1, 10, 100, 1 kHz; duty rate-10, 25, 50, 75, 90%	*E. coli* ATCC 11229, coliphage MS2 ATCC 15597-B1		No significant difference in the microbial inactivation observed between treatments with continuous light and light pulses emitted from the LEDs	Song, Taghipour, & Mohseni (2018)
Dechlorinated tap water	UV LED (285 nm)	Heterotrophic plate count (HPC)		UV LED treatment showed decreased HPC for 5 days storage; *Methylobacterium* species was UV resistant	Oguma et al. (2018)
Bacterial suspension in sterile distilled water	UV-A (365 nm) and UV-C (265 nm) LEDs; treatment times- 20 or 30 min (UV-A) and 5-16 min (UV-C); sample volume- 15 mL	*E. coli* (ATCC 25922, ATCC 700891, ATCC 15597 and ATCC 700891)	UVA pre-radiation showed: no effect in photo repair of bacteria; suppressed dark repair; no role of hydroxyl radical in the inactivation; improved CPD formation only in *E. coli* ATCC 15597	Synergistic effect of UV-A and UV-C was effective for *E. coli* (ATCC 11229, ATCC 15597 and ATCC 700891)	Xiao et al. (2018)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Microbes in appropriate buffers	UV LEDs (265, 280 and 300 nm)	*Pseudomonas aeruginosa* and *Legionella pneumophila, E. coli, Bacillus subtilis* spores, and bacteriophage Qb		Energy consumption was least for 280 nm LED for 3 log reduction; linear curve observed for *L. pneumophila* and bacteriophage Qb; sigmoidal curve observed for *E. coli*, *P. aeruginosa*, and *B. subtilis* spores	Rattanakul, & Oguma (2018)
UHT skim milk (<0.5% fat)	405 (NUV-Vis), 433 and 460 nm (blue) LEDs; illumination temperature- 5-15°C; treatment time- 0-90 min	*E. coli* ATCC 25922	No effect on physicochemical properties of the LED treated milk	Highest inactivation at higher temperature and lower wavelengths; 406 nm LED treatment at 13.8°C for 37.83 min can yield 5 log reduction with minimal colour change	Srimagal, Ramesh, & Sahu (2016)
Clear and cloudy apple juice	Four UV LEDs emitting wavelengths 254 (0.3 mW/cm^2, 707.2 mJ/cm^2), 280 (0.3 mW/cm^2, 771.6 mJ/cm^2), 365 and 405 nm; working distance- 1 cm; sample volume- 3 mL	*E. coli* K12 (ATCC 25253)	Highest inactivation of PPO enzyme obtained by 280/365 and 280/405 nm LED treatment; lowest colour difference observed with 280/365 nm LED combination	UV LEDs most effective in clear apple juice; highest inactivation in cloudy apple was ~2 log CFU/mL by 280 nm and 280/365 nm LEDs; 280 nm LED produced 4.4 log reduction in clear apple juice	Akgün, & Ünlütürk (2017)

Table 2.3 (continued)

Tested liquid system	LED used	Tested Microorganisms	Quality and mode of action	Major findings	References
Colored beverages and two different commercially available orange juices (A and B)	UV-A LED (365 nm); intensity- 70 mW/cm^2; Coloring pigment concentrations- 0.001, 0.01, 0.1 and 1.0%; Treatment time- 30 min; Dose- 126 J/cm^2	*E. coli* DH5α	Increasing the concentration of coloring agents decreased the antibacterial effect	Maximum log reduction was 1.75 log CFU/mL in the beverage containing 0.001% β carotene; orange juices (A and B) showed 0.35 and 1.58 log reduction, respectively	Lian et al. (2010)
Orange juice	Blue (460 nm) LED; Irradiances used- 92, 147.7, and 254.7 mW/cm^2; Illumination temperatures- 4, 12 and 20°C	Cocktail of *Salmonella enterica serovars* Gaminara, Montevideo, Newport, Typhimurium, and Saintpaul	Significant color changes observed	2-5 log reduction observed in *Salmonella* cocktail; best treatment conditions obtained was an irradiance of 92 mW/cm^2 for 13.58 h corresponding to dose of 4500 J/cm^2 at 12°C	Ghate et al. (2016)

Blue light and UV-C combined with UV-A LEDs has shown synergistic effects in terms of bacterial inactivation and the preservation of food quality. The nature of liquid foods (particle size, turbidity, and color), the dose, the time irradiated, and the temperature should be optimized when performing LED decontamination of liquid foods. LEDs combined with other non-thermal technologies, or with mild thermal treatments, should be explored to improve decontamination efficacy.

2.3.3 LED treatment of water

Safe drinking water is of global importance, particularly in countries with limited resources. Around 1.2 billion people do not have access to uncontaminated drinking water (Qu, Alvarez, & Li, 2013). Millions of people die every year from waterborne diseases (Bohn et al., 2009). Waterborne microorganisms cause intestinal infections such as diarrhea, typhoid, cholera, dysentery, amebiasis, salmonellosis, shigellosis, and hepatitis A (Hapke, 1988). Conventional approaches to treat wastewater involve the application of chemicals and considerable energy, which makes them expensive and inaccessible for many societies. Advanced water treatments in developed countries are also costly, involving thermal treatments, chemical disinfections (chlorination, ozone, chlorine dioxide, chloramination), and metals ions (Ag and Cu) to reduce the microbial content (Kim et al., 2002; Chen et al., 2005; Bergmann et al., 2008; Szabo, & Minamyer, 2014; Marchesi et al., 2012; Bitton, 2014). Besides being expensive, conventional methods of water disinfection are often ineffective and unsustainable. Thus, efficient, economical, and robust technologies that have minimal detrimental effects on the environment continue to be investigated for their application to water disinfection and decontamination (Bohn et al., 2009).

More than 7000 municipal UV disinfection systems have been installed worldwide (Song, Mohseni, & Taghipour, 2016), and small disinfection systems are available for domestic use (Brownell et al., 2008). Water disinfection using UV light has several advantages over conventional disinfection approaches. UV light has antimicrobial efficacy, produces minimal residue and by-products, has low environmental impact, and is compatible with current industrial processes (Choi, & Choi, 2010; Aoyagi et al., 2011; Dotson et al., 2012; Masschelein, & Rice, 2016; Chen et al., 2017). Unlike chemical water treatments, UV water treatment does not produce drug resistant bacteria (Mori et al., 2007). Disadvantages of conventional UV sources include easy breakage and a need for careful disposal, as the mercury lamp can pollute the environment.

Song et al. (2018) reported the inactivation of microorganisms such as *E. coli* and coliphage MS2 in laboratory water, and *E. coli* and total coliform in wastewater, after treatments with continuous light and light pulses emitted from 265 nm LEDs. The inactivation levels of all microorganisms were similar for the treatments with both the continuous light and the light pulses emitted from LEDs at different pulse patterns under equivalent UV energy dose. The treatments with the light pulses emitted from the LEDs inactivated microorganisms as effectively as pulses produced by conventional xenon lamps, providing high output thermal management for water disinfection. Inactivation of pathogenic bacteria *(Legionella pneumophila*, *Pseudomonas aeruginosa*) and surrogate species (*Bacillus subtilis* spores, bacteriophage Qβ, *E. coli*) was reported with UV-LEDs emitting light of different wavelengths (265, 280, and 300 nm) and compared with bacterial inactivation with a conventional low pressure UV (LPUV) lamp emitting light at 254 nm. The kinetics of microorganism inactivation were determined mathematically with the help of LED energy response curves at different wavelengths using a multitarget model. The inactivation profile of each species showed either a linear or sigmoidal survival curve. LED

treatments were more efficient than LPUV treatment for the inactivation of *P. aeruginosa*, *L. pneumophila*, and surrogate microorganisms in water. The 265 nm LED exhibited the most effective energy efficacy based on the inactivation rate constant of all the tested microorganisms except for *E. coli*. The 280 nm LED treatment consumed the least electrical energy to obtain a 3 log reduction of the microorganisms tested (0.15-1.11 kWh/m^3) compared to 265 and 300 nm LEDs (0.24-17.4 kWh/m^3) (Rattanakul, & Oguma, 2018).

Li et al. (2017) evaluated the inactivation of *E. coli* with 265 and 280 nm LED treatments, individually and in 265, 280 (50%) nm and 265, 280 (75%) nm combinations. A comparative study of *E. coli* photoreactivation and dark repair was also quantitatively conducted with LEDs and LPUV. The results showed that a 265, 280 nm LED combination did not have any synergistic effect on *E. coli* inactivation. Reactivation of the 265 nm LED treated bacteria was comparable to the LPUV treated bacteria. *E. coli* treated with 280 nm LEDs at 6.9 mJ/cm^2 showed the lowest percentage of photoreactivation and dark repair. This study concluded that, in water, the 280 nm LED inactivated *E. coli* more efficiently than the 265 nm LED due to the additional output power of the former and its better inhibition of bacterial reactivation. The synergistic antimicrobial efficacy of 260 nm and the 280 nm LEDs was evaluated against *E. coli*, *B. pumilus* spores, MS2 coliphage, and human adenovirus type 2 (HAdV2), and its efficacy was compared with mercury vapor lamps at low and medium pressures. The 260 nm LED was the most suitable for the inactivation of MS2 coliphage, whereas a medium pressure UV lamp inactivated HAdV2 and *B. pumilus* more efficiently than other UV sources (Beck et al., 2017b). Similar observations were made by Sholtes et al. (2016), where the inactivation of *E. coli* B, *B. atrophaeus*, and MS2 were subjected to a 260 nm LED and LPUV lamps. *E. coli* B and MS-2 inactivation kinetics were similar with LED and LPUV treatments. For all UV radiation sources, the doses required for a 4 log

reduction in microorganisms were higher for *B. atrophaeus* and MS2 than for *E. coli* B. Chatterley et al. (2010) treated *E. coli* in water with a 265 nm LED and conventional LPUV. The LED provided a higher antimicrobial efficacy than LPUV lamps but resulted in a higher disinfection cost. Gross et al. (2015) reported water disinfection using a 280 nm LED to inactivate *E. coli* and *B. subtilis* with two different glass (soda lime and quartz) guided lights to increase the disinfection efficiency. Almost all the radiated light was guided to the samples due to total reflection. The rate and efficiency of disinfection of *B. subtilis* and *E. coli* were improved by this light-guided method.

E. coli inactivation was tested with respect to exposure time and LED fluence between batch and flow-through reactors at peak emissions of 265, 280, and 310 nm. Light wavelength combinations (265/310, 265/280/310, 280/310, and 265/280 nm) were tested for their inactivation efficacy (Oguma et al., 2013). The time-dependent inactivation efficacy was a maximum with 280 nm LEDs, while 265 nm LEDs exhibited the highest fluence dependent efficiency. In the batch system, 265 and 280 nm LEDs required a dose of 10.8 and 13.8 mJ/cm^2 for achieving 4 log reduction in *E. coli*. The 310 nm LED required 56.9 mJ/cm^2 dose for just 0.6 log CFU/mL inactivation. Lower inactivation efficacy and decreased output power were observed with combined emissions at 265/280, 265/310, 280/310, and 265/280/310 nm in a flow-through reactor. The 265 nm LED treatment efficiency in water disinfection was also time dependent (Nelson et al., 2013). The results indicated that the sample turbidity influenced the bacterial inactivation, and better efficiency was achieved in less turbid water samples. These results suggest that particle accumulation in liquids can protect microorganisms from UV light exposure.

Hamamoto et al. (2007) disinfected water with UV-A LEDs (365 nm) and a low pressure UV-C lamp (254 nm). Inactivation of *Staphylococcus aureus*, *Vibrio parahaemolyticus*, enteropathogenic *E. coli*, and *E. coli* DH5α was greater than 3 log CFU/mL after 80 min of high

energy UV-A LED treatment. This observation was supported in a study by Mori et al. (2007), in which a 365 nm (UV-A) LED showed antimicrobial effects against *E. coli* DH5α, Enteropathogenic *E. coli*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* Enteritidis. Vilhunen et al. (2009) observed the effect of 269 and 276 nm on *E. coli* inactivation in two photolytic batch reactors differing in the wavelength emitted with different test media, including ultrapure water, nutrient, and water, and nutrient and water with humic acids. The LEDs were efficient for *E. coli* destruction even at low optical power. The study showed that the LED wavelengths were effective for *E. coli* inactivation, but the test medium did not have much impact on the inactivation.

Several studies have shown that UV LED can substitute for conventional treatment methods of water disinfection and that it provides benefits absent in conventional treatments. The most studied UV spectrum region for water disinfection is between 200-300 nm, with a wavelength of 265 nm being the most commonly used wavelength and *E. coli* the most studied microorganism. Water disinfection with a single wavelength was compared with water disinfection using a combination of two wavelengths. However, the data were not consistent, so there was no conclusion made. The fact that different microorganisms respond differently to light energy of same wavelength can be ascribed to the UV light source, the fluence rate, the UV dose, and the exposure time. There is a need to develop a standard operating method to determine the dose required for microbial inactivation in water (Song et al., 2016), and to determine the mechanism of LED microbial inactivation.

2.4 Food quality changes during LED treatment

Bacterial disinfection using LEDs in the UV or blue light range is a new non-thermal method for food processing. Most of the research has focused on microbial inactivation, with less emphasis

on food quality and structural changes in food components. LED light produces reactive oxygen species (ROS) by photosensitizing light-absorbing molecules in the bacteria, which causes damage to lipids, proteins, cell membranes, and DNA, and results in cell injury and death (Luksiene, & Brovko, 2013). However, ROS generated by LED light can modify the structure of food molecules, affecting their nutritional and sensory properties.

Kim et al. (2017c) evaluated the quality changes on the surface of fresh-cut mango treated with a 405 nm LED to test its antibacterial effects against *E. coli*, *Listeria monocytogenes*, and *Salmonella*. There was no significant difference observed between treated and untreated mango in terms of color, antioxidant capacity, ascorbic acid content, *β*-carotene, and flavonoids, regardless of the storage temperature. Likewise, there was no significant difference in the physicochemical properties of untreated milk and milk treated with a 406 nm LED (13.8°C, 37.8 min). However, color changes in the treated milk were observed (Srimagal, Ramesh, & Sahu, 2016). Ghate et al. (2015) noted variations in orange juice color after exposure to a 460 nm LED. The authors ascribed the color change to the oxidative degradation of carotenoids, which have an absorption spectrum between 400 and 500 nm. Akgün, & Ünlütürk (2017) showed that UV LED treatment of apple juice led to microbial and enzymatic inactivation. The study also noticed apple juice color changes during LED treatment, with the lowest total color change being observed when 280 and 365 nm LEDs were combined in a single treatment.

A pulsed UV light energy dose of more than 2.1 J/cm^2 produced a deterioration in the sensory quality of meat products (Hierro et al., 2012). LED light induced unfavorable flavor development in milk samples due to its reaction with photosensitive compounds (Chang, & Dando, 2018). Research of functional and structural changes in food after LED decontamination

treatments are scarce, and more investigation of food quality changes after LED illumination are needed.

2.5 Mechanisms of inactivation

LEDs emitting visible light can excite light-sensitive compounds, e.g., porphyrins, present in the bacterial cell wall (Figure 2.2). These excited compounds collide with, and transfer energy to oxygen molecules, producing ROS such as hydroxyl radicals, hydrogen peroxide, and singlet oxygen. ROS further react with cellular components causing cell death (Luksiene, & Zukauskas, 2009; Ghate et al., 2013; Ghate et al., 2015).

Light energy in the 320-400 nm range can produce oxidative stress, protein damage, and inhibition or delay of growth without killing the microorganisms irradiated (Rüd et. al., 2017). For instance, 405 nm LED treatment showed antibacterial activity against the gram-positive bacteria *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*, damaging the bacterial membrane, although the bacterial DNA was not damaged by the oxidative stress (Kim et. al., 2015). Kim et al. (2016b) also reported no DNA degradation in gram-positive and gram-negative bacteria after treatment of *L. monocytogenes, E. coli* O157:H7, *Salmonella* Typhimurium, and *Shigella* sonnei with 405 nm LED. However, DNA oxidation and damages to efflux pump activity and the glucose uptake system due to ROS production were observed after LED treatment of *Salmonella* spp. with 405 nm LED at refrigerated conditions. In similar studies, refrigerated bacteria showed membrane damage, apart from the damage caused by the LED treatment (Kim, & Yuk, 2017). ROS produced by LED illumination is believed to oxidize the guanine bases in DNA and peroxidate lipids in the cell membrane. However, no lipid peroxidation was observed, whereas DNA oxidation was observed when fresh-cut papaya infected with *Salmonella* was subjected to a 405 nm LED treatment; in the same experiment, the LED did not produce any

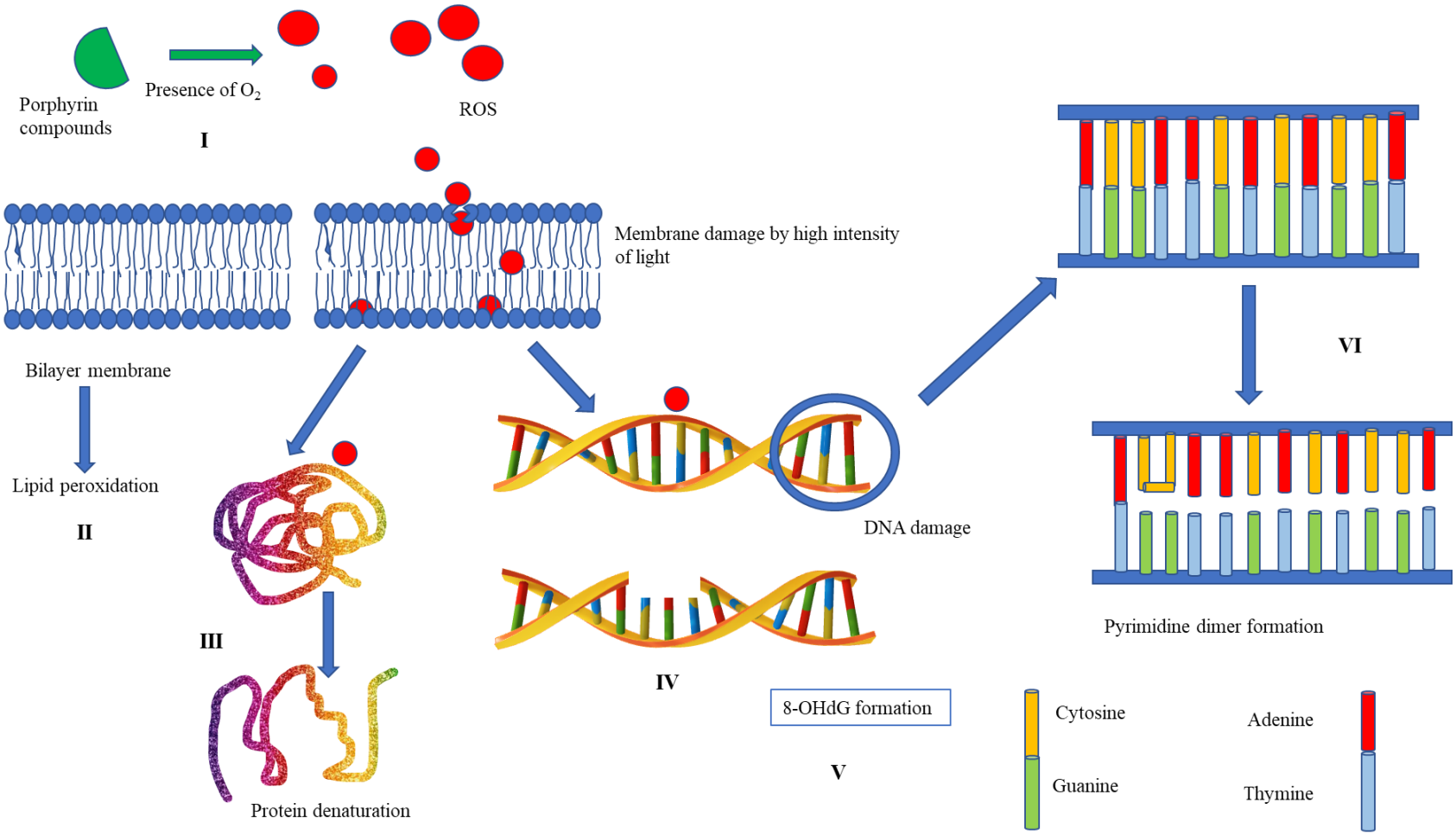


Figure 2.2: Effect of LED treatments on bacteria, (I) porphyrin compounds in the bacterial cell wall absorb light and undergo photosensitization producing reactive oxygen species (ROS) in the presence of oxygen; ROS leads to (II) lipid peroxidation in the bacteria; (III) protein denaturation; (IV) DNA damage; (V) the ROS can oxidize the guanine bases leading to the production of 8-hydroxy-2'-deoxyguanosine (8-OHdG) indicating oxidative stress produced by LED treatment; and (VI) UV-C light can lead to the formation of pyrimidine dimers which can lead to inhibition of DNA replication.

significant effect on the organoleptic properties of fruit at refrigeration temperature (Kim, Bang, & Yuk, 2017a). Kim, & Kang (2018a) observed no significant loss in the membrane integrity of gram-negative bacteria *E. coli* O157:H7 and gram-positive bacteria *L. monocytogenes* treated with light pulses emitted from UV-C LEDs emitting light of wavelength 280 nm, whereas the membrane potential values were significantly changed. Moreover, a high membrane lipid peroxidation was observed in both the strains, but it was higher in *L. monocytogenes*. The LED treatments might reduce the activity of succinate-coenzyme Q, an electron transport chain enzyme involved in the production of energy and cell proliferation in bacterial cells (Kim, & Kang, 2018a). This possibility was supported by LED illumination of *Pseudomonas aerugenosa* with wavelengths of 464 nm (blue), 528 nm (green), and 636 nm (red). The red and green LEDs did not inactivate the bacteria. A catalase A (enzyme that detoxifies hydrogen peroxide) mutant strain showed more sensitivity than the wild strain to the blue LED treatment, and overexpression of catalase A increased the sensitivity of the wild strain to the irradiation, indicating that hydrogen peroxide was a major ROS during the LED treatment (Orlandi et al., 2018). Addition of the hydroxyl radical scavenger mannitol did not affect the inactivation of *E. coli* DH5α when treated with UV-A followed by UV-C LED, indicating that the hydroxyl radical might not have any role in the inactivation. On the contrary, it was observed that production of the hydroxyl radical and hydrogen peroxide had a major role in UV-A LED inactivation, as the reduction of *E. coli* DHα decreased with the addition of mannitol (Hamamoto et al., 2007). The cytotoxic response of bacteria to 405 nm LED treatment was further studied in *S. epidermidis*, where the antimicrobial effect of the LED was significantly decreased in the presence of sodium pyruvate (hydrogen peroxide scavenger), but a decrease was not observed in the presence of dimethyl thiourea (hydroxyl scavenger). This suggests that hydrogen peroxide played a greater role than the hydroxyl

radical in the microbial inactivation (Ramakrishnan et al., 2016). HPLC analysis by reversed-phase chromatography of *P. aeruginosa* samples showed expressed coproporphyrin III, suggesting the production of endogenous porphyrins in the bacteria, which would explain the photodynamic inactivation (PDI) effect of blue light (Amin et al., 2016).

Bacteria use defence mechanisms, such as DNA repair, photoreactivation, dark repair, biofilm formation, in response to the damage inducing UV LEDs. For instance, cyclobutene pyrimidine dimer (CPD) formation was enhanced in *E. coli* ATCC15597 due to a 265 and 280 nm LED hybrid irradiation. Also, the recA protein, a core protein in the repair of the cell after an SOS response, was overexpressed (Xiao et al., 2018). CPD generation in *E. coli* DHα was more pronounced with UV-C irradiation than with UV-A irradiation, which produced minimal *E. coli* DHα CPD, indicating that UV-A treatment induced less damage than UV-C treatment to *E. coli* DHα DNA (Hamamoto et al., 2007). LED treatment with 280 nm light inhibited the photoreactivation and dark repair compared to the more germicidal 265 nm light in normal conditions and these LED treatments further resisted the compromised DNA repair mechanisms in the bacteria (Li et al., 2017). These results are supported by an earlier study on the oxidative stress by NUV light on *S.* Typhimurium, where long exposure to low-intensity NUV light resulted in bacterial demise, probably due to oxidative stress and inhibition of the oxyR regulon, which plays a major role in triggering bacterial defence against stress. Long exposure to low-intensity NUV light also rendered the cells sensitive to further sterilization techniques (Kramer, & Ames, 1987).

Synergistic antimicrobial effects of sequential treatments with LEDs emitting light at different wavelengths have also been reported. Pre-treatment of different strains of *E. coli* with a UV-A (365 nm) LED before UV-C (265 nm) LED treatment increased the level of UV-C

inactivation (Xiao et al., 2018). Although the photoreactivation ability was not influenced by the pretreatments, the dark repair was inhibited. The hybrid 260/280 nm LED treatment caused no DNA or RNA damage and did not inactivate *E. coli* K12, MS2 coliphage, human adenovirus type-2, or *Bacillus pumilus* spores (Beck et al., 2017a).

Visible light (400-700 nm) sensitized with curcumin and toluidine blue increased the inactivation of *Streptococcus mutans* more than light treatment or photosensitizers alone (Paschoal et al., 2014). Red and blue LED treatment at 24 J/cm^2 sensitized with 0.75 mM and 25 μM curcumin/toluidine blue mixtures resulted in almost complete inactivation of *S. mutans* (Paschoal et al., 2015). Red and blue visible light produced more ROS when photosensitizers were added, supporting the higher inactivation observed (Bouillaguet et al., 2010). Similarly, the addition of ultrasound to UV-LED (254 nm) treatment enhanced the inactivation of *E. coli* ATCC15997 and also reduced the photoreactivation of the microorganisms tested (Zhou et al., 2017).

The antimicrobial effects of LED varied with the bacteria treated. Gram-negative bacteria are encased in a thin peptidoglycan layer sandwiched between an inner and relatively impermeable outer membrane, which maintains the rigidity of the cytoplasmic membrane, whereas a thick peptidoglycan layer and a single membrane encase gram-positive bacteria (Murray, Steed, & Elson, 1965; Shockman, & Barrett, 1983). At high pH, the cytoplasmic membranes of gram-negative bacteria leaked, while gram-positive bacteria were resistant (Mendonca et al., 1994). Ghate et al. (2015) found that the gram-negative bacteria *Salmonella* and *E. coli* were sensitive to 461 nm LED treatment in alkaline pH, while the gram-positive bacteria *L. monocytogenes* were sensitive to 461 nm LED treatment in acidic conditions. *E. coli* O157:H7, *S.* Typhimurium, and *Shigella sonnei* were sensitive to bile salts after 405 nm LED treatment due to the loss of membrane integrity. The lack of an outer membrane probably made the gram-positive bacteria sensitive to

acidic conditions and the solubilization of the outer membrane of the gram-negative bacteria made it more sensitive to the alkaline pH (Kim et al., 2016b). *S. enterica* Enteritidis that contaminated cooked chicken were subjected to 405 nm LED on agar plates supplemented with antibiotics specific to cell wall, protein, DNA, and RNA. The 405 nm LED made these sites more sensitive to the antibiotics and the *S. enterica* Enteritidis were metabolically inhibited by 32.5, 24.2, 30.1, 44.1%, suggesting that the antibacterial efficacy was linked to cellular damage (Kim et al., 2017b). The inactivation of *E. coli* and the production of 8-hydroxy-2'-deoxyguanosine (8-OHdG) after UV-A (365 nm) LED treatment indicated that the presence of oxygen played a major role in the DNA damage, as the DNA damage was reduced significantly when the same experiment was performed under anaerobic conditions (Hamamoto et al., 2007).

Possibly, light inactivates bacteria by activating prophages (bacteriophages in a dormant state) present in the bacterial genome. In methicillin-resistant *S. aureus*, 460 nm LED treatment induced upregulation of phage-related genes, activating prophages into phages and thus causing cell lysis (Yang et al., 2017). Adenovirus, a resistant waterborne pathogen residing in both treated and wastewater systems, consists of core proteins that play a major role in the adenovirus infection of a host (Russell, 2009). Possibly, UV light produces its antimicrobial effects by damaging viral proteins. This hypothesis was supported when UV light of less than 240 nm from a germicidal UV spectrum lamp damaged viral proteins, inducing protein aggregation in adenovirus 2 (Beck et al., 2017a). The maximum reductions were observed in hexon and penton proteins. Proteins tend to absorb light of lower wavelengths, whereas nucleic acids tend to absorb light of higher wavelengths. Bacteriophage MS2 was more sensitive than Adenovirus type 41 to UV treatment. Adenovirus type 41 was resistant to UV light at 254 nm and its inactivation required a much higher dose, 225 mJ/cm^2 (Ko, Cromeans, & Sobsey, 2005). The addition of titanium dioxide (TiO_2) as a

photocatalyst to UV-A light increased its antimicrobial efficacy against the murine norovirus, a surrogate of the human norovirus, while UV-B alone was effective in inactivating murine norovirus activity. Although a reduction in infectious viral particles was observed, there were no significant changes observed in viral nucleic acids, indicating that the effect on other components, including proteins, in such viruses (Lee, & Ko, 2013). Although UV-LEDs emitting 285 nm light showed promising viral inactivation (Oguma et al., 2015), more extensive research at different wavelengths is needed.

Both dormant and germinating stages of fungi show involvement of ROS and a requirement for the presence of oxygen, as the sensitivity of these fungi to 405 nm LED was reduced in the absence of oxygen and in the presence of ROS scavengers. All three fungi − *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus niger* (conidia) − showed the presence of intracellular porphyrin, indicating the involvement of PDI in fungi. *S. cerevisiae* showed inactivation even in anaerobic conditions in the presence of ROS scavengers and showed significantly higher inactivation in aerobic conditions, suggesting the involvement of components other than porphyrins in *S. cerevisiae* inactivation (Murdoch et al., 2013). Synergistic use of UV-A (365 nm) light and riboflavin produced phenotypic changes in the fungi *C. albicans* and *Fusarium solani*, such as a lower growth of biofilm and color variations, where biofilm formation is a synergistic microorganism response to any stress (Kashiwabuchi et al., 2013).

LED light of different wavelengths inactivates microorganisms, mainly by activating the production of ROS and inhibiting microorganism defence mechanisms. The extent of LED damage depends on the microorganism targeted, the wavelength and dose of the light, and surrounding conditions. More research on the inactivation mechanisms of LEDs at different wavelengths is required.

2.6 Other applications of LED technology

2.6.1 UV-C treatment

LEDs emitting light in the UV-C range, 200–280 nm, have been developed since 2010 (Yeh et al., 2015), and have been applied in air disinfection systems to inactivate nebulized viruses, bacteria, and fungi (Kim, & Kang, 2018b). Ultraviolet germicidal irradiation lamps reduced viable microorganisms and endotoxins in the central ventilation system of an office (Menzies et al., 2003). A UV-C LED in the 275-285 nm range showed antimicrobial efficacy against *E. coli* W3110, *P. aeruginosa* PAO001, *S. marcescens* NBRC 3046, *S. aureus* NBRC 12732, and *C. albicans* IFM 40009, microorganisms that frequently contaminate solutions used for intravenous infusions (Omotani et al., 2018). Longer treatment times are required when UV-C LEDs are used in low dose, however, the development of higher power UV-C LEDs will lead to their wider use in the disinfection of water and food products.

2.6.2 UV-B treatment

UV-B LEDs (280-320 nm) are applied in the phototherapy of psoriasis, a common skin disease. UV-B light has also reduced the powdery mildew in cucumber due to the pathogen *Podosphaera xanthii* (Suthaparan, Sciences, & Stensvand, 2014).

2.6.3 UV-A treatment

LEDs emitting light at 365 nm (UV-A) have been used to disinfect air. Two stable currents of UV-A LED (1.2 mW/cm^2, 0.5 A or 0.2 mW/cm^2, 1.0 A) applied for 75 min resulted in a 3 log reduction of *E. coli* DH5α in air (Gadelmoula et al., 2009). UV-A LED light sources are also being developed for suitable therapeutic applications in human skin (Pirc et al., 2019). UV-A LEDs have found

applications in curing polymers, in medicine, and in air disinfection. UV-A LED light at 320-400 nm emits low heat, an advantage in curing applications.

2.6.4 Near-UV-visible LED treatment

UV light near the visible region (~395-405 nm) (NUV-Vis) has several applications. NUV-Vis LEDs emitting light at 405 nm have been used for tooth bleaching (Klaric et al., 2015). LEDs in the NUV range are being developed for curing, as an alternative to mercury lamps (Martin et al., 2002). Their curing ability has also found use in 3D printing and adhesive curing.

2.6.5 Visible LED treatment

LEDs emitting light in the visible range have been evaluated in dental implantations, wound healing, and algaculture. Titanium dioxide (TiO_2) is generally used as a photocatalyst in dental applications when UV light is used (Suketa et al., 2005; Pantaroto et al., 2018). TiO_2 was codoped with nitrogen and bismuth to increase its antimicrobial activity when the LED was used in the visible range of 420 to 690 nm. TiO_2 doped with Bi showed promising antimicrobial effects on the biofilm-producing bacteria *Streptococcus sanguinis* and *Actinomyces neaslundii* on the surface of dental implants (Nagay et al., 2019).

Visible LEDs have been shown to improve algaculture. Increases in biomass production of *Pichoclorum atomus* were the highest when irradiated with red, followed by blue, yellow, purple, and green LEDs, while the green LED produced the maximum lipid content from the algal species (Hun et al., 2016). Blue, green, red, and white LEDs increased the biomass production of a *Spirulina* sp. LEB 18 culture (food supplement) (Prates et al., 2018). The ability of green LED treatment to yield high lipid content in microalgae cultures has been reported extensively (Ra et al., 2016; Sirisuk et al., 2018). A red LED increased the biomass and a blue LED enhanced oil

formation in a mixed culture of *Chlorella* sp. and *S. cerevisiae* (Shu et al., 2012). The ability of visible LEDs to enhance algaculture makes them suitable for biodiesel production. Applications of visible LEDs are discussed in sections 2.6.6, 2.6.7, and 2.6.8.

2.6.6 Blue LED treatment

LEDs made of indium gallium nitride (InGaN) and gallium nitride (GaN) emit blue light of 450-500 nm. Blue LEDs are used to treat water with or without photocatalysts (Cheng et al., 2011) and to disinfect medical instruments. A blue LED emitting light of 455 ± 30 nm enhanced the antimicrobial effect of curcumin in the oral cavity (Leite et al., 2014). An LED emitting blue light showed antimicrobial effects against the periodontopathic species *Porphyromonas gingivalis* and its biofilm when used in combination with 0.1% riboflavin as a photosensitizer. However, this antimicrobial effect was significantly less than that of a red LED (Bärenfaller et al., 2016). Blue LEDs with a color intensity of 96.8 µmol photon/m^2/s facilitated maximum biomass production from *Chlorella vulgaris* microalgae (Fozer et al., 2019). Blue LEDs improved biomass production in *Synechococcus nidulans* LEB 115 cultures by 80% and improved lipid production in *Chlorella fusca* LEB 111. Chlorophyll pigments and carotenoid accumulation in the latter increased with an increase in light intensity (Duarte, & Costa, 2018). *Chlorella vulgaris* showed maximums in specific growth rate and lipid production when treated with at 200 µmole/m^2/s with a blue LED and a 12:12 h L/D photoperiod (Atta et al., 2013). A blue LED fostered the highest specific growth in *Nanochloropsis* spp. followed by white, green, and red LEDs (Das et al., 2011). The potential of blue LEDs in medical applications needs to be supported by data regarding their mode of action. However, they have proven to be effective in enhancing the specific growth of microalgae, which can be used for biofuel production.

2.6.7 Red LED treatment

Red light from 610 to 760 nm is emitted by LEDs with aluminium gallium arsenide semiconductors. The germicidal effect of red LEDs has been found to accelerate the wound healing process in mice, and is used to disinfect appliances (Vinck et al., 2003; Ribeiro et al., 2015). A red LED (660 nm) in combination with toluidine blue O (TBO) reduced *Streptococcus oralis* in dental plaques in a dose-dependent manner (Ichinose-tsuno et al., 2014). After treatment with 0.25% hydrogen peroxide, *Porphyromonas gingivalis* biofilms (associated with periodontitis) were reduced by LED light in the red spectrum (625-635 nm) (Eick et al., 2013). As an algaculture application, a red LED (660 nm) was more effective than blue and white LEDs in improving the specific growth rate and increasing the cell concentration of *Chlorella* sp. (Choi et al., 2013).

2.6.8 Coupling different LEDs

The synergistic effect of LEDs emitting light of different wavelengths has been tested in biodiesel production and algaculture. Abomohra et al. (2019) combined blue and red LEDs to enhance biodiesel production and lipid productivity in the microalga *Scenedesmus obliquus*. *Isochrysis galbana* is food for several bivalve larvae and has been studied for biomass and lipid production in a two-phase system by Che et al. (2019). The authors used a 50:50 ratio of blue (465 nm) and red (640 nm) LEDs in the first phase for biomass culture and a green (520 nm) LED in the second phase for lipid production. Maximum biomass and lipid content were obtained at a light intensity of 400 μmol/m^2/s and a photoperiod of 18.6 h L/D (light/dark) cycle. Hun et al. (2018) also observed that a combination of blue and red LEDs improved biomass production and photosynthetic pigments and that a green LED improved lipid production in four microalgae (*Phaeodactylum tricornutum*, *Isochrysis galbana*, *Nannochloropsis salina*, and *Nannochloropsis*

oceanica). In a three phase culture study of *Nannochloropsis oceanica*, a blue LED (465 nm) was used in the first phase to study the microalgal growth parameters, a green LED (550 nm) was used in the second phase for lipid production, and temperature stress was used in the third phase to increase the production of mono and polyunsaturated fatty acids (Sirisuk et al., 2018). The synergistic effects of combined blue and red LEDs for biomass production, and combined blue, red, and green LEDs for lipid production have been established. However, the antimicrobial efficacy of combining multiple wavelengths in areas other than food and water disinfection needs more attention. LEDs emitting light in the UV range are replacing mercury lamps in curing applications, while red, blue, and green LEDs are frequently applied in biodiesel production and algaculture.

2.7 Challenges and opportunities

LEDs are mercury-free and their consistent light irradiance and high efficiency is an improvement on the performance of the traditional UV lamp. Efficient UV-A, NUV, and visible LEDs are available for research and industrial work, but UV-C LEDs need to be improved with respect to output degradation of sticking resin and adhesive die, and reduced reflection of reflectors (Muramoto, Kimura, & Nouda, 2015). The heat generated within LED devices during operation can cause device damage and wavelength shift. Continuous irradiation results in an increase in the LED temperature, necessitating a large heat sink to control temperature. Treatment with light pulses (1 to 20 pulses per s) can significantly reduce the rate of temperature rise (Song, Taghipour, & Mohseni, 2018). Radiant energy supplied in pulses can be changed based on need.

Since LED technology is a surface decontamination method, the shadowing effect of multiple layers of bacteria in a treated material can result in a lower bacteria inactivation rate. Thus, proper exposure of a bacterial sample to the LED light during treatment must be ensured

(Lee, Jin, & Hong, 2018). The penetration depth of UV light is only few millimeters and depends on the surface and optical properties of the target. Therefore, the experimental design must consider the LED antimicrobial efficacy, particularly when targeting solid/liquid foods. The design of UV-A LED food/liquid treatments must also consider the high capability of microorganisms to regenerate after treatment (Lui et al., 2016).

The high LED doses required to kill microorganisms in food can have a detrimental effect on the quality of the treated products. A dose of more than 2.1 J/cm^2 of pulsed UV light energy resulted in sensory quality deterioration in meat products (Hierro et al., 2012). As light reacts with photosensitive compounds, and a high-intensity light source can cause a temperature rise in the target, unfavorable flavors in target foods can develop during UV LED treatments; this is frequently observed in the treatment of milk (Chang, & Dando, 2018).

UV-C LEDs provided non-thermal food treatments that resulted in few changes in color, flavor, and vitamins during treatments of fruit juices (Keyser et al., 2008; Santhirasegaram et al., 2015). Apart from providing microbial decontamination, subjecting foods to blue LEDs improved the chlorophyll levels in pea seedlings, and red LEDs were observed to increase β-carotene in the leaves and stems of pea seedlings (Wu et al., 2007). The use of a combination of LEDs emitting light at different wavelengths can improve the antimicrobial effect. For instance, UV-C induces pyrimidine dimer formation and UV-A delays the DNA repair mechanism of microorganisms, and can also kill microorganisms by inflicting oxidative damage (Nakahashi et al., 2014; Chevremont et al., 2012). The use of chlorine with a UV-LED improved the inactivation rate of *B. subtilis* spores by approximately two-fold compared to the use of the UV-LED alone (Li et al., 2018). Thus, other decontamination methods and photosensitizers can be used in combination with UV-LEDs to improve the inactivation of microorganisms and spores.

LEDs require further research to improve their ability to disinfect water. UV-C LEDs are the most commonly utilized to disinfect water, but their low power output, low energy conversion efficiency, and high cost has hindered a large scale adoption of UV-C LEDs. In the conversion of electrical energy to light energy, a high proportion of UV-C LED electrical energy is converted to heat, which must be immediately removed to cool down the LED junction. The heat production of LEDs during operation wears out system components and subjects the targets to damaging heat. Therefore, durable, heat resistant, cost-effective LED components need to be carefully selected, and the LED system must be designed to release heat efficiently to avoid operational failure.

Previous research has focused on LED treatments of stagnant water. LED treatments of large volumes of flowing water would be more useful for real life conditions. Water depth plays a major role in decontamination. Longer wavelengths can penetrate deeper to achieve microbial inactivation. The turbidity of the targeted sample plays a major role in the overall efficiency of the LED treatment. Inorganic matter in a water sample absorbs the light, reducing the light available for disinfection. The particles that constitute the turbidity also shield the microorganism targets of the LED. Such factors are familiar to LED researchers and optimization studies are ongoing. The light wavelength used must provide enough energy to eliminate the LED target in spite of being inhibited by turbidity and the absorption of light by nontargeted components in the medium.

2.8 Concluding remarks

Light emitting diodes (LEDs) are an emerging technology for various applications in food processing, including disinfection of solid and liquid food products and water; this technology offers several benefits to food processors including, no toxic waste generation, durability, robustness, monochromatic light production, customization depending on the final application, compared to conventional sources of light. UV-C LEDs are mainly used to kill microorganisms in

water, and UV-C LED units for portable applications such as water sterilisation bottles, disinfection of medical equipment, are available in the marketplace. The selection of LEDs for economical water disinfection is based on microorganism inactivation efficacy and low electrical energy consumption. Simultaneous and sequential LED treatments, utilizing different combinations of LEDs, are used to achieve maximum disinfection levels in water and foods. Microorganism species respond differently to light at different wavelengths, and more research is needed to understand this differential response, and to select the LED treatment that fits the conditions at hand. Changes in food macromolecules during LED treatments are also under continued investigation. While, LEDs could be added as an additional treatment for microbial inactivation in food products and water in the future, the important factors influencing the disinfection efficacy of LEDs emitting light with different wavelengths and the mechanisms involved need further investigation.

There are limited studies focussing on evaluating the inactivation efficacy of LED treatments in low a_w foods. Further research is required to understand the antimicrobial efficacy of the LEDs emitting light pulses in the ultraviolet spectrum; 275 (UV-C), 365 (UV-A); 395 (NUV-Vis) nm and visible light; 455 nm (blue) in low a_w foods. This research gap is addressed in Chapters 3, 4 and 5, where *Salmonella* inactivation efficacies of 365, 395 and 455 nm light pulses emitted from LEDs were evaluated at low a_w conditions and in low a_w pet food pellets. The improvement in *Salmonella* inactivation efficacy of a sequential treatment of 275 nm and 455 nm light pulses in low a_w foods like pet foods was evaluated (Chapter 4). Chapter 5 addressed the research gap of understanding the underlying mechanisms of the antibacterial efficacy of the 365, 395 and 455 nm LEDs against *Salmonella* in low a_w conditions. Also, there are limited studies

focussing on the antibiofilm efficacy of LED treatment in food contact surfaces, which is addressed in Chapter 6.

Chapter 3: Inactivation of *Escherichia coli* and *Salmonella* using 365 and 395 nm high intensity light pulses emitted from light emitting diodes

3.1 Introduction

Growth of foodborne pathogenic microorganisms in foods requires a high a_w. Low-a_w foods with $a_w < 0.85$ do not support their growth, but these microorganisms may survive in the dry state throughout the storage life of the foods (Cordier, 2014). Dry foods including pet foods, cereals, and spice powders can cause foodborne illnesses after contamination with *Salmonella enterica* or Shiga-toxin producing *Escherichia coli* (Carrasco et al., 2012; Gurtler et al., 2014). Some low-a_w foods including nuts were contaminated by *Salmonella enterica* spp. before harvest, while in products such as spray dried milk powder, contamination can occur from product handling or processing environment (Carrasco et al., 2012). A decrease in a_w increases the heat resistance of the foodborne pathogens and makes them more resistant to conventional decontamination methods, including thermal treatments (Laroche, Fine, & Gervais, 2005; Villa-Rojas et al., 2013). Pathogen inactivation on dry foods thus requires prolonged thermal treatments, which adversely affect the quality and nutritional composition of the food products. Hence, alternate food decontamination technologies are necessary to achieve elimination of pathogens in dry foods without compromising food quality. Four decontamination technologies for dry foods like almonds were approved by the FDA, which involve the application of propylene oxide, hot oil, hot water, or steam (Pan et al., 2012).

Pulsed light treatment is an efficient antimicrobial technology, which uses short, intense pulses of light ranging from Ultraviolet (UV) to Near Infrared (NIR) (Chen et al., 2015). The antibacterial efficacy of pulsed light has been attributed mainly to the UV spectrum, which is

divided into UV-A (320–400 nm), UV-B (280–320 nm), UV-C (200–280 nm) and far UV (100–200 nm) (Elmnasser et al., 2007; Kim, Kim, & Kang, 2016a; Kramer, Wunderlich, & Muranyi, 2017; Sánchez-Maldonado et al., 2018). However, the lamps emitting the pulsed light require long warm-up times and have reduced efficiency at low temperatures (Elmnasser et al., 2007; Kim, Kim, & Kang, 2016a).

Light emitting diodes (LED) emitting monochromatic light are an emerging alternative for decontamination of food. LED technology uses semiconductors that release energy in the form of light with a specific wavelength that depends on the type of semi-conductor material used (Kim, Kim, & Kang, 2016a; Song et al., 2016). The LED system does not require warm-up time to start and produce a uniform irradiation (Kim et al., 2016b; Li et al., 2017). Moreover, the longer life span, easy incorporation to the existing processing line owing to its compact design and low voltage requirement may make it a cost-effective alternative in the future (D'Souza et al., 2015; Matafonova, & Batoev, 2018).

UV-C technology is used for disinfection of air and water, and for sterilization of food surfaces (Adhikari et al., 2015; Syamaladevi et al., 2013, 2014, 2016a). UV light with lower wavelengths <315 nm (UV-B and UV-C) are hazardous to skin and causes sunburn and mutations, while UV-A is safer to handle (Gruijl, 2002; Shirai, Watanabe, & Matsuki, 2017). UV-A LED treatment is an emerging microbial intervention process for water treatment (Hamamoto et al., 2007; Li et al., 2010). UV light with wavelengths close to visible region (~395 nm) is called Near UV-Visible (NUV-Vis) light, which has also been proposed as a reliable antimicrobial technology. The 395 nm LED reduced cell counts of *E. coli* O157:H7 in liquid suspension and wheat flour (Birmpa et al., 2014; Du et al., 2020). The antibacterial efficacy of the LEDs could be due to the

oxidative stress produced in the bacteria. Moreover, visible light could induce photodynamic inactivation in the bacteria (Kim et al., 2016b; Kim et al., 2017b; Kumar et al., 2016).

Although previous studies reported antimicrobial potential of 365 and 395 nm light emitted from LEDs, information about their antimicrobial efficacy at low-a_w conditions and for the treatment of foods is limited. Therefore, understanding the antimicrobial efficacy of high intensity light pulses emitted from the LEDs in high- and low-a_w conditions would help in developing a novel technology for surface sanitation of high- and low-a_w food products. The main objective of this study was to evaluate the antibacterial effectiveness of 365 and 395 nm LED system against *E. coli* and *Salmonella enterica* spp. in both high- and low-a_w conditions and to evaluate their efficacy in low-a_w foods such as pet foods. The influence of selected product (sample type, water activity) and process (power levels, treatment dose) parameters on the inactivation efficacy of the light pulses emitted from the LEDs were also analyzed.

3.2 Materials and Methods

3.2.1 Sample Preparation

Escherichia coli AW1.7, a heat resistant food isolate strain encoding the locus of heat resistance (Mercer et al., 2015), *Salmonella enterica* Typhimurium ATCC13311, the heat resistant *S. enterica* Senftenberg ATCC43845 (Mercer et al., 2017), and the waste water isolates *Salmonella enterica* FUA1946, FUA1934, FUA1955 were used in this study. The heat resistant food and wastewater isolates of bacteria were chosen for the study to evaluate whether these strains can survive LED treatments as long-term LED treatments also generate heat. Tryptic soy agar plates (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 0.6% yeast extract (YE; Fischer Bioreagents, Geel, Belgium) were used to restore the frozen bacterial stock cultures. This

was followed by two consecutive transfers in 5 mL sterile tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 0.6% YE and incubation at 37°C for 18–24 h.

Dried samples of *E. coli* AW1.7 and *S. enterica* ATCC13311 were prepared according to the procedure reported previously with some modifications (Chen, Temelli, & Gänzle, 2017; Uesugi, Danyluk, & Harris, 2006). After restoration of the strains from the stock cultures, 100 µL of culture was spread on TSAYE plates and incubated for 24 h at 37°C. The bacterial lawn was washed with 1.5 mL of 0.1% peptone water (Fischer Bioreagents, Geel, Belgium) and collected in 1.5 mL Eppendorf tubes followed by centrifugation and another washing step with 1 mL of 0.1% peptone water. The initial cell count of the suspension was 10^{12} CFU/mL. These cell suspensions were then transferred to sterile glass vials (2 mL in each vial) and air dried in a biosafety cabinet for 4–5 days which resulted in 1.56 log reduction in bacterial cell counts, followed by drying over silica gel for 24 h. The dried cells were equilibrated for 7 days to a a_w of 0.75 inside an air-tight equilibration chamber containing a supersaturated sodium chloride solution. The a_w was confirmed by using the water activity meter (4TE, patent number 5816704, Aqualab, Pullman, WA, USA). The final concentration of the cells after equilibration was 10^9–10^{10} CFU/g resulting in an overall log reduction of 1.82 CFU/mL.

To test the antibacterial efficacy of LED treatments on *Salmonella* inoculated pet foods, dry pet food pellets with initial a_w of ~0.54 were obtained (Orijen fit and trim, Champion Pet Foods, Edmonton, AB, Canada). Each pellet weighed ~0.15 g and was ovular in shape and concave on one side. The pet foods were composed of 44% crude protein, 15% fat, 10% moisture and minerals, as mentioned n the product label. Fifteen µL of a five-strain cocktail of *Salmonella enterica* (prepared in a similar manner as mentioned above for the preparation of cell suspension

for dry bacteria) was inoculated on the concave side of each pellet and air dried for 45–60 min in the biosafety cabinet to facilitate the attachment of cells on the surface of pet foods, which resulted in 0.95 log CFU/g reduction of bacteria, followed by equilibration to 0.75 a_w by storing the samples in the equilibration chamber for 3–4 days resulting in an overall cell count reduction by 1.52 log CFU/g. The final cell count of *Salmonella enterica* in pet foods after equilibration was 10^9 CFU/g.

3.2.2 Light Emitting Diode (LED) System

The LED system consisted of a bench-top controller (CF3000, Clearstone Technologies Inc., Hopkins, MN, USA) compatible with the JL3 series LED heads (111 × 70 × 128 mm^3; 6 high intensity LEDs), emitting the light with wavelengths of 365 and 395 nm with an irradiance of 0.05 and 0.23 W/cm^2 (when the distance was 4 cm from the sample) and 0.114 and 0.55 W/cm^2 (when the distance was 2 cm from the product), respectively, at 60% power level. The total doses (J/cm^2) of 365 and 395 nm LEDs during treatments were determined using a laser energy meter (7Z01580, Starbright, Ophir Photonics, A Newport Company, Har Hotzvim, JRS, Israel), connected to a photodiode irradiance and dose sensor (PD300RM-8W, Ophir Photonics, A Newport Corporation Brand, Har Hotzvim, JRS, Israel). The distance between the LED head and the sensor was maintained at 4 and 2 cm to determine the dose per unit surface area. The spectra of the 365 and 395 nm LEDs were confirmed using the StellarNet Inc. spectrometer (Black Comet C-25, Tampa, Florida, USA). The frequency of both the LEDs was 100 Hz and the dose values depended on the power level/duty cycle selected. For example, a power level of 60% corresponds to the 'ON' and 'OFF' times of 6 and 4 ms, respectively. Pet foods were treated at a 2 cm distance (the depth of the concave side of the pet foods were minimal) and the other samples were treated with a 4 cm distance from the LED head. These treatment heights were selected based on the preliminary experiments. The surface temperature of the samples during the treatments was determined using

a thermocouple connected to a digital thermometer (1507726, Fischer Scientific, Hampton, NH, USA) and by keeping the thermocouple on the surface of the sample during the LED treatments. Doses of each LED varied based on the power level output set on the controller system, ranging from 0 to 100%, which is related to the pulse width of the LED treatment (Tables 3.1 and 3.2).

3.2.3 Inactivation of *E. coli* and *Salmonella enterica* cells in Phosphate Buffered Saline

Overnight culture of *E. coli* AW1.7 and *S. enterica* ATCC13311 (1 mL) were mixed with 5 mL phosphate buffered saline, pH 7.4 (PBS; Gibco, Life Technologies, Waltham, MA, USA). One mL of this solution was spread in a petri dish with area of 28.3 cm^2 and treated with the LEDs. The treatments were conducted with 365 and 395 nm LEDs at power levels ranging from 20, 40 and 60% for *E. coli* and 60% for *Salmonella enterica* and treatment times between 10 and 60 min (365 nm) or 5 and 60 min (395 nm). The doses of the treatments ranged from 9.6 to 188.07 J/cm^2 and 22.6 to 834.43 J/cm^2 for 365 and 395 nm LED, respectively (Tables 3.1 and 3.2). The addition of PBS resulted in ~0.06 log reduction in the cell counts due to the dilution effect. The sample in PBS and without the LED treatment was taken as the control for comparison of the effect of power levels on the inactivation efficacy of the LED treatments. The treated and untreated samples were serially diluted in sterile TSBYE (Tryptic Soy Broth with 0.6% Yeast extract) and the viable cell counts were obtained by spread plating on TSAYE (Tryptic Soy agar with 0.6% Yeast Extract) plates and incubating the plates for 20–24 h at 37°C. The detection limit was 2 log CFU/mL.

Table 3.1: The total dose (J/cm^2) reached by 365 nm Light Emitting Diode (LED) for different treatment times at selected power levels of 20, 40, 60 and 100% used in this study at 4 cm height between the sample and the LED source.

Treatment Time (min)	Power Levels			
	20% *	**40%**	**60%**	**100%**
10	9.6	19.2	28.9	48.1
20	19.8	39.6	59.2	99.0
30	30.3	60.6	90.9	151.5
45	46.3	92.8	139.1	231.8
60	62.6	125.4	188.1	313.4

* A power level of 20, 40 and 60% was adjusted by treatment with 2, 4 and 6 ms, respectively, at a frequency of 100 Hz; 100% corresponds to exposure to continuous light.

Table 3.2: The total dose (J/cm^2) reached by 395 nm LED for different treatment times at selected power levels of 20, 40 and 60% used in this study at 4 cm height between the sample and the LED source.

Treatment Time (min)	Power Levels		
	20%	**40%**	**60%**
5	22.6	45.2	67.8
10	46.2	92.6	138.8
15	69.9	139.9	209.9
17	79.5	159.2	238.7
20	92.9	186.2	279.2
30	139.4	279.3	418.7
45	208.9	418.4	627.3
60	277.9	556.6	834.4

3.2.4 Inactivation of dry *E. coli* and *Salmonella enterica*

Dried and equilibrated cells of *E. coli* AW1.7 and *S. enterica* ATCC13311 (10 mg) with 0.75 a_w were spread on a small disk made up of plant-based and biodegradable PLA (polylactic acid)

filament with an area of 7.07 cm^2. The samples were kept at 4 cm from the LED head and treated at power levels of 60% with 365 and 395 nm LEDs. For *S. enterica* with 365 nm LED, an additional power level of 100% was used. The treatment times chosen were 10, 20, 30, 45 and 60 min for both the LEDs. Sample without LED treatment was taken as control. To determine the effect of varying initial inoculum levels (10^8, 10^7 and 10^6 CFU/cm^2) of *Salmonella enterica*, the bacteria dried on a coverslip equilibrated to 0.75 a_w was used for the treatment with 365 and 395 nm LED with a similar dose of 139 J/cm^2, corresponding to treatment times of 45 and 10 min, respectively, at 60% power level and at 4 cm from the LED head. The equilibrated sample without LED treatment was taken as control. Viable cell counts were determined by surface plating of serial dilutions in 0.1% peptone water as described in section 3.2.3.

3.2.5 Inactivation of *Salmonella* cells on low-a_w pet food pellets

Four pellets of the equilibrated dry pet foods, weighing 0.6 g were treated with 365 and 395 nm LED with a total dose of ~658 J/cm^2 in a benchtop humidity chamber (BTL-433, ESPEC North America Inc., Hudsonville, MI, USA). The treatment temperature and relative humidity maintained inside the humidity chamber were 25°C and 75%, respectively, and the distance between the sample and the LED head was kept at 2 cm. A cooling fan (DC Brushless fan, 50 × 50 × 15 mm, model BB5015H12, HK fans, Shenzen, China) with a voltage of 7 V supplied by a DC power supply (KD3005D, Digital Control DC linear power supply, Korad, Shenzen, China) was placed 2.7 cm away from the samples during the treatment with both the LEDs to reduce the increase in the surface temperature. Re-humidification of the pet foods to a a_w of 0.75 at the end of the LED treatment was carried out by incubating the treated samples inside the humidity chamber set to 25°C and 75% relative humidity for 30 min. To understand the effect of intermittent LED treatments, pellets were treated in two ways: (1) continuous treatment that involved the

treatment of samples continuously for a total dose of ~658 J/cm^2, corresponding to a treatment time of 20 min for 395 nm and 96 min for 365 nm, followed by re-humidification to 0.75 a_w and (2) non-continuous treatment, which involved the treatment of the pellets for a dose of ~329 J/cm^2, corresponding to 10 min for 395 nm and 48 min for 365 nm LED, followed by spraying of 15 µL of autoclaved water on each pellet and immediately followed by another LED treatment for a dose of ~329 J/cm^2 and a final re-humidification to 0.75 a_w. The equilibrated pet food pellets without any LED treatment were taken as the control. For enumeration, the treated and untreated pet foods were homogenized in a stomacher bag with 100 mL of autoclaved 0.1% peptone water by using the stomacher (Seward, London, UK). Then, 100 µL of the homogenized samples was used for serial dilution and enumeration was done, as described in section 3.2.3.

3.2.6 Weight loss, water content and water sorption isotherms of the treated samples

Weight loss due to evaporation of water during the LED treatments was determined by measuring the weights of samples before and after the treatments. To determine the initial water content of dry *S. enterica* equilibrated to 0.75 a_w, 1 g of sample was dried in a gravity convection oven (Heratherm OGS60, Thermo Scientific, Waltham, MA, USA) in triplicates at 105°C for 8 h and the dry weight of the bacteria was determined, and the water content (dry basis) was calculated. Similarly, the water content (dry basis) for pet foods equilibrated to 0.75 a_w was analyzed by drying 3.5 g of pet food pellets in triplicates in the convection oven at 105°C until constant final weight was achieved. The change in the a_w after the treatments were recorded using a water activity meter (4TE, patent number 5816704, Aqualab, Pullman, WA, USA).

The desorption isotherms for *S. enterica* were prepared by using a Vapor Sorption Analyzer (VSA, Meter group, Inc., Pullman, WA, USA) (Syamaladevi et al., 2016a; Syamaladevi, Tang, & Zhong, 2016b) at ambient (~20°C) and treatment temperatures corresponding to 365 and 395 nm

LED treatments (i.e., 32 and 55°C, respectively). These treatment temperatures were selected based on the temperature increase observed in the samples during the LED treatments. To develop the isotherms, dry *Salmonella* samples (approximately 0.6 g) were exposed to selected relative humidity values corresponding to the water activities 0.1 to 0.8 inside the VSA. The equilibrium water contents at the selected water activities were determined automatically by monitoring the mass of the samples at equilibrium conditions. To map the drying process of *Salmonella* samples, their desorption isotherms were used to determine their approximate final a_w, using the water content data. The isotherm modeling was done by using the Guggenheim-Anderson-de Boer (GAB) (Equation 3.1) as a best fit (Blahovec, & Yanniotis, 2007):

$$\frac{X}{X_m} = \frac{CKa_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \tag{3.1}$$

where X is the water content (dry basis), X_m is the water content of the monolayer (dry basis) and C, K and X_m are the temperature dependent parameters, which can be expressed as given in Equations 3.2, 3.3 and 3.4, respectively:

$$C = C_0 \exp\left(\frac{\Delta H_c}{RT}\right) \tag{3.2}$$

$$K = K_0 \exp\left(\frac{\Delta H_k}{RT}\right) \tag{3.3}$$

$$X_m = X_{mo} \exp\left(\frac{\Delta H_x}{RT}\right) \tag{3.4}$$

where R corresponds to gas constant and T is the temperature. In Equation 3.2, ΔH_c is the difference in enthalpy between monolayer and multilayer sorption and is generally positive. In Equation 3.3, ΔH_k is the difference between heat of condensation of water and the heat of sorption of multimolecular layer. In Equation 3.4, ΔH_X is the constant that expresses the temperature dependence of X_m (Quirijns et al., 2005; Syamaladevi et al., 2016a).

3.2.7 Statistical analysis

The experiments were done independently in triplicates ($n = 3$). The statistical analysis was done using the SAS version 5.1.26 (SAS Institute Inc., Cary, NC, USA) and the significant differences between means was performed by Tukey's LSD test ($p < 0.05$). The effect of both the LED treatments on the dry bacterial samples were assessed by three-way ANOVA. Comparison of the effect of different power levels for each LED treatments, the effect of LED treatments on pet foods and the effect of LED treatments on the water loss were analyzed by two-way ANOVA.

3.3 Results

3.3.1 Antibacterial efficacy of 365 and 395 nm LED treatments

To compare the effects of LED treatment on high- and low-a_w *E. coli* and *Salmonella*, the cells were suspended in PBS or equilibrated to a_w 0.75 and treated with 365 or 395 nm light pulses. Bacterial cells suspended in PBS were more sensitive than the dry bacteria. The 365 and 395 nm LED treatments produced a reduction of ~8 log CFU/g (below the detection limit) in *E. coli* and *Salmonella enterica* cells suspended in PBS compared to a maximum reduction of ~1 to 2 log CFU/g in dried bacterial cells (Figure 3.1). Treatments with the dose of 139 J/cm^2 were performed at both wavelengths, and thus, allowed for direct comparison. The 395 nm LED required higher energy input compared to 365 nm for achieving the same microbial inactivation level. For example, for the same dose of 139 J/cm^2, the 395 nm LED treatment produced 1.13 and 1.46 log reduction compared to 8.12 and 8.63 log reduction with 365 nm LED treatment in *E. coli* and *Salmonella enterica* suspended in PBS, respectively. Similarly, the 365 nm LED showed significantly ($p < 0.0001$) better antibacterial efficacy compared to the 395 nm LED for the same

dose in the dry *Salmonella enterica* and *E. coli* cells. Dried cells of *E. coli* were moderately more resistant to 395 nm LED treatments than *S. enterica*.

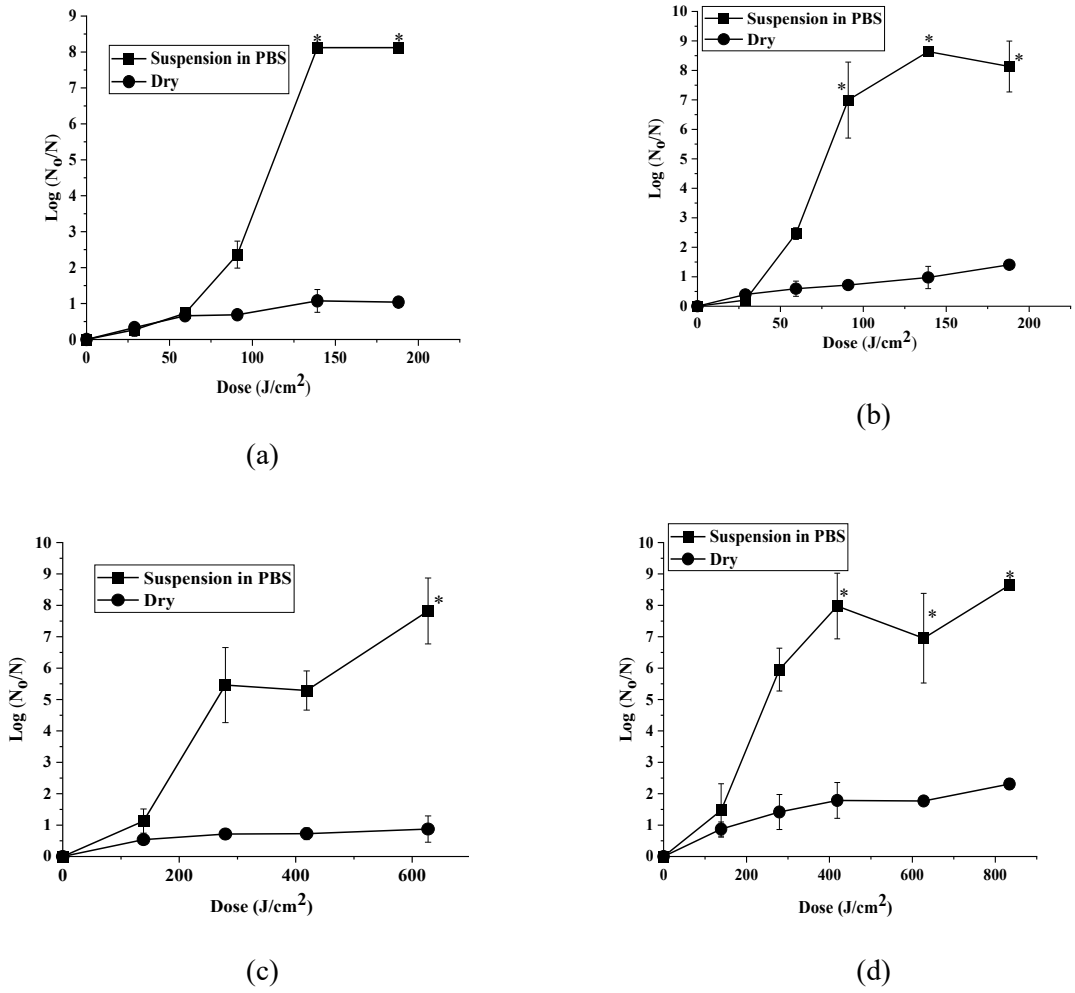
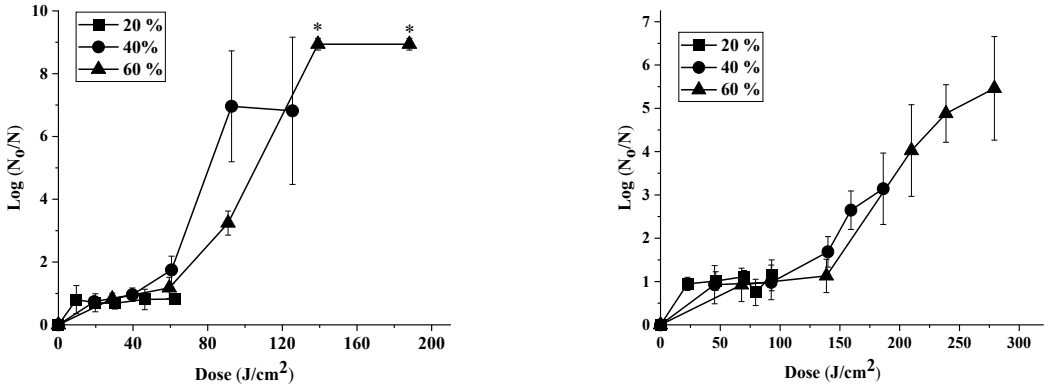


Figure 3.1: The efficacy of 365 nm LED against *E. coli* (a), *Salmonella enterica* (b) and the efficacy of 395 nm LED against *E. coli* (c), *Salmonella enterica* (d) suspended in PBS (phosphate buffered saline, pH 7.4) and dried bacteria for different dose treatments at 60% power level with 4 cm height between the sample and the LED head. The doses used for 365 nm were 28.9 (10 min), 59.2 (20 min), 90.9 (30 min), 139.1 (45 min) and 188.1 (60 min) J/cm^2. The doses used for 395 nm were 138.8 (10 min), 279.2 (20 min), 418.7 (30 min), 627.3 (45 min) and 834.4 (60 min) J/cm^2. Here, N_0 represents the CFU/mL in control and N represents the CFU/mL in the treated samples of bacteria suspended in PBS. For dry bacteria, N_0 represents the CFU/g in control and N represents the CFU/g in the treated samples. Error bars indicate the standard deviation (n = 3). An asterisk (*) indicates the reduction of cell counts below the detection limit.

The 365 nm LED treatment produced significantly ($p < 0.0001$) higher antibacterial effect in *E. coli* cells suspended in PBS compared to 395 nm for the same dose. For instance, treatments with ~92 J/cm^2 reduced cell counts by about 7 log CFU/mL after treatments with 365 nm but only by about 1 log CFU/mL after treatment with 395 nm. After treatment of dry *Salmonella enterica* cells with 365 or 395 nm, the reduction of cell counts remained less than 1 log CFU/g even at an energy input of 139 and 138.8 J/cm^2, respectively (Figure 3.1). Overall, the dose had a major effect on the antibacterial effect of both the LEDs than the pulse width (power levels) of the light produced.

3.3.2 Effect of power levels on the antibacterial efficacy of the LEDs

Increasing the power level of the LED system increased the dose and pulse of the light (Tables 3.1 and 3.2), and hence increased the antibacterial efficacy at higher power levels. To understand the effect of power levels, *E. coli* suspended in PBS were treated with 20, 40 or 60% power levels with 365 or 395 nm LEDs. For the low power level of 20%, different doses did not produce any effect on the inactivation of *E. coli* suspended in PBS for both 365 and 395 nm LEDs. However, increasing the dose above 60 J/cm^2 at 40% and above 139 J/cm^2 at 60% power levels resulted in the significant ($p<0.0001$) effect of dose on the antibacterial efficacy of both 365 and 395 nm LED treatments (Figure 3.2). For the similar dose of 91 J/cm^2, treatment with 365 nm LED produced a reduction of 6.96 log CFU/g at 40% power level (45 min) compared to 3.24 log CFU/g at 60% power level (30 min), indicating that there was no linear effect of power level and the reduction was not influenced by power setting but by the overall dose or energy input.



(a) (b)

Figure 3.2: Effect of power level on the inactivation efficiency of 365 nm (a) and 395 nm (b) LED against *E. coli* AW1.7 suspended in PBS, subjected to different doses of LED treatment. The doses used for 365 nm LED treatments corresponding to treatment times of 10, 20, 30, 45 and 60 min were 9.6, 19.8, 30.3, 46.3 and 62.6 J/cm^2 for 20% power level, 19.2, 39.6, 60.6, 92.8 and 125.4 J/cm^2 for 40% power level and 28.9, 59.2, 90.9, 139.1 and 188.1 J/cm^2 for 60% power level, respectively. The doses used for 395 nm LED treatments corresponding to treatment times of 5, 10, 15, 17 and 20 min were 22.6, 46.2, 69.9, 79.5 and 92.98 J/cm^2 for 20% power level, 45.2, 92.6, 139.9, 159.2 and 186.2 J/cm^2 for 40% power level and 67.8, 138.8, 209.9, 238.7, 279.2 J/cm^2 for 60% power level, respectively. Here, N_0 represents the CFU/mL in the control and the N represents the CFU/mL in the treated samples. Error bars indicate the standard deviation (n = 3). An asterisk (*) indicates the reduction of cell counts below the detection limit.

3.3.3 Antibacterial efficacy of LED in low-a_w pet foods

The effectiveness of both 365 and 395 nm LEDs against a *Salmonella enterica* cocktail of five strains inoculated on dry pet foods equilibrated to 0.75 a_w treated at the same total dose of ~658 J/cm^2 in an enclosed humidity chamber set at 25°C and 75% relative humidity were determined. The 395 nm LED treatment produced significantly (p = 0.007) better inactivation of *Salmonella enterica* on pet foods with the non-continuous treatment (method 2) compared to the continuous treatment (method 1), (Figure 3.3). Overall, 395 nm LED showed better antimicrobial effectiveness on pet foods compared to 365 nm LED, contrary to the trend observed with dry

bacteria, indicating that the LED's antibacterial efficacy was dependent on the product parameters and the strains used for the treatment.

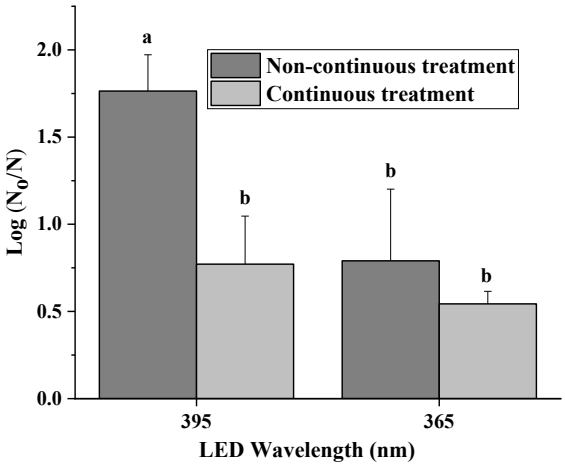


Figure 3.3: The efficacy of 365 and 395 nm LED against 5 strain cocktail of *Salmonella enterica* spp. in low-a_w pet food pellets equilibrated to 0.75 a_w when the height between the sample and the LED head was maintained at 2 cm. The non-continuous treatment involved the treatment of the pellets at a dose of ~329 J/cm^2, corresponding to 10 min for 395 nm and 48 min for 365 nm LED, followed by spraying of 15 µL of autoclaved water on each pellet and immediately followed by another LED treatment for a dose of ~329 J/cm^2 and a final re-humidification to 0.75 a_w. The continuous treatment involved the treatment of samples continuously for a total dose of ~658 J/cm^2, corresponding to a treatment time of 20 min for 395 nm and 96 min for 365 nm, followed by re-humidification to 0.75 a_w. Here, N_0 represents the CFU/g in control and the N represents the CFU/g in the treated pet foods. Bars with same letter are not significantly different ($p < 0.05$). Error bars indicate the standard deviation ($n = 3$).

3.3.4 Change in temperature during LED treatment

The surface temperature changes were monitored during LED treatments of bacterial samples at high- and low-a_w conditions. The initial temperature of the untreated high- and low-a_w bacterial samples was 21–23°C. The temperature of *E. coli* and *Salmonella enterica* cultures suspended in PBS during 365 nm LED increased to 25–27°C after treatment with 188.07 J/cm^2 dose; treatments with 395 nm at 418.7 J/cm^2 dose increased the temperature to 34–36°C after 30 min. In dry

bacterial samples, a higher surface temperature increase compared to the bacterial suspension in PBS was observed. For example, 365 nm LED treatment with 188.07 J/cm^2 dose (60 min) increased the temperature of the bacteria to 33°C while the 395 nm LED treatments increased the temperature to maximum of 53–55°C with 834.43 J/cm^2 dose (60 min).

Similarly, in pet foods, a dose of ~329 J/cm^2 with 365 nm LED treatments increased the temperature to 34–35°C and a continuous treatment with ~658 J/cm^2 increased the temperature to 32–35°C from 25°C, at 60% power level. However, the 395 nm LED treatment increased the temperature from 25°C to 57–62°C when the dose was ~329 J/cm^2 (10 min) and to 66–67°C with continuous treatment with ~658 J/cm^2 (20 min) dose. Overall, a greater surface temperature increase was observed with 395 nm LED treatments compared to 365 nm LED treatments at the same treatment times and power level.

3.3.5 Change in water content and water activity of bacterial samples and pet foods during LED treatments

The LED treatments resulted in weight loss, which is associated with water loss due to drying of the samples during both 365 and 395 nm LED treatments. The water content (dry basis) of the dry *Salmonella enterica* cells equilibrated to 0.75 a_w was 0.168 kg water/kg dry solids. The 395 nm LED treatments reduced the water content of *Salmonella enterica* more when compared to 365 nm LED treatments. The maximum reduction in water contents after 365 and 395 nm LED treatments were 15.5 and 50.6%, respectively (Table 3.3).

During the 60 min (834.4 J/cm^2) treatment with 395 nm LED, the suspension of bacteria in PBS was dried up entirely, due to the high light energy dose and the temperature increase during the LED treatments, resulting in the evaporation of water. To determine the extent of drying

Table 3.3: The water content of the dry *Salmonella enterica* Typhimurium ATCC13311 after the 395 and 365 nm LED treatments at 60% power level and the water activity from desorption isotherm.

Treatment Time (min)	Water Content (kg water/kg dry solids) with 395 nm LED	Water activity values determined from desorption isotherm with 395 nm LED	Water content (kg water/kg dry solids) with 365 nm LED	Water activity values determined from desorption isotherm with 365 nm LED
0	0.168 ± 0.0124 ^{a}	0.74 ± 0.0133 ^{a}	0.168 ± 0.0124 ^{a}	0.74 ± 0.0133 ^{a}
10	0.137 ± 0.0171 ^{ab}	0.698 ± 0.2115 ^{a}	0.154 ± 0.0069 ^{ab}	0.675 ± 0.0204 ^{bc}
20	0.113 ± 0.0243 ^{bc}	0.594 ± 0.1058 ^{ab}	0.155 ± 0.0093 ^{ab}	0.678 ± 0.0254 ^{bc}
30	0.131 ± 0.0162 ^{abc}	0.677 ± 0.0574 ^{a}	0.158 ± 0.0042 ^{ab}	0.686 ±0.0113 ^{b}
45	0.083 ± 0.0267 ^{c}	0.374 ± 0.0566 ^{b}	0.142 ± 0.0053 ^{b}	0.637 ± 0.0184 ^{c}
60	0.101 ± 0.0528 ^{bc}	0.545 ± 0.2377 ^{ab}	0.154 ± 0.0146 ^{ab}	0.675 ±0.0431b ^{c}

Values are given as means ± standard deviation (n = 3). Values in each column with the same letter are not significantly different ($p < 0.05$).

in dry bacteria, the desorption isotherms of *Salmonella enterica* cells and their water contents obtained from weight loss data after LED treatments were used to determine the final a_w values of cells after LED treatments (Figure 3.4). The isotherm data was fitted with the GAB equation (Equation 3.1) and the a_w values after the LED treatments were determined using this equation (Table 3.3). To use the desorption isotherm in a_w calculations, the existence of a pseudo water vapor equilibrium at the interface of the bacterial surfaces and the surrounding air during long LED treatments (especially for long treatments, i.e., after 60 min) was assumed. Even though drying is a dynamic process, this important assumption was made to use the desorption isotherms of samples at ambient and LED treatment temperatures to determine the final a_w values of the bacterial samples after LED treatments. The determined a_w values of bacterial samples using the desorption isotherms showed that, after 365 and 395 nm LED treatments, the a_w values were

reduced significantly (p = 0.0054) (Table 3.3). For the same dose of 139 J/cm^2 with 365 and 395 nm LEDs corresponding to 45 and 10 min, the a_w was reduced to 0.64 and 0.7, respectively (Table 3.3). In Figure 3.4, after 60 min (834.43 J/cm^2) treatments with the 395 nm LED with 60% power level, the water content and a_w values were reduced from 0.168 kg water/kg dry solids and 0.75 (Box A) to 0.1 kg water/kg dry solids and 0.54 (Box B), respectively (see black arrow).

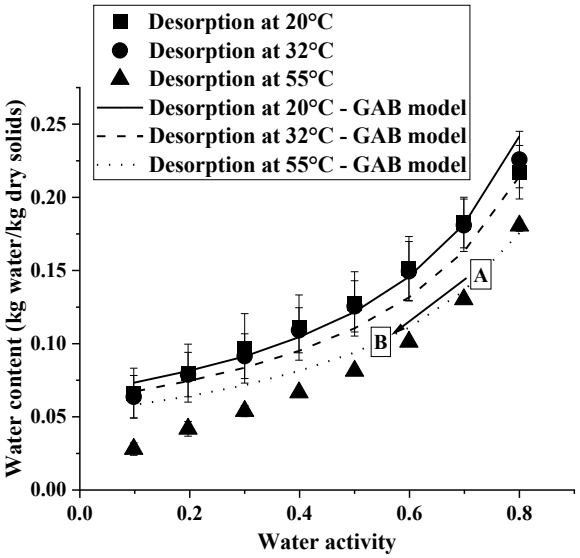


Figure 3.4: The desorption isotherm of dry *Salmonella enterica* Typhimurium ATCC13311 at 20, 32 and 55°C. The scatter plots correspond to the desorption isotherm developed by the Vapor Sorption Analyzer (VSA). The curves represent the desorption isotherms as predicted using the GAB (Guggenheim-Anderson-de Boer) model (Equation 3.1). Error bars indicate the standard deviation (n = 2).

The initial water content (dry basis) of pet food pellets was obtained as 0.131 kg water/kg dry solids. The changes in their water content and a_w during continuous and non-continuous LED treatments were determined (Figure 3.5). Here, the non-continuous treatment involved sequential LED treatments and re-humidification using water spray. The maximum weight loss observed in the LED treated pet foods was 6–7% in the case of 395 nm treatment and 3.3–4.5% with 365 nm.

Consequently, the water content of the pet foods was significantly (p = 0.001) reduced by 3 and 5.4%,

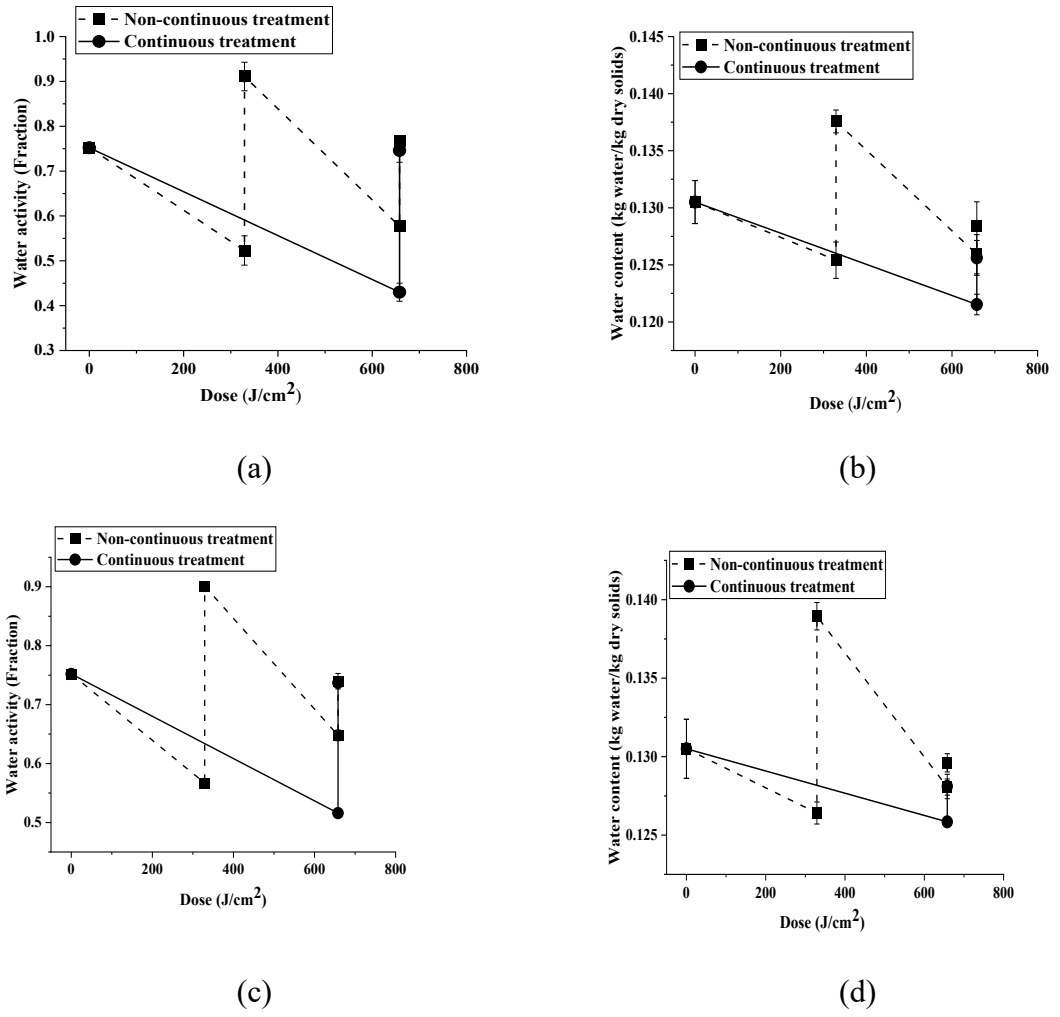


Figure 3.5: The mapping of a_w (a) and water content (dry basis) (b) of low-a_w pet foods equilibrated to 0.75 a_w in both continuous and non-continuous treatments with 395 and a_w (c) and water content (dry basis) (d) with 365 nm LED. The non-continuous treatment involved the treatment of the pellets for a dose of ~329 J/cm^2, corresponding to 10 min for 395 nm and 48 min for 365 nm LED, followed by spraying of 15 µL of autoclaved water on each pellet and immediately followed by another LED treatment for a dose of ~329 J/cm^2 and a final re-humidification to 0.75 a_w in the humidity chamber. The continuous treatment involved the treatment of samples continuously for a total dose of ~658 J/cm^2 corresponding to a treatment time of 20 min for 395 nm and 96 min for 365 nm, followed by re-humidification to 0.75 a_w in the humidity chamber. Error bars indicate the standard deviation (n = 3).

respectively, with continuous and non-continuous treatments after 395 nm LED treatments, while the 365 nm LED treatments reduced the water content by 2 and 3%, respectively (Figure 3.5). The reduction in the a_w of the pet foods was higher in the case of continuous treatment with 395 nm LED treatment compared to 365 nm LED treatment. During the non-continuous treatment, the a_w and water contents of pet foods reduced during the first treatment with ~329 J/cm^2 dose of 365 and 395 nm LEDs, then increased with water spray and again decreased during subsequent LED treatments with a dose of ~329 J/cm^2. However, the final a_w observed after the second LED treatment was higher than the final a_w observed after the first LED treatment (Figure 3.5).

3.4 Discussion

The LED system is cost-effective and its implementation to the existing processing lines is relatively simple, owing to their small size and convenience in using them (Ghate et al., 2015). In this study, it was observed that the treatments with 365 and 395 nm light pulses emitted from the LEDs reduced bacterial cell counts in their suspension in PBS, while the LED treatments were much less effective in inactivating dry cells. This observation confirms the results of another study, where the treatment of *E. coli* suspension with 365 nm LED for 75 min (315 J/cm^2 dose) reduced cell counts by ~5.7 log CFU/mL (Hamamoto et al., 2007). The energy input and the LED wavelength were the most important factors influencing treatment lethality. The distance of the sample from LED source, dose, treatment time and sample type additionally influence the inactivation efficiency.

Gram-negative bacteria such as *E. coli* and *Salmonella enterica* survive and remain infectious in low-a_w environments for extended periods of time (Shachar, & Yaron, 2006). The antimicrobial efficacy of 405 nm LED treatment has been extensively reported in relatively high-

a_w food products, such as fresh cut papaya and ready-to-eat salmon (Kim et al., 2016b; Kim, Bang, & Yuk, 2017a; Li, et al., 2018a). Kim et al. (2017c) studied the inactivation of *E. coli* and *Salmonella enterica* on fresh cut mango by 405 nm LED treatment, where 36 h treatment resulted in cell count reductions of 1 log CFU/cm^2 with a maximum dose of 3.6 kJ/cm^2. *E. coli* K12 was susceptible to 395 nm LED in a dose-dependent manner (Birmpa et al., 2014). However, only a few studies reported the use of LED treatment in low-a_w foods. Better sensitivity of the suspension of bacteria in PBS than dry bacteria observed in this study could be attributed to the low penetration capacity of the light emitted by the LED or shadowing of cells in the dry powdered bacteria and the low a_w of the dry bacterial cells, which can influence the resistance of the bacteria towards the LED treatments. Decreasing the inoculum level from 10^8 to 10^6 CFU/cm^2 did not increase in the inactivation of *Salmonella enterica* (Figure A1, Appendix 1), indicating that a shadowing effect is not critical, or that the non-uniform layer of the bacteria on the boundary of the inoculum had a higher cell density (Mampallil et al., 2018).

The maximum log reduction obtained in dry *E. coli* and *Salmonella enterica* was 1.36 and 2.3 log CFU/g, respectively. This low reduction is justifiable in this study as the strains used were already highly resistant and were ideal to study the effect of LED treatment in dry conditions. These strains were subjected to further stress as the bacterial cells were dried before their equilibration to 0.75 a_w. Under stress, accumulation of trehalose, heat shock proteins, etc. might occur as part of the adaptation mechanism of the bacterial cells, which might result in the increased resistance of these cells to further stress conditions or antimicrobial treatments (Laroche et al., 2005).

The 365 and 395 nm LEDs were effective in the inactivation of *Salmonella enterica* cocktail in pet food pellets in this study. However, higher dose treatments were required, probably

due to the collective resistance of five strains of *Salmonella enterica* compared to a single strain tested in the study with the dry bacteria. *Salmonella enterica* FUA1946, FUA1934, FUA1955, especially, were determined to be highly resistant to thermal treatment and high pressure CO_2 (Mercer et al., 2017; Schultze et al., 2020). Additionally, surface characteristics such as roughness and the composition of pet foods might have interfered with the LED treatment and may have contributed to the increased resistance of *Salmonella enterica* on the pellets. In food systems, higher energy may be generally required to achieve the same level of reduction in comparison to pure microbial cells (Kim & Kang, 2018a; Oliver, Jayarao, & Almeida, 2005). Similarly, the 405 nm LED treatment on *Salmonella enterica* cocktail inoculated almonds (a low-a_w food) produced 0.49 to 0.64 log reduction in almonds (Lacombe et al., 2016). In this study, the non-continuous treatment of pet foods led to better inactivation with 395 nm LED (Figure 3.3). Addition of water increased the susceptibility of the bacterial cells to the LED treatment and probably aided in more production of reactive oxygen species (ROS) than continuous treatment. Additionally, changes in the a_w of the pet foods during non-continuous treatment might have produced an osmotic stress on the cells. The dehydrated cells during the LED treatment might undergo increased membrane permeabilization and cell shrinkage followed by formation of membrane vesicles, previously observed in *E. coli* (Mille et al., 2002). Moreover, rehydration of the cells might not help the cells to recover from this permeabilization and changes in membrane functionality and integrity. The sudden dehydration and rehydration might also affect the cell viability (Mille et al., 2002; Simonin, Beney, & Gervais, 2007), causing further inactivation in the bacterial cells. Similarly, other decontamination methods that involve subjecting raw almonds to high pressures in water followed by drying at high temperatures resulted in the reduction of *Salmonella enterica* to undetectable levels (Willford, Mendonca, & Goodridge, 2008). Overall, the 365 nm LED treatments were better

than 395 nm LED for dry bacteria, but the trend was opposite for their inactivation in the pet foods, and this observation deserves further research.

The 365 nm LED treatments showed a smaller increase in the temperature of liquid suspension or dry bacteria compared to 395 nm LED treatments. Similarly, an increase in the surface temperature was observed with 405 nm LED treatment of fresh-cut papaya (Kim, Bang & Yuk, 2017a). The greater temperature increases in the case of 395 nm compared to 365 nm LED treatments could be due to its higher intensity at the same power levels and treatment times, which resulted in an increased drying rate in bacterial samples. The temperature increase during LED treatment might have contributed to the inactivation observed in addition to the effect of light emitted. Drying with reduction in weight and water content of the dry *Salmonella enterica* samples after both the LED treatments were observed. The higher intensity of 395 nm increased the drying rate and water loss. The inactivation efficacy of the high intensity light pulses emitted from the LEDs could be due to the drying observed as well.

Here, the LED treatments were conducted in open laboratory conditions, which resulted in the drying of samples and loss of light energy to the surroundings. Therefore, preventing the loss of LED light during the treatments might improve the antibacterial effect of the LED. Moreover, the limited penetration of the light in the solid matrix as well as the intrinsic resistance of dry cells resulted in a relatively low reduction of cell counts. The high intensity light pulses emitted from the LEDs probably would work better when combined with an intermediate drying or rehydration step. Therefore, the antibacterial effectiveness of LED could be further improved by the addition of water in dry food as an intermediate step indicating the probability of improving the decontamination efficacy of LED when used with some other technology while maintaining the

food quality. Development of such antibacterial technology is important to deal with the highly resistant microorganisms in food that can cause illnesses even when present in small numbers.

In conclusion, the 365 and 395 nm LED treatments reduced *E. coli* and *Salmonella enterica* populations significantly at high- and low-a_w conditions. LED treatments showed antimicrobial effect in pet foods at 0.75 a_w, based on the treatment conditions and the dose used. The 395 nm LED had a higher irradiance compared to 365 nm LED treatments, which resulted in faster reduction in bacterial population for the same treatment times. The antibacterial efficacy of both the LEDs varied significantly. There was a variation in the susceptibility of the bacteria towards the LED treatments, based on the strain and the condition (liquid suspension or dry powder) of the bacteria. Water loss and reduction in the a_w were observed during the LED treatments, indicating the drying of the treated bacterial samples, which was confirmed from the desorption isotherm of bacterial cells. This study showed the potential application of the 365 and 395 nm LEDs as an antibacterial technology to reduce foodborne pathogen population in high- and low-a_w conditions.

Chapter 4: Antimicrobial activity and drying potential of high intensity blue light pulses (455 nm) emitted from LEDs

4.1 Introduction

Salmonella has consistently caused food-borne outbreaks in low water activity (a_w) foods, including breakfast cereals, ready-to-eat dried meat products, spices, shell eggs, and pet foods (Bedinghaus, & Ockerman, 1995; Beuchat et al., 2011; Finn et al., 2013). *Salmonella* survives under low a_w conditions for over weeks or months and the infectious dose is low for sensitive individuals (Gurtler et al., 2014). For example, *Salmonella* survived in dry dog foods under low a_w conditions for up to 19 months (Lambertini et al., 2016). At low a_w conditions, *Salmonella* resists decontamination methods that are lethal to *Salmonella* at high a_w (Finn et al., 2013). Current methods for decontamination of dry foods include heating to more than 120°C or the use of oxidizing chemicals, which negatively impact food quality (Bari et al., 2009; Hasani et al., 2020); therefore, alternative methods, which reduce the contamination with *Salmonella* without negative impact on food quality are required. Novel non-thermal technologies that have shown efficacy in reducing cell counts of *Salmonella* on contaminated foods include irradiation, cold plasma (Niemira, 2012), high-pressure gas-phase CO_2 (Schultze et al., 2020) and treatment with light pulses emitted from light emitting diodes (LEDs) (Du et al., 2020; Chapter 3). LEDs emitting lights of wavelengths 266, 270, 275, 365 and 395 nm have been shown to have inactivation efficacy against food products like, sliced camembert cheese, pet foods, wheat flour, etc. (Du et al., 2020; Kim et al., 2016a; Chapter 3)

LEDs can be easily incorporated into the existing process lines, attributed to their multiple advantages, including their compact size, absence of warm-up time, and the emission of

monochromatic light (D'Souza et al., 2015; Kebbi et al., 2020; Li et al., 2017; Chapter 2). The LED consists of semiconductor material, which is doped with impurities that is responsible for the emission of light of specific color and wavelength in the presence of electric current (Held, 2009; Chapter 2). LEDs emitting light in the blue spectrum (400-470 nm) has been found to exhibit antimicrobial effect in medical applications by means of photodynamic inactivation (Wang et al., 2017). The light absorbing molecules like porphyrin compounds act as endogenous photosensitizers in bacteria and absorb light in the range of 400-500 nm (Plavskii et al., 2018). These compounds produce reactive oxygen species (ROS) on collision with oxygen molecules, which triggers certain cytotoxic responses like cell membrane damage, DNA oxidation, DNA degradation, eventually leading to cell death (Luksiene, & Zukauskas, 2009).

Previous studies focused on understanding the antimicrobial efficacy of blue light in liquids like fruit juices and high-moisture foods including fresh-cut fruits and seafoods (Ghate et al., 2017; Ghate et al., 2016; Josewin et al., 2018; Bhavya et al., 2021). For example, the 460 nm LED treatment with the irradiance of 92 mW/cm^2 (7950 J/cm^2 dose) at 25°C resulted in the maximum reduction of 1.21 log CFU/g in fresh-cut pineapples (Ghate et al., 2017). Also, the potential bactericidal effect of 465 nm LED has been reported in fancy carps (Roh et al., 2018). In dry foods, 455 nm light showed a limited reduction of *Salmonella* in wheat flour with high dose (Subedi et al., 2020). Therefore, it is necessary to combine the 455 nm LED with drying and/or with other wavelength to enhance its bactericidal effect. Moreover, the drying and quality changes are specific for specific foods. Therefore, it is necessary to understand the effect of the 455 nm LED in other dry foods.

One product of concern are pet food pellets, which can cause human illness after contamination of the product post-extrusion and through handling of the contaminated pet food

pellets by humans. Understanding the antimicrobial and drying potential of high intensity light pulses, emitted from LEDs can help in the development of an alternate surface decontamination technology with the benefit of drying to produce low a_w foods. The main objective of this study was to evaluate the drying and antibacterial efficacy of light pulses of 455 nm wavelength emitted from the LEDs against *Salmonella* under low a_w conditions. The impact of varying the initial inoculum concentration in low a_w pet foods and the effect of post-treatment storage of pet foods on the inactivation effect of the 455 nm light pulses were also studied. The lipid oxidation of low a_w pet foods due to the treatment with the 455 nm light pulses was also quantified. The potential effects of pre-treatment of low a_w pet foods with the light pulses emitted from the 275 nm LEDs on the inactivation efficacy of the 455 nm LED light pulses were analyzed.

4.2 Materials and Methods

4.2.1 The 455 nm light emitting diode (LED) system

The LED system used in this study is the same as the one used in Chapter 3 (section 3.2.2). Here, the LED head of JL3 series (111×70×128 mm^3) emitting light of the wavelengths 275 and 455 nm at a frequency of 100 Hz. The irradiance and the total dose 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-8W, Ophir Photonics, A Newport Corporation Brand, Har Hotzvim, JRS, Israel) by maintaining a distance of 4 and 2 cm from the LED head. Similarly, the irradiance of the 275 nm LED was measured using a radiometer (ILT2400, International Light Technologies, Peabody, MA, USA) attached to an intensity sensor at 2 cm from the LED head. The distance of the 5-strain cocktail inoculated pet food pellets from the LED head was maintained at 2 cm based on the preliminary research. Remaining treatments

were conducted at 4 cm gap from the LED head at a power level of 60%. The power level/duty cycle controlled the light pulses (Chapter 3). The irradiance of the blue light pulses (455 nm) from LEDs was measured as 0.53 and 0.291 W/cm^2 at 60% power level for the distance of 2 and 4 cm from the LED head, respectively, and the irradiance was 0.391 W/cm^2 at 80% power level for the distance of 4 cm from the LED head. The irradiance of 275 nm LED at 60% power level and distance of 2 cm from the LED head was 0.0126 W/cm^2. The high treatment doses of the 455 nm LED ranged from 174.6-1047.6 J/cm^2 for the sample distance of 4 cm from the LED head and from 358.8-2152.8 J/cm^2 for 2 cm distance from the LED head.

4.2.2 LED treatment of *S.* Typhimurium at low a_w conditions

4.2.2.1 Preparation and LED treatment of dried *S.* Typhimurium

Dried *Salmonella enterica* Typhimurium ATCC13311 was prepared from cultures on agar plates as described in Chapter 3 (section 3.2.1) (Chen et al., 2017; Uesugi et al., 2006). Frozen culture of *S.* Typhimurium was retrieved, and the inoculum was prepared as mentioned in Chapter 3 (section 3.2.1). Viable cells in the cell suspension were enumerated in tryptic soy agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates supplemented with 0.6% yeast extract (YE; Fischer Bioreagents, Geel, Belgium) and cell counts of ~10^{12} CFU/mL were obtained. Two milliliters of suspension were transferred into glass vials for air drying in a biosafety cabinet for 4-5 days followed by drying on silica gel for 24 h. The dried bacteria were incubated in an air-tight equilibration chamber containing supersaturated sodium chloride solution for 7 days to obtain 0.75 a_w. The final cell count of the equilibrated *Salmonella* was 10^9-10^{10} CFU/mL (Chapter 3, section 3.2.1).

To evaluate the inactivation efficacy of 455 nm light pulses against dry *S.* Typhimurium equilibrated to 0.75 a_w, 10 mg of dry bacteria were placed on a small disc (7.07 cm^2) made up of

polylactic acid filament. The sample was placed at 4 cm from the LED head and treated at 60 or 80% power level corresponding to different irradiances. The treatment times were 10, 20, 30, 45 and 60 min for 60% power level and 10, 15, 20, 30 and 40 min for 80% power level. The effect of the environment condition on the LED treatments was evaluated by conducting the same experiments inside a humidity chamber (BTL-433, ESPEC North America Inc., Hudsonville, MI, USA), where the temperature and relative humidity (RH) were controlled. The conditions used for this study included 20°C and 75% RH, and 60°C and 75% RH and the treatment time was 30 min (523.8 J/cm^2) at 60% power level. Untreated dry bacteria served as control. Viable bacterial cells were enumerated by serial 10-fold dilutions in 0.1% peptone water followed by spread plating in the TSAYE plates and incubation at 37°C for 24 h.

4.2.2.2 Preparation and LED treatment of *S.* Typhimurium on pet food

To study the inactivation effect of LED on *Salmonella* inoculated pet food pellets having an initial a_w of 0.54, the inoculum was prepared as described in Chapter 3 (section 3.2.1). The pet food pellets were procured from a local store, which were ovular in shape and consisted of 44% crude protein, 15% fat, 10% moisture and minerals (as mentioned on the product label). Fifteen microliters of cells of *S.* Typhimurium with a cell count of 10^{11} CFU/mL were prepared as described section 3.2.1, inoculated on the surface of the concave side of each pellet and air-dried in the biosafety cabinet for 45 min. The samples were then incubated in an air-tight equilibration chamber consisting of supersaturated sodium chloride solution for 3-4 days to obtain a final a_w of 0.75. The final cell counts obtained were 10^8-10^9 CFU/g.

To study the effect of 455 nm LED on *S.* Typhimurium inoculated on the pet food pellets, 0.6 g of low a_w pet foods (4 pellets) were treated at 60% power level and 4 cm distance from the LED head. The treatment times chosen were 10, 20, 30, 45 and 60 min and the experiments were

conducted in the open system (room conditions; ~23°C, ~48% RH). Viable cell counts were evaluated by homogenizing the pet food pellets in 100 mL of 0.1% peptone water in a stomacher bag using a stomacher (Seward, London, UK). This was followed by serially diluting 100 µL of the homogenized solution in 0.1% peptone water followed by spread plating on TSAYE plates and incubating at 37°C for 24 h. Untreated pet foods served as control.

4.2.2.3 Effect of initial microbial concentration in low a_w pet food

To test the effect of varying the initial microbial concentration of *S.* enterica Typhimurium ATCC13311, 500 µL of the initial bacterial suspension with 10^{11} CFU/mL were added to 4.5 mL of 0.1% peptone water to obtain a cell count of 10^{10} CFU/mL, which was further diluted in a similar manner to obtain the cell counts of 10^9 CFU/mL. These three concentrations were inoculated on pet foods and equilibrated to 0.75 a_w. The final cell counts obtained after the equilibration were ~10^8, ~10^7 and ~10^6 CFU/g, respectively. Four pellets (~0.6 g) were treated at 4 cm distance from the LED head at 60% power level in open conditions and treatment times of 10, 20, 30 and 60 min were selected. Untreated pet food pellets of each concentration served as control. Microbial enumeration was conducted by soaking the treated and untreated pet food pellets in 6 mL of 0.1% peptone water in a 50 mL falcon tube for 30 min followed by vortexing for 1 min. Hundred µL of this homogenized culture was used for the serial dilutions and viable cell counts were obtained as mentioned in section 4.2.2.2.

4.2.2.4 Inactivation of *S.* Typhimurium on pet food during storage

The initial microbial concentration of 10^8 and 10^6 CFU/g of *S.* Typhimurium ATCC13311 were used for inoculating the low a_w pet food pellets for the storage study. Four pellets (0.6 g) of inoculated low a_w pet foods were treated with 455 nm LED at 60% power level and 4 cm distance from the LED head in open conditions. The treatment time chosen was 30 min (~ 523.8 J/cm^2) and

the treated and untreated pet food pellets were stored in a dark air-tight chamber at room conditions for a maximum of 21 days. The *S.* Typhimurium cells were enumerated at the 0^{th}, 7^{th}, 14^{th} and 21^{st} days of storage period and untreated low a_w pet food pellets stored at the 0^{th}, 7^{th}, 14^{th} and 21^{st} days for each microbial concentration were taken as control, respectively. Viable cell counts were done as mentioned in section 4.2.2.3. The cell counts for controls were 8.32±0.35, 8.28±0.19, 8.42±0.16 and 7.67±0.13 log CFU/g after 0, 7, 14, and 21 days, respectively, for the initial microbial concentration of 10^8 CFU/g. The cell counts of controls were 6.03±0.18, 5.52±0.21, 5.48±0.16 and 5.28±0.03 log CFU/g after 0, 7, 14, and 21 days, respectively, for initial microbial concentration of 10^6 CFU/g.

4.2.3 Transmission electron microscopy (TEM) of *S.* Typhimurium

Transmission electron microscopy analysis was used to understand the effect of 455 nm wavelength light pulses emitted from LEDs on the powdered *Salmonella* cells treated at different environmental conditions. Ten milligrams of dry *S.* Typhimurium cells equilibrated to 0.75 a_w were treated with 455 nm LEDs at 4 cm distance with 523.8 J/cm^2 dose at 60% power level in open conditions and inside the humidity chamber at 20°C and 75% RH, and 60°C and 75% RH. Also, powdered *S.* Typhimurium treated with 455 nm LED for 60 min, corresponding to the treatment dose of 1047.6 J/cm^2 (the highest treatment time and dose used for the treatment of dry bacteria in this study) were also used in the analysis. The untreated dry bacteria were used as a control.

Treated and untreated bacteria were fixed overnight in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After draining off the fixative followed by washing multiple times with the buffer, cells were post-fixed in 1% osmium tetroxide for 1.5 h. The cells were again washed with buffer and dehydrated followed by mixing and embedding in the SPURR resin. The resins were stained with uranyl acetate and lead citrate stains after curing, followed by

analysis using Philips-FEI Morgagni 268 transmission electron microscope (FEI Co., Hillsboro, OR, USA).

4.2.4 LED treatment of a five-strain cocktail of *Salmonella* on low a_w pet food

Five strains of *Salmonella* including *Salmonella enterica* Typhimurium ATCC13311, *S. enterica* Senftenberg ATCC43845 and wastewater isolates *Salmonella* Enteritidis FUA1946, *S.* Bareilly FUA1934, and *S.* Thompson FUA1955 were used in this study (Mercer et al., 2017; Schultze et al., 2020). The frozen bacterial cultures were restored as mentioned in section 3.2.1. For inoculation of pet food pellets with 5-strain cocktail of *Salmonella*, the bacterial suspension of each strain was prepared as described in section 3.2.1 and equal volumes of each strain were mixed in a 15 mL falcon tube. Fifteen microliters of the cocktail were inoculated on the concave side of the pet food surface and airdried and equilibrated to 0.75 a_w as described in section 4.2.2.2. The final cell counts of the *Salmonella* cocktail on the pet foods after equilibration was 10^9 CFU/g. The a_w of the samples were checked by using a water activity meter (4TE, Aqualab, Pullman, WA, USA). Four pellets (~0.6 g) of these pet foods were treated with 455 nm LED at 60% power level and at 2 cm distance from the LED head in open conditions. The treatment times chosen were 10, 20, 30, 45 and 60 min and a small cooling fan (DC Brushless fan, 50×50×15 mm, model BB5015H12, HK fans, Shenzen, China) operated at 5 V by a DC power supply (KD3005D, Digital Control DC linear power supply, Korad, Shenzen, China) was used next to the samples during the LED treatment to control the temperature increase in the sample. The untreated equilibrated pet food pellets were taken as control and the viable cell counts were determined by following the method mentioned in section 4.2.2.2 for single strain inoculated pet foods by using the stomacher.

To investigate the combined effect of 275 (UV-C) and 455 (Blue) nm light pulses from LEDs against 5-strain cocktail of *Salmonella* in low a_w pet food, four pellets (~0.6 g) were first

treated for 10 min (7.56 J/cm^2 dose) at 2 cm distance from the LED head with 275 nm LED followed by 10 min (358.8 J/cm^2 dose) treatment with 455 nm LED as mentioned above. Apart from the combined treatments, four pellets of equilibrated pet foods were treated for 20 min (15.12 J/cm^2 dose) with 275 nm LED at 60% power level at 2 cm from the LED head. The untreated pet food pellets were used as control. Microbial enumeration for the treated and untreated samples were performed by following the method mentioned in section 4.2.2.2.

4.2.5 Changes in a_w, weight, and temperature during LED treatment

The 455 nm LED treatment resulted in the reduction of weight, a_w, and increase in the surface temperature of the treated samples. For analyzing the change in the water content of the treated samples, the weight of the dry *Salmonella* and pet food pellets, before and after the LED treatments were recorded. For evaluating the reduction in the a_w of the samples, the a_w of the samples before and after the treatments were recorded using the water activity meter. The initial water content (dry basis) of dry *Salmonella* and pet food pellets equilibrated to 0.75 a_w were obtained as mentioned in Chapter 3 (section 3.2.6). The desorption isotherm of the dry bacteria was generated at the treatment temperatures (highest temperature increase obtained with the 455 nm LED treatment) using a vapor sorption analyzer (VSA, Meter group, Inc., Pullman, WA, USA) as mentioned in Chapter 3 (section 3.2.6) to predict the approximate a_w of dry *Salmonella* after the LED treatments. The isotherm data was fitted with Guggenheim-Anderson-de Boer (GAB) (Equation 3.1, Chapter 3).

The surface temperature of the treated dry bacteria and low a_w pet food was recorded by keeping the thermocouple connected to a digital thermometer (1507726, Fischer Scientific, Hampton, NH, USA) on the surface of the samples during the treatment. A temperature profile was formed by monitoring the temperature increase on the surface of the pet food pellets and dry

bacteria for a total period of 60 min at every 5 min during the 455 nm LED treatment at 60% power level and at 4 cm distance from the LED head in triplicates in open conditions.

4.2.6 Determination of lipid oxidation in pet food during LED treatment

To study the effect of the 455 nm LED treatment on the quality of the low a_w pet food pellets, lipid oxidation was determined by the thiobarbituric acid (TBA) assay as reported by Bedinghaus, & Ockerman (1995) and Yadav et al. (2020) with some modifications. The uninoculated pet food pellets were equilibrated to 0.75 a_w by incubation in an air-tight equilibration chamber. Five pellets (~0.75 g) of low a_w pet foods were treated by blue light pulses at 4 cm distance from the 455 nm LED head at 60% power level in open condition for 10, 20, 30, 45 and 60 min. The treated and untreated samples were ground using a mortar and pestle, and final weight of the samples taken for the study was ~0.62 g, which was homogenized by using a homogenizer (POLYTRONR PT 10–35GT, Kinematica AG, Luzern, Switzerland) at 15000 rpm for 90 s in 5 mL of 20% trichloroacetic acid (TCA) supplemented with 1.6% phosphoric acid. This was followed by centrifugation at 5311 g for 8 min at 4°C using a centrifuge (Allegra 25R centrifuge, Beckman coulter Inc. Indianapolis, IN, USA). One millilitre of the supernatant was taken in a 15 mL falcon tube and was mixed with 1 mL of 0.02 M TBA by vortexing for 20 s. The solution was kept in a boiling water bath at 90°C for 35 min and was immediately transferred for cooling on ice for 10 min. Fluorescence intensity of the samples were then measured by transferring 200 µL into a 96-well Corning flat bottom microtiter plate in duplicates. A spectrophotometer (Variskon flash, Thermo Electron Corporation, Nepean, ON, Canada) was used at an excitation and emission wavelength of 532 and 553 nm, respectively. The lipid oxidation was reported in mg equivalent of malondialdehyde (MDA) per kg of pet food pellets.

4.2.7 Statistical analysis

The experiments were done in triplicates (n = 3). The statistical analysis was done using the SAS version 5.1.26 (SAS Institute Inc., Cary, NC, USA) and the significant differences between means was performed by Tukey's LSD test ($p < 0.05$). The comparison of inactivation efficacy of 455 nm LED in dry bacteria and pet foods; initial microbial concentration in pet foods; storage study was performed using two-way ANOVA. The effect of 455 nm LED treatment on the surface temperature and lipid oxidation was analyzed by one-way ANOVA.

4.3 Results

4.3.1 LED treatment of *S.* Typhimurium at low a_w conditions after drying in peptone water, or after drying on pet foods

Dried *S.* Typhimurium equilibrated to 0.75 a_w showed sensitivity to blue light pulses (455 nm) from LEDs with increasing light energy dose. Inactivation of *Salmonella* in low a_w foods is difficult as they become resistant to decontamination methods like heat treatment and can survive for long periods in such foods. Therefore, to test the effect of 455 nm LED treatment in a low a_w food system, *S.* Typhimurium was inoculated on the surface of pet foods and equilibrated to 0.75 a_w for treatment. The LED treatment produced significantly higher reduction in pet foods compared to dry bacteria and a significant interaction of the sample type (dry powder or pet food pellets) and treatment dose was observed ($p=0.0004$). The maximum log reduction of dried *S.* Typhimurium was 1.6 log CFU/g; after inoculation on pet foods, the maximum reduction of the same strain at the same a_w was more than 3 log CFU/g (Figure 4.1). The increase in dose did not increase the log reduction significantly above 523.8 and 349 J/cm^2 doses in the case of dry *Salmonella* and *Salmonella* on pet food, respectively.

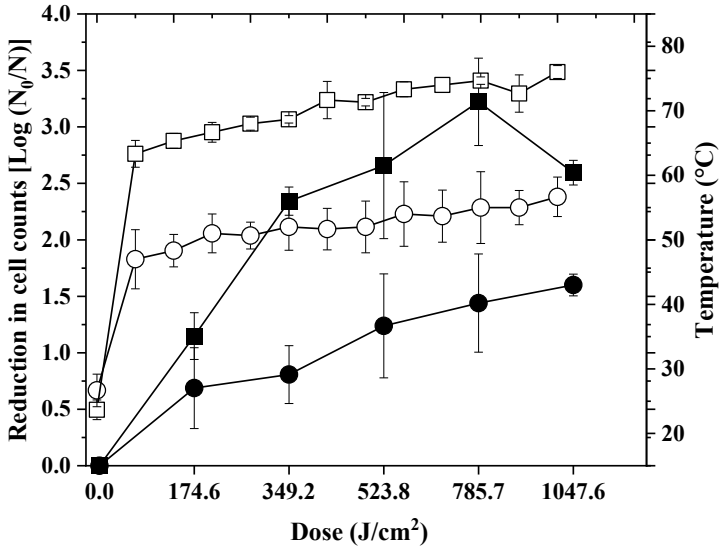


Figure 4.1: Reduction in cell counts [log(N_0/N)] of *S.* Typhimurium ATCC13311 after drying, equilibration to a_w 0.75 and treatment with 455 nm LED (closed symbols). The treatment dose is expressed as J/cm^2. *S.* Typhimurium cells were dried in 0.1% peptone water (closed circles) or on pet food (closed squares). The temperature profile of *Salmonella* Typhimurium in dry powdered form (open circle symbols) and on pet food pellets (open square symbols) is also shown. The samples were treated at a 4 cm distance from the LED head. Results are shown as means ± standard deviation of triplicate independent experiments.

The temperature increases of low a_w pet food surface (inoculated with *S.* Typhimurium) and dried *S.* Typhimurium were monitored at a sample distance of 4 cm from the LED head with 455 nm LED treatment at 60% power level. The increase in the surface temperature of the pet foods and dry bacteria during the LED treatment was unstable and it varied with increase in the dose during the treatment (Figure 4.1). However, the surface temperature of pet foods increased to 76°C, while the surface temperature of dry bacteria increased to 56°C for the same treatment dose of 1047.6 J/cm^2 (Figure 4.1), indicating the role of temperature increase on the varied inactivation of *Salmonella* in dry powdered form and on pet foods. Overall, the increase in the treatment dose significantly increased the inactivation effect by 455 nm LED treatment. The local surface

temperature increases due to the LED treatment, initial irradiance or power level used and sample composition also influenced the inactivation effect of the 455 nm LED. The increase in the irradiance (or power level) of 455 nm LED treatment of dry *Salmonella* resulted in a significant ($p=0.0019$) increase of the reduction of cell counts (Table 4.1). Hence the photodynamic and photothermal effects of blue light treatments or their synergistic or additive antimicrobial effect contributed to the *Salmonella* inactivation. However, more research is required to confirm the influence of photodynamic and photothermal effects on the inactivation efficacy of the light pulses emitted from the LEDs.

Table 4.1: Reduction in cell counts [$\log(N_0/N)$], water content (dry basis) and water activity of dry *Salmonella enterica* Typhimurium after the 455 nm LED treatments at 60 and 80% power level with 0.291 and 0.391 W/cm^2 irradiance, respectively, when samples were kept at a distance of 4 cm from the LED head. *S.* Typhimurium was dried in 0.1% peptone water and equilibrated to a_w 0.75 prior to treatment.

Irradiance (W/cm^2)	Treatment time (min)	Dose (J/cm^2)	Reduction in cell counts[1] (Log N_0/N)	Water Content (kg water/kg dry solids)	Water activity[2]
0.291	0	0	0^{a}	0.168 ± 0.012^{a}	0.740 ± 0.013^{a}
	10	174.6	0.687 ± 0.358^{b}	0.101 ± 0.032^{cb}	0.504 ± 0.188^{bc}
	20	349.2	0.807 ± 0.256^{b}	0.114 ± 0.015^{b}	0.606 ± 0.067^{ab}
	30	523.8	1.24 ± 0.46^{bc}	0.081 ± 0.013^{cd}	0.381 ± 0.118^{c}
	45	785.7	1.44 ± 0.44^{c}	0.093 ± 0.007^{cb}	0.494 ± 0.050^{bc}
	60	1047.6	1.59 ± 0.09^{c}	0.050 ± 0.014^{d}	0.135 ± 0.049^{d}
0.391	0	0	0^{a}	0.168 ± 0.012^{a}	0.740 ± 0.013^{a}
	10	234.6	1.02 ± 0.24^{b}	0.096 ± 0.016^{b}	0.459 ± 0.104^{b}
	15	351.9	1.47 ± 0.18^{c}	0.084 ± 0.008^{b}	0.383 ± 0.072^{b}
	20	469.2	1.29 ± 0.31^{bc}	0.076 ± 0.021^{bc}	0.275 ± 0.246^{bc}
	30	703.8	1.69 ± 0.24^{c}	0.049 ± 0.023^{c}	0.097 ± 0.086^{c}
	40	938.4	2.29 ± 0.36^{d}	0.068 ± 0.026^{bc}	0.245 ± 0.216^{bc}

Values are given as means ± standard deviation (n = 3). Values in each column with the same letter are not significantly different ($p < 0.05$).

[1] Log reduction corresponding to the irradiance of 0.291 W/cm^2 is also shown in Figure 3.1.

[2] Water activity values were determined from the desorption isotherm

To understand the effect of the environmental conditions on the antibacterial efficacy of 455 nm LED treatments, dry *S.* Typhimurium were treated inside a closed humidity chamber at two conditions i.e., 20°C, 75% RH and 60°C, 75% RH. A significant (p=0.02) effect of surrounding condition on the inactivation efficacy of 455 nm LED was observed at 60°C, 75% RH (2.04 log CFU/g) compared to open (1.24 log CFU/g) and 20°C, 75% RH (0.95 log CFU/g) with 523.8 J/cm^2 dose treatment (Figure 4.2). In this study, controlled environmental temperature and RH inside the closed chamber influenced the *Salmonella* reduction due to local temperature increase and RH changes during high intensity LED treatments.

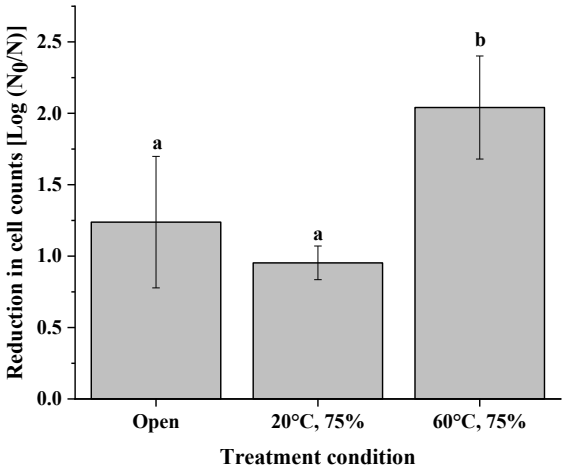


Figure 4.2: Comparison of the inactivation efficacy of 455 nm LED against dry *S. enterica* Typhimurium equilibrated to 0.75 a_w when treated with a dose of 523.8 J/cm^2 in different treatment conditions including open system (23°C, 48% relative humidity) and inside the humidity chamber, set at 20°C, 75% relative humidity and 60°C and 75% relative humidity. Results are shown as means ± standard deviation of triplicate independent experiments. Different letters a and b indicate the significant difference ($p<0.05$).

4.3.2 Effect of initial microbial concentration on LED inactivation efficacy

Treatment with the light pulses emitted from the LEDs is a surface decontamination method with limited penetration capacity, which might restrict its antibacterial effect in solid foods. To

understand the effect of possible shadowing of the bacteria, the pet food pellets were inoculated with three different concentrations of *S.* Typhimurium, i.e., 8, 7 and 6 log CFU/g. It was observed that the highest concentration of 8 log CFU/g showed higher sensitivity to 455 nm LED treatment than the lower concentrations. The difference in initial counts, i.e., 7 and 6 log CFU/g on pet foods did not influence the inactivation efficacy of 455 nm LED treatment (Figure 4.3). This indicates that lowering the initial number of *S.* Typhimurium may not increase the inactivation efficacy of 455 nm LED treatment. The irregular surface of the pet food pellets might protect the cells present in the crevices of the pet food surface from the light treatment, which might have resulted in the inconsistent exposure of *Salmonella* to the light pulses, in addition to the shadowing of the active cells in the lower layer by the damaged cells on the surface during the LED treatments. The log

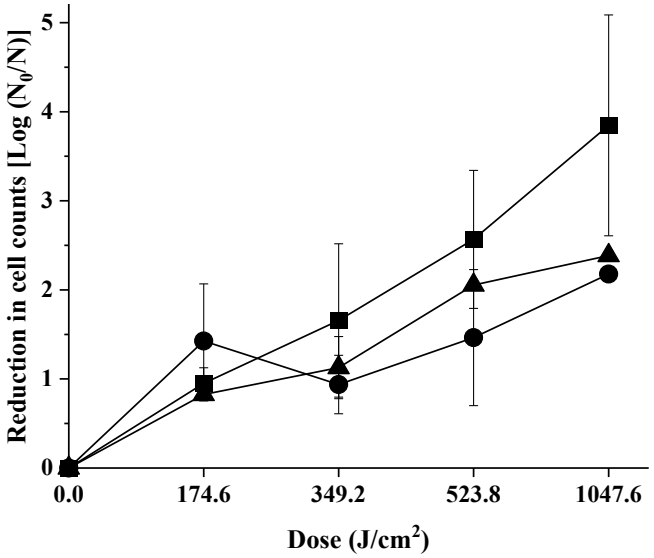


Figure 4.3: Reduction in cell counts [log(N_0/N)] of *S.* Typhimurium ATCC13311 after drying, equilibration to a_w 0.75 and treatment with 455 nm LED. *S.* Typhimurium was inoculated on pet foods to an initial cell count of 8.32 log CFU/g (square symbols); 7.12 log CFU/g (circle symbols) and 6.03 log CFU/g (triangle symbols)]. The treatment dose is expressed as J/cm^2. The samples were treated at a 4 cm distance from the LED head and the treatment times selected were 10, 20, 30 and 60 min. Results are shown as means ± standard deviation of triplicate independent experiments.

reductions in *S.* Typhimurium with selected initial concentrations on pet food was dependent on the light energy dose (Figure 4.3). Overall, increasing the dose to 523.8 J/cm^2 and above produced significant effect in the inactivation for all the three concentrations ($p<0.0001$).

4.3.3 Inactivation of *S.* Typhimurium on pet food during storage

The effect of storage on bacterial inactivation after cold plasma treatment and high-pressure processing was previously reported (Yadav et al., 2020; Marcos et al., 2008). However, the inactivation efficacy of 455 nm LED in low a_w foods during the post treatment storage is unknown. Therefore, the effect of 455 nm LED on *S.* Typhimurium inoculated on low a_w pet foods was analyzed for a storage period of 21 days after the LED treatment. It was observed that the reduction of *Salmonella* on pet food was influenced by storage after 455 nm LED treatment and two initial concentrations [(8 and 6 log CFU/g] of *Salmonella*. A significant interaction between the initial concentrations and storage period ($p<0.0001$) was found. The log reduction decreased significantly upon storage for 7 and 21 days as compared to the reduction observed on day 0 for initial microbial concentration of 8 log CFU/g (Figure 4.4). For initial microbial concentration of 6 log CFU/g, the log reduction reduced significantly during the storage compared to log reduction at day 0 ($p=0.0002$). Therefore, post treatment storage of low a_w pet foods showed a significant recovery of *Salmonella* for initial inoculum of 6 log CFU/g and a similar trend was observed for 7 and 21 days storage for initial inoculum of 8 log CFU/g. This increase in the *Salmonella* cell counts could be due to the recovery of sublethally injured cells in the presence of favorable conditions during the storage period. However, further studies on the recovery mechanisms of bacteria during the post-treatment storage in low a_w pet foods would be required to understand the effect of 455 nm LED treatments.

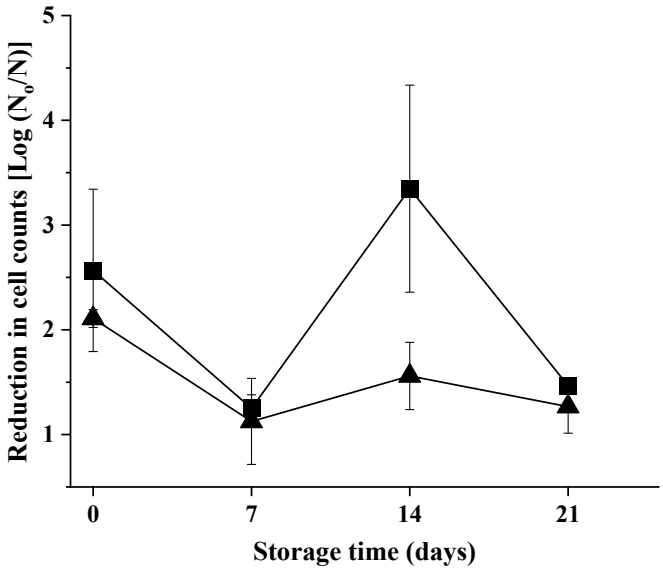
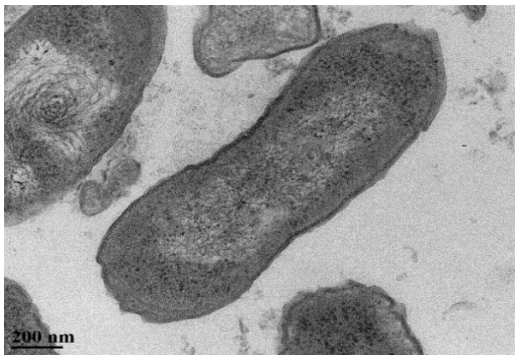


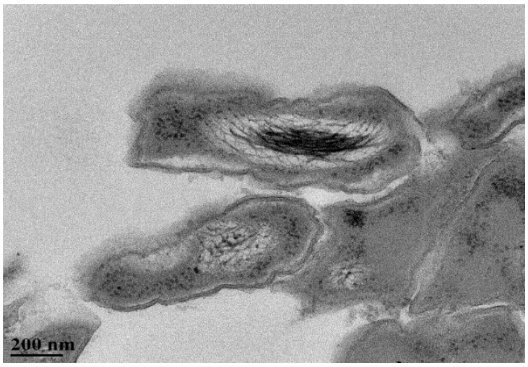
Figure 4.4: Reduction in cell counts [log(N_0/N)] of *S.* Typhimurium ATCC13311 during storage of pet food after drying, equilibration to a_w 0.75 and treatment with 455 nm LED. *S.* Typhimurium was inoculated on pet foods to an initial cell count of 8.32 log CFU/g (square symbols); and 6.03 log CFU/g (triangle symbols). The treatment dose was 523.8 J/cm^2 and samples were treated at a 4 cm distance from the LED head. After LED treatment, samples were hermetically sealed and stored for up to 21 days at an ambient temperature of about 20°C. Results are shown as means ± standard deviation of 3 to 5 independent experiments.

4.3.4 Structural changes in *S.* Typhimurium during LED treatment

To understand the underlying mechanisms of the *Salmonella* inactivation by 455 nm LED, TEM images were analyzed. The untreated *S.* Typhimurium cells showed intact cells in the TEM images, while the 455 nm LED treatment seemed to have produced cell rupture in dry *Salmonella* (Figure 4.5). The treatment with high intensity pulses from 455 nm LEDs and the two drying steps involved in the preparation of the bacteria might have produced damage to the *Salmonella* cells. However, some cells in the control samples might have also been damaged due to long drying and equilibration processes involved. Therefore, more research on understanding the mode of action of light pulses emitted from 455 nm LED would help in understanding the exact cause of cell damage observed in this study.



(a)



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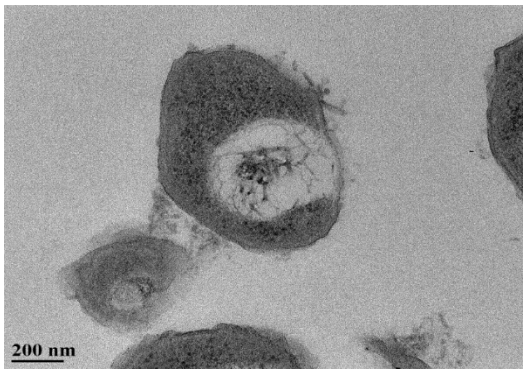
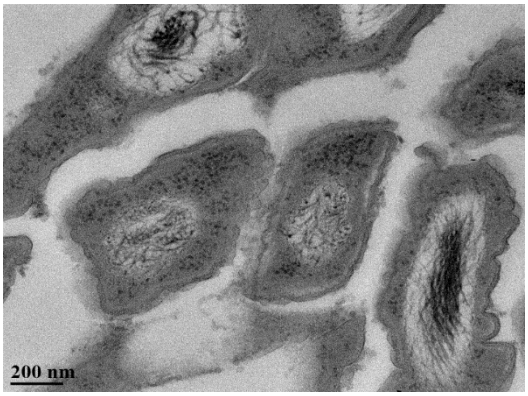
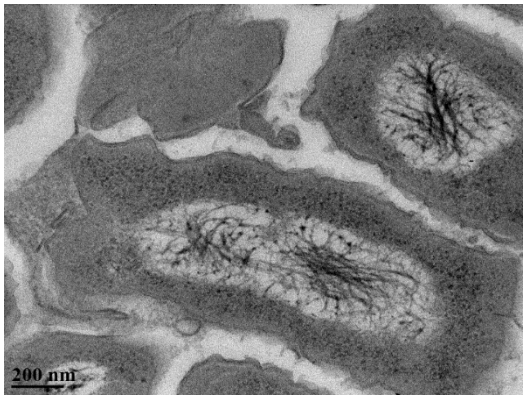


(c)



(d)



(e)

Figure 4.5: Transmission electron microscopy images at 56 kV. These images represent cellular rupture of the dry *S.* Typhimurium ATCC13311 cells due to the 455 nm LED treatment at a distance of 4 cm from the LED head at 60% power level under different treatment conditions. The treatments include (a) Control; (b) 523.8 J/cm^2 (30 min) treatment in open condition; (c) 1047.6 J/cm^2 (60 min) treatment in open condition; (d) 523.8 J/cm^2 (30 min) treatment in 20°C and 75% RH; (e) 523.8 J/cm^2 (~30 min) treatment in 60°C and 75% RH.

4.3.5 Inactivation of five strain cocktail of *Salmonella* on low a_w pet food

Five strain *Salmonella* cocktail was inoculated on low a_w pet foods, to study their influence on the bactericidal effect of the 455 nm LED. Pet foods inoculated with *Salmonella* cocktail were treated with higher dose by reducing the distance of the sample from the LED head to 2 cm and a cooling fan was used to reduce the surface temperature increase during the LED treatments at 60% power level. The 455 nm LED treatments showed antibacterial effect on *Salmonella* cocktail inoculated low a_w pet foods. However, higher dose of 2152.8 J/cm^2 (60 min) was required to produce a log reduction of 1.71 log CFU/g as compared to *S.* Typhimurium inoculated low a_w pet foods and powdered *S.* Typhimurium (section 4.3.1) (Table 4.2).

Table 4.2: Reduction in cell counts [log(N_0/N)] of a 5 strain cocktail of *Salmonella* after drying on pet food, equilibration to a_w 0.75 and treatment 455 nm LED at 60% power level. The samples were treated at 2 cm distance from the LED head.

Treatment time (min)	Dose (J/cm^2)	Reduction in cell counts (Log N_0/N)
0	0	0^{a}
10	358.8	0.661 ± 0.417^{b}
20	717.6	0.959 ± 0.147^{b}
30	1076.4	0.804 ± 0.324^{b}
45	1614.6	1.22 ± 0.57^{bc}
60	2152.8	1.71 ± 0.42^{c}

Values are given as means ± standard deviation (n = 3). Values with the same letter are not significantly different ($p < 0.05$).

4.3.6 Drying of pet foods after 455 nm LED treatment

The 455 nm LED treatment resulted in drying of treated samples by temperature increase during the treatments. The initial water content (dry basis) of dry *Salmonella* equilibrated to 0.75 a_w was

0.168 kg water/kg dry solids. The maximum reduction in water content (dry basis) after LED treatment was 70% for the dry *Salmonella* treated with 1047.6 J/cm^2 dose (Table 4.1). The desorption isotherm (Figure 3.4) of dry *Salmonella* at 55°C (closer to the surface temperature of the samples during 455 nm LED treatment) was used to determine the a_w of the treated bacteria (Chapter 3, section 3.2.6). The initial a_w of dry *Salmonella* was 0.74 and it reduced to 0.135 after treatment with 455 nm LED with 1047.6 J/cm^2 dose (60 min) (Table 4.1). Increasing the irradiance of the 455 nm LED to 0.391 W/cm^2 (80% power level) from 0.291 W/cm^2 (60% power level), resulted in the maximum reduction of water content and a_w to 0.049 kg water/kg dry solids and 0.097 with 703.8 J/cm^2 dose treatment, respectively (Table 4.1).

The initial a_w and the water content (dry basis) of pet foods after equilibration was 0.749 and 0.131 kg water/kg dry solids, respectively. For single strain *Salmonella* on pet foods treated at 4 cm height, the water content of the pet foods reduced to 0.120 kg water/kg dry solids after 455 nm LED treatment with 1047.6 J/cm^2 dose (Figure 4.6). The significant effect of dose was observed when it was increased beyond 349.2 J/cm^2 ($p<0.0001$). The a_w of the pet foods reduced significantly ($p<0.0001$) with an increase in the treatment dose and the maximum reduction was obtained with 1047.6 J/cm^2 dose treatment where a_w was reduced to 0.203 (Figure 4.6). Varying the initial microbial concentration did not have any significant effect on the reduction of water content and a_w. For five strain cocktail study in pet foods at 2 cm, the water content and a_w of the pet foods reduced to 0.118 kg water/kg dry solids and 0.116, respectively for treatment with 2152.8 J/cm^2 dose (Figure 4.6).

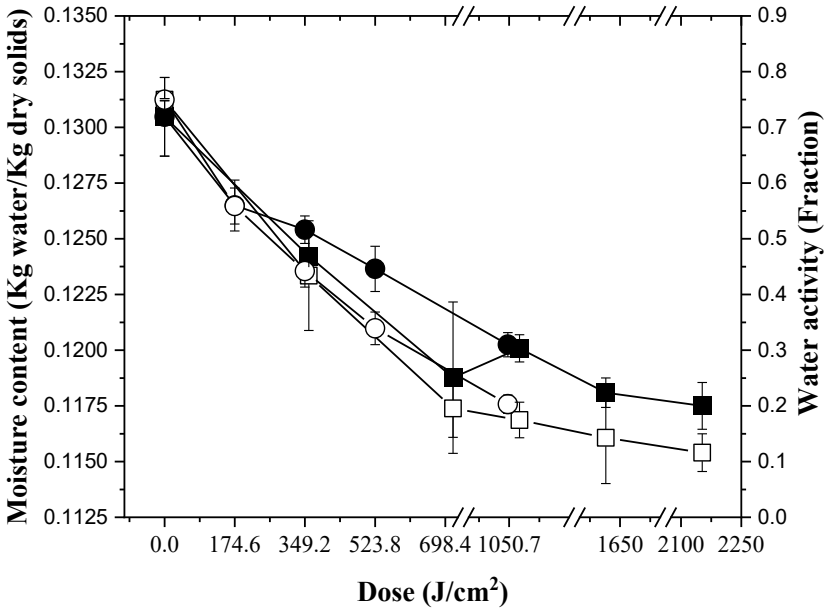
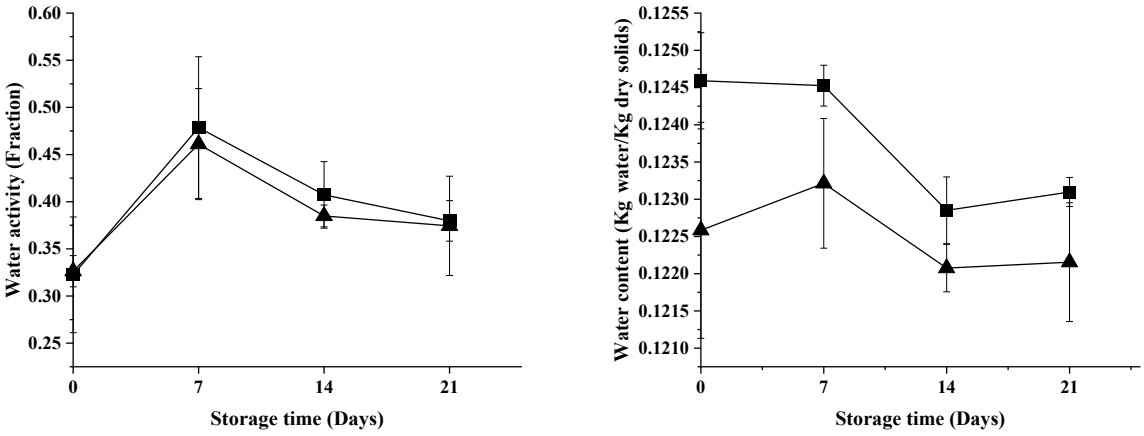


Figure 4.6: Change in the moisture content (closed symbols) and the water activity (open symbols) observed in pet foods after drying, equilibration to 0.75 a_w and treatment with 455 nm LED at 60% power level. The change in the moisture content (closed squares) and water activity (open squares) in pet foods inoculated with 5 strain cocktail of *Salmonella* and treated with 455 nm LED at 2 cm are represented by squares. The change in the moisture content (closed circles) and water activity (open circles) in pet foods inoculated with single strain of *Salmonella* Typhimurium ATCC13311 and treated with 455 nm LED at 4 cm are represented by circles. The treatment dose is expressed as J/cm^2. Results are shown as means ± standard deviation of triplicate independent experiments.

Storage of the 455 nm LED treated pet foods showed variation in the a_w and water content values. The initial a_w and water content (dry basis) after treatment with 523.8 J/cm^2 dose before storage period (Day 0) was 0.323, 0.124 kg water/kg dry solids, and 0.326, 0.123 kg water/kg dry solids for initial microbial concentration of 8 and 6 log CFU/g, respectively. The a_w of the pet foods increased significantly ($p<0.0001$) to 0.479 and 0.460 at Day 7 for initial microbial concentration of 8 and 6 log CFU/g, respectively, and then it reduced significantly ($p<0.0001$) at day 14 to 0.407 and 0.385, and to 0.379 and 0.374 at Day 21, respectively (Figure 4.7a). Also, the a_w of the LED treated pet foods at Day 7, 14 and 21 were significantly higher than a_w of the treated pet foods at Day 0 in both the concentrations indicating the absorption of moisture in the pet foods

during the storage period. Varying the initial microbial concentration did not have a significant effect on the a_w during storage (Figure 4.7a). Overall, varying the initial microbial concentration had a significant effect (p=0.017) on the water content (dry basis) in pet foods during storage but no variation was observed in the a_w during the storage period (Figure 4.7a & b). However, the storage period of the treated pet foods did not produce any significant effect in their water content (dry basis). The inactivation effect of 455 nm LED might be influenced by the simultaneous drying observed during the treatments with high intensity light pulses.



(a) (b)

Figure 4.7: Change in the water activity (a) and water content (b) observed in the pet foods inoculated with two different initial microbial concentrations [8.32 log CFU/g (square symbols); 6.03 log CFU/g (triangle symbols)] of *S. enterica* Typhimurium during storage for 21 days. The samples were treated with 455 nm LED with 523.8 J/cm^2 dose at 4 cm distance from the LED head. Results are shown as means ± standard deviation of triplicate independent experiments.

4.3.7. Sequential treatment of five strain cocktail of *Salmonella* on low a_w pet food with UVC$_{275}$-Blue$_{455}$ LED

To minimize the requirement for high dose for inactivation of *Salmonella* cocktail on pet foods and the associated drying, the effect of the sequential treatment of 275 and 455 nm light pulses on

the inactivation of *Salmonella* cocktail in low a_w pet foods was analyzed. Pre-treatment of *Salmonella* inoculated pet foods equilibrated to 0.75 a_w with 275 nm LED light for 10 min (7.56 J/cm^2) before 455 nm LED treatment for 10 min (358.8 J/cm^2) significantly improved the inactivation caused by the individual (275 or 455 nm) treatments (Figure 4.8). For instance, the additive effect of UVC_{275}-$Blue_{455}$ LED treatment of 20 min (10 min each) resulted in the log reduction of 1.47 log CFU/g as compared to 0.456 and 0.959 log CFU/g reductions after 20 min treatment with 275 (15.12 J/cm^2) and 455 (717.6 J/cm^2) nm LED, respectively (Figure 4.8). Also, 275 nm LED treatments with 15.12 J/cm^2 dose only increased the surface temperature of the pet foods from 24°C to 30°C.

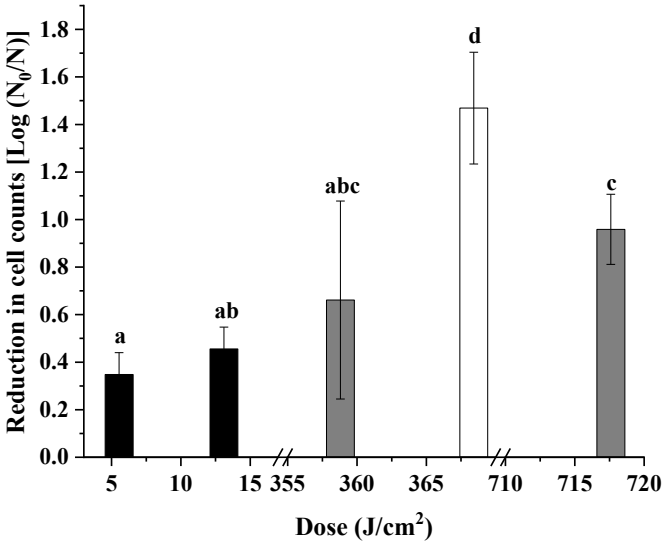


Figure 4.8: Reduction in cell counts [log(N_0/N)] of a 5 strain cocktail of *Salmonella* after drying on pet food, equilibration to a_w 0.75 and treatment with 275 nm and/or 455 nm LED. The reduction of cell counts of the *Salmonella* cocktail in pet foods after treatment with 275 nm LED alone is shown by black bars and with 455 nm LED alone is shown by dark gray bars. The reduction of cell counts of the *Salmonella* cocktail with the sequential treatment of UVC_{275}-$Blue_{455}$ LED is represented by a white bar. The treatment dose is expressed as J/cm^2. Samples were treated at 2 cm distance from the LED head. Results are shown as means ± standard deviation of triplicate independent experiments. Values differ significantly ($P<0.05$) if they do not share a common superscript.

4.3.8 Lipid oxidation in pet food during LED treatment

Lipid oxidation in the 455 nm LED treated low a_w pet food pellets was observed. The untreated pet food had 0.34 mg equivalent of MDA per kg of pet food pellets and it significantly (p=0.0105) increased to 1.28 mg equivalent of MDA/kg of pet food with 174.6 J/cm^2 dose (10 min) of 455 nm LED treatment at 60% power level. However, increasing the treatment dose did not increase the MDA content significantly and the maximum value obtained was 1.95 mg equivalent of MDA/kg of pet food for LED treatment with 523.8 J/cm^2 dose (30 min) (Figure 4.9). The light pulses from the LEDs emitting light in the visible region produce ROS in the presence of oxygen and lead to oxidation of lipids present in the pet food pellets used in the study.

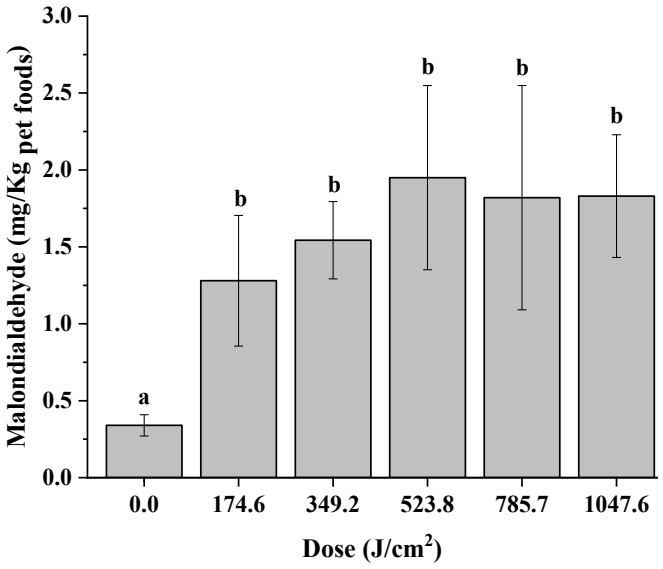


Figure 4.9: Lipid oxidation as determined by TBARS after treatment of pet food at 455 nm with samples kept at a distance of 4 cm from the LED head. Results are shown as means ± standard deviation of triplicate independent experiments. Values differ significantly (P<0.05) if they do not share a common superscript.

4.4. Discussion

The LED technology has drawn attention for microbial inactivation in food as it can be easily incorporated into existing processing lines owing to its compact size and continuous operation (Matafonova, & Batoev, 2018). The bactericidal effect of 455 nm LED, however, is limited. FDA regulations require that pathogen intervention methods achieve a reduction of cell counts of *Salmonella* by 4 log (7 CFR 981.442(b)) but treatments with 455-460 nm LED typically result in a reduction of cell counts of *Salmonella* in foods of ~2.5 log CFU/g or less (Ghate et al., 2017; Subedi et al., 2020). In comparison to LED emitting light at lower wavelengths, treatment with 455 nm LED has a higher energy input (Chapter 2) and can thus be used for combination treatments that achieve heating or drying in conjunction with bacterial inactivation (Subedi et al., 2020)**.** This study provides an integrated assessment of the treatment of pet foods with 455 nm LED alone or together with 275 nm LED on the inactivation of *Salmonella*, moisture loss, and lipid oxidation.

Treatment of food with Blue (455-470 nm) LED is associated with a temperature increase that contributes to the bactericidal effect (Ghate et al., 2017; Subedi et al., 2020)**;** this temperature increase depends on the treatment parameters and the type of food (Ghate et al., 2013; Srimagal, Ramesh, & Sahu, 2016; Chapter 3)**.** A difference in the sensitivity of *S.* Typhimurium in dry powdered form and in pet foods was observed. A significant effect of increasing the initial irradiance was also observed in this study. The efficacy of the 455 nm LED treatment might have been reduced by the shadowing effect of damaged dry *Salmonella* cells on the surface, on the active cells in the lower layers resulting in the uneven treatment of the lower layer of cells (Gomez-Lopez et al., 2005; Kim et al., 2015). The drying steps involved in the preparation of dry bacteria were longer (air-drying- 4 to 5 days and silica gel drying- 1 day) compared to the preparation of *S.* Typhimurium inoculated low a_w pet foods, where only air-drying was performed for 45 min.

Also, the equilibration of the dried *S.* Typhimurium to 0.75 a_w was performed for 7 days compared to 4-5 days in the case of pet food pellets. Hence, longer preparation time of dried *S.* Typhimurium might have contributed to their increased resistance to the 455 nm LED treatments compared to *S.* Typhimurium on pet food pellets. The treatment energy is delivered only on the surface in solid foods, while light penetration in liquid foods distributes the energy on a larger volume. Accordingly, the temperature increase in liquid broth and the bactericidal effect of 461 nm LED treatment at 596.7 J/cm^2 against *Salmonella* (Ghate et al., 2015) was less pronounced when compared to the temperature increase and bactericidal effect observed in this study (Figure 4.1).

The choice of the target strain and the drying conditions also impact the resistance of *Salmonella.* Drying conditions produce osmotic stress in *Salmonella* that triggers the accumulation of compatible solutes as an immediate response in the bacteria (Csonka, 1989). Additionally, *Salmonella* might induce filamentation or enter into the viable but non-culturable state upon exposure to drying and other stress conditions (Finn et al., 2013; Oliver, 2010; Stackhouse et al., 2012). The preparation of dry *Salmonella* in this study involved two drying steps, i.e., air-drying and equilibration inside a sealed chamber with silica gel, while inoculated pet foods were air-dried for a short duration during their preparation, which might have increased the resistance to stress conditions in dry bacteria and reduced their sensitivity to 455 nm LED pulses. This study also showed the significant effect of illumination temperature on the inactivation effectiveness of 455 nm LED treatment of dry *Salmonella* equilibrated to 0.75 a_w which is supported by a previous study by Ghate et al. (2017).

In this study, different initial inoculum concentrations of *S.* Typhimurium on low a_w pet foods surface were treated with 455 nm LED, to reduce the layers of bacteria exposed to the treatment to minimize shadowing effect. However, the 455 nm LED treatment did not show any

significant effect of reducing the initial inoculums. This observation is supported by a previous study by Aurum, & Nguyen (2019), where inactivation efficacy of 465 nm LED in combination with curcumin as a photosensitizer was not affected by varying the inoculum levels of *E. coli*.

The validation of novel pathogen intervention technologies necessitates the use of validated strain cocktails rather than the use of single strains to ensure treatment efficacy against all strains of the target species (Álvarez-Ordóñez et al., 2015; Breidt, Andress, & Ingham, 2018; Garcia-Hernandez, McMullen, & Gänzle, 2015; Ingham et al., 2010) The strains of *Salmonella* used in this study were previously identified as highly resistant to drying and high pressure carbon dioxide (Schultze et al., 2020). The same cocktail of *Salmonella* also showed resistance to 395 nm LED treatment in wheat flour (Du et al., 2020)**.** Also, a maximum reduction of just 0.61 log CFU/g in *Salmonella* cocktail with LED treatment dose of 7950 J/cm^2 (254.7 mW/cm^2 irradiance) was obtained in the fresh-cut pineapples (Ghate et al., 2017). The present study confirms by treatment with 455 nm LED that cocktail is more resistant than single strain and should be used for validation.

Salmonella survives on pet foods for more than a year (Lambertini et al., 2016). A significant recovery during the post-treatment storage was observed in this study. Treatment with 455 nm LED induces sublethal injury of *Salmonella*, which involves loss of some cellular functions and makes them non-detectable in non-selective medium (Ghate et al., 2015; Ghate et al., 2013; Gilbert, 1984). Sublethally injured cells recover under favorable conditions but are killed under stress conditions and the inactivation of sublethally injured cells during post-treatment storage is exploited in other novel non-thermal processing methods to enhance the bactericidal treatment effect (Schottroff et al., 2018).

The energy input of 455 nm LED required to produce a ~3 log CFU/g reduction in *S.* Typhimurium on pet foods used in this study was ~6 kJ/g (calculated by considering a pet food pellet surface as circle of diameter ~1.2 cm) (Figure 4.1), which is higher in comparison to other non-thermal pathogen intervention technologies but sufficient for drying of the foods. A significant loss of water in the 455 nm LED treated pet foods along with an increase of the surface temperature was observed. The maximum temperature increase observed in the pet foods treated at 2 cm from the LED head was from 23°C to 68°C for the 455 nm LED treatment with 2152.8 J/cm^2 dose. Previous studies have reported the drying effect and temperature increase in the LED treated low a_w wheat flour (Du et al., 2020; Subedi et al., 2020). However, this temperature increase is dependent on wavelength and the nature of the food product.

LED treatment at different wavelengths has a different mode of action and different energy input (Beck et al., 2017a; Hamamoto et al., 2007; Xiao et al., 2018). LEDs emitting blue light are in the absorption range of light absorbing molecules like porphyrins in bacteria, which produces bactericidal effect by producing ROS. LED treatment at 461 nm (blue light), produced better inactivation effect against bacteria when compared to 521 nm LED treatments and required high energy input for the inactivation (Ghate et al., 2015; Ghate et al., 2013). Therefore, in this study, the additive efficacy of combined treatments was confirmed**.** Additionally, the pre-treatment with a low dose of 275 nm LED helped in balancing the heating and drying effects caused in pet foods by the treatment with 455 nm LED alone by reducing the need for high doses of 455 nm light pulses, while improving its inactivation efficacy. The synergistic effect of 288/271 nm LED lights were also reported against *E. coli*, *S.* Typhimurium and *Staphylococcus epidermis* (Lu et al., 2021). However, the impact of the pretreatment of *E. coli* with UV-A LED followed by UV-C LED treatment varied based on the strain of *E. coli* (Xiao et al., 2018).

The 455 nm LED treatment resulted in the significant lipid oxidation of treated low a_w pet foods (Figure 4.9). This observation is supported by a previous study by Lennersten, & Lingnert (2000), where light in the range of 410-470 nm produced significant lipid oxidation in mayonnaise. Moreover, the temperature increase and drying caused during the 455 nm LED treatments might also have contributed to the lipid oxidation (Tran, Hendriks, & Poel, 2011). This Lipid oxidation affects the sensory attributes of the pet food pellets (Lin et al., 1998a). However, the extent of lipid oxidation also depends on the quantity of fatty acids and other ingredients present in pet foods (Lin, Hsieh, & Huff, 1998b). Therefore, more research involving the effect of lipid oxidation produced by 455 nm LED on the nutrient and sensory attributes of the pet food pellets are necessary.

In conclusion, treatments using 455 nm light pulses emitted from LEDs showed promising results on *Salmonella* reductions in low a_w conditions. The 455 nm LED was more effective in low a_w pet foods as compared to dry powdered form of *S.* Typhimurium and this efficacy was reduced against 5-strain cocktail of *Salmonella*. A sequential treatment with 275 and 455 nm light pulses for 20 min was more effective in reducing *Salmonella* in pet food compared to individual treatments using 275 or 455 nm LED treatments. Significant surface temperature increase and drying were observed in the 455 nm LED treated samples. The 455 nm LED treatments resulted in the significant lipid oxidation in low a_w pet foods. Overall, this study showed the potential application of the treatments with 455 nm light pulses emitted from the LEDs as a drying and microbial load reduction method in low a_w foods.

Chapter 5: Understanding the antibacterial mechanisms of 365, 395 and 455 nm light pulses emitted from light emitting diodes

5.1 Introduction

Salmonellosis is a disease caused by *Salmonella*, which infects humans by the consumption of contaminated foods or can be transmitted through pets. The increasing cases of foodborne outbreaks due to *Salmonella* in low water activity (a_w) foods like pet food pellets, breakfast cereals, spices, dried coconuts, etc., (Beuchat et al., 2011; CDC, 2018; FDA, 2019) is a major concern. *Salmonella* can contaminate dried food products in any stage of their preparation and can survive in low a_w foods for over a year (Carrasco et al., 2012; Gurtler et al., 2014; Lambertini et al., 2016). They develop resistance to the traditional decontamination methods like heat treatments or use of oxidizing chemicals in the food industry by developing certain defence mechanisms like accumulation of compatible solutes in the cell to maintain their turgor pressure under stress conditions (Bari et al., 2009; Csonka, 1989; Luo et al., 2022). This necessitates the need for exploring alternative decontamination technologies for low a_w foods.

The light emitting diode (LED) technology has gained attention due to its several advantages, including cost-effectiveness, ease of incorporation to the existing processing lines, monochromatic light, small size, absence of warm up time and others (D'Souza et al., 2015; Kebbi et al., 2020). This technology can produce antibacterial effect by photodynamic inactivation (PDI), which involves generation of reactive oxygen species (ROS) upon absorption of light by chromophores like porphyrin compounds in the presence of oxygen. This triggers cytotoxic responses in cellular components like DNA, RNA, protein, etc., eventually leading to cell death (Luksiene, & Zukauskas, 2009).

Several mechanisms attributed to the inactivation efficacy of LED treatments have been reported in high a_w conditions, especially in cell suspensions (Beck et al., 2017a; Hamamoto et al., 2007; Kim, & Kang, 2018a; Kim, Bang, & Yuk, 2017a; Li et al., 2017; Xiao et al., 2018). For instance, LEDs emitting light of wavelengths 260 and 280 nm showed inactivation efficacy against *E. coli* suspension by inducing DNA and RNA damage (Beck et al., 2017a). Also, the 365 nm (UV-A; Ultraviolet-A) LED treatment of *E. coli* DH5α suspension showed DNA damage, DNA oxidation and generation of hydroxyl radical and hydrogen peroxide in the bacterial cell (Hamamoto et al., 2007). Similarly, the LEDs emitting blue light (~460 nm) have shown significant oxidative stress and loss of cell membrane integrity in bacteria, when combined with exogenous photosensitizers like curcumin, riboflavin (Bhavya, & Hebbar, 2019; Li et al., 2021).

The light pulses emitted from the LEDs has been shown to have antibacterial efficacy in low a_w food systems also. For instance, LED emitting light of wavelength 405 nm previously showed its inactivation efficacy against *E. coli* and *Salmonella* in shelled almonds (Lacombe et al., 2016). Previous studies reported the antibacterial effect of 365 (UV-A), 395 (NUV; Near Ultraviolet-Visible) and 455 nm (Blue) LEDs against *Salmonella* in low a_w foods like wheat flour and pet food pellets (Du et al., 2019; Subedi et al., 2020; Chapters 3 & 4). However, studies focussing on understanding the antibacterial mode of action of light pulses emitted from the LEDs in low a_w conditions are limited. This study focused on understanding the *Salmonella* inactivation mechanisms of 365, 395 and 455 nm light pulses emitted from LEDs in the low a_w environment.

5.2 Materials and Methods

5.2.1 The Light Emitting Diode (LED) system

The LED heads of JL3 series (111×70×128 mm^3; 6 high intensity LEDs) emitting light of wavelengths 365 (Ultraviolet-A), 395 (Near-Visible Ultraviolet) and 455 (Blue) nm compatible

with a controller unit (CF3000, Clearstone Technologies Inc., Hopkins, MN, USA) were used in this study. The irradiance of the LEDs were measured using a laser energy meter (7Z01580, Starbright, Ophir Photonics, Har Hotzvim, JRS, Israel) connected to an irradiance and dose sensor (PD300RM-8 W, Ophir Photonics, A Newport Corporation Brand, North Logan, UT, USA) at a distance of 4 cm from the LED head and at 60 or 80% power level as mentioned in Chapters 3 and 4. The 365, 395 and 455 nm LEDs emitted light with irradiance values of 0.06, 0.30 and 0.35 W/cm^2, respectively at 80% power level and the irradiance values of the 365 and 395 nm LEDs at 60% power level were 0.05 and 0.23 W/cm^2, respectively.

5.2.2 Determination of cell membrane damage in low a_w *S.* Typhimurium

Salmonella enterica serovar Typhimurium ATCC13311 strain was used in this study. The low a_w *S.* Typhimurium for determination of cell membrane damage was prepared as described in Chapter 3 (section 3.2.1). The frozen culture was restored and the inoculum was prepared as mentioned in Chapter 3 (section 3.2.1). Two millilitres of the prepared cell suspension were transferred in each glass vial. The cell counts of the inoculum was ~10^{12} CFU/mL in each glass vial. These glass vials were air-dried in the biosafety cabinet for 4-5 days, followed by drying in a desiccator containing silica gel for 1 day. The cell counts of the dried *S.* Typhimurium was ~10^{11} CFU/g. These dried *S.* Typhimurium in the glass vials were incubated in an airtight chamber containing supersaturated sodium chloride solution for 7 days under room conditions (~23°C), for equilibration to 0.75 a_w. The final cell counts of the equilibrated dried bacteria was obtained as 10^9-10^{10} CFU/g as described in Chapter 3 (section 3.2.1).

For the determination of cell membrane damage in low a_w *S.* Typhimurium, LIVE/DEAD Baclight kit (L7012, Molecular Probes Inc, Eugene, OR, USA) consisting of SYTO9 (labels viable

cells) and propidium iodide (PI; labels damaged and dead cells) was used. Ten milligrams of low a_w *S.* Typhimurium was treated with the LEDs emitting light pulses of wavelengths 365 and 395 nm at 60% power level and at 4 cm from the LED head. The treatment times chosen were 10 and 60 min corresponding to a treatment dose of 28.9 and 188.1 J/cm^2 for 365 nm LED, and 138.8 and 834.4 J/cm^2 for 395 nm LED treatments, respectively. The LED treated low a_w *S.* Typhimurium was washed with 2 mL 0.85% sodium chloride (NaCl) solution and the resultant pellet was resuspended in 2 mL 0.85% NaCl solution and mixed with 20 mL of the same salt solution and incubated at ~23°C for 30 min, while vortexing every 10 min. After the incubation, the cell suspensions were centrifuged and washed with 20 mL 0.85% NaCl solution and the pellet was resuspended again in 10 mL of the same salt solution. Ten milligrams of low a_w *S.* Typhimurium were taken as control, while 10 mg of bacteria heat treated at 85°C for 1 h followed by incubation in 70% isopropanol for 30 min was considered as positive control. The cell suspensions were diluted with sterile deionized water to maintain the cell numbers per second in the range of 300 to 3000 events per second. For staining, equal volumes of the dyes were mixed and 3 µL of the dye mixture was mixed with 1 mL of the cell suspension followed by incubation at room conditions for 15 min in dark. Flow cytometry was performed using BD LSR Fotessa X-20 (BD Biosciences, San Jose, CA, USA) with a 488 nm excitation from a blue air laser at 50 mW and a 561 nm excitation from a yellow air laser at 50 mW to excite green (530 ± 30 nm) and red fluorescence (586 ± 15 nm), respectively. Sample injection and acquisition was performed simultaneously, and the data were recorded until 10,000 events. The data were analyzed using the FlowJo software (version 10.7.1, Becton Dickinson & Company (BD), San Jose, CA, USA).

5.2.3 LED treatment of low a_w *S.* Typhimurium prepared by shorter drying and equilibration period

To limit the cell membrane damage and oxidation of *S.* Typhimurium due to long drying and equilibration period involved in section 5.2.2, the low a_w *S.* Typhimurium was prepared as described by Fang, McMullen, & Gänzle (2020) with slight modifications. The inoculum was prepared as mentioned in section 5.2.2 in this study. The cell counts of the resultant cell suspension was ~10^{11} CFU/mL. Two hundred and fifty microlitres of this culture was transferred to glass vials and was dried in a vacuum chamber containing silica gel for 18-20 h. The cell counts of the vacuum dried bacteria was ~10^{12} CFU/g. The glass vials were then transferred into an airtight chamber containing supersaturated sodium chloride solution and incubated for 20-24 h for equilibration to 0.75 a_w at room conditions. The cell counts of the resultant low a_w *S.* Typhimurium was ~10^{12} CFU/g**.**

Ten milligrams of low a_w *S.* Typhimurium was weighed in a small disc (7.07 cm^2) composed of polylactic acid filament and treated with the light pulses emitted from the LEDs at room conditions (~23°C, 48% relative humidity). The dried bacteria were treated with treatment doses of ~217, ~217 and ~250 J/cm^2 corresponding to 60, 12 and 12 min treatment times with 365, 395 and 455 nm LEDs, respectively, at 80% power level and at 4 cm distance from the LED heads. Ten milligrams of low a_w *S.* Typhimurium with no LED treatment was considered as control. Enumeration of the viable cell counts was performed by 10-fold serial dilution in 0.1% peptone water and spread plating in TSAYE plates, followed by incubation at 37°C for 24 h.

5.2.4 Determination of intracellular ROS in low a_w *S.* Typhimurium

The production of intracellular ROS in the bacterial cell was evaluated using 5-(and-6)-carboxy-2′7′-dichlorodihydroflourescein diacetate (carboxy-H2DCFDA) dye (Invitrogen, Eugene, OR, USA) by following the manufacturer's instruction with slight modifications. The 365, 395 and 455 nm LED treated low a_w *S.* Typhimurium were washed with phosphate buffered saline, pH 7.4 (PBS; Gibco, Life Technologies, Waltham, MA, USA) and the pellet was resuspended in PBS containing carboxy-H2DCFDA dye with concentration of 10 µM in 1 mL of cell suspension. The solution was incubated at 37°C for 30 min in dark to facilitate the staining of the bacteria. The stained cell suspension was centrifuged, and the resultant pellet was resuspended in PBS making the final volume as 1 mL. Two hundred microlitres of the cell suspension were loaded in each well in 96 well microtiter plate (Costar, Corning, NY, USA) and 4 wells were loaded for each sample. Ten milligrams of untreated low a_w *S.* Typhimurium was considered as control. Ten milligrams of vacuum dried *S.* Typhimurium were also analyzed for intracellular ROS production due to drying. Fluorescence values were measured using a spectrophotometer (Variskon flash, Thermo Electron Corporation, Nepean, ON, Canada) at excitation wavelength of 495 nm and emission wavelength of 525 nm. Here, the intracellular ROS generation was reported as an arbitrary value I, calculated as $I = (I_t-I_o)/I_o$, where I_t and I_o are the mean fluorescent values of the treated and untreated low a_w *S.* Typhimurium as mentioned by George et al. (2019).

5.2.5 Determination of membrane lipid oxidation of low a_w *S.* Typhimurium

To determine the membrane lipid oxidation of low a_w *S.* Typhimurium due to the treatments with light pulses emitted from the LEDs, C_{11}-BODIPY$^{581/591}$ dye (Invitrogen, Eugene, OR, USA) was used in this study. The assay was performed according to the procedure reported previously with

slight modifications (Fang et al., 2020; Wang et al., 2020). The 365, 395 and 455 nm LED treated low a_w *S.* Typhimurium was washed twice with 1 mL of 50 mM Tris×HCl (pH 8.0) containing 20% (wt/vol) sucrose and the resultant pellet was resuspended in 1 mL of Tris×HCl buffer. The cell suspension was mixed with 200 µL lysozyme (5 mg/mL in 0.25 M Tris×HCl, pH 8.0) and 400 µL of ethylenediaminetetraacetic acid (EDTA; 0.25 M, pH 8.0) to facilitate the disruption of the outer cell membrane and was incubated in 37°C shaker (200 rpm) for 30 min. This was followed by centrifugation, resuspension of the pellet in 1 mL of 10 mM citrate buffer (pH 7) and 10 µM C_{11}-BODIPY$^{581/591}$ dye in dimethyl sulfoxide (DMSO) and incubation in 37°C shaker (200 rpm) for 30 min in the dark for staining. Low a_w *S.* Typhimurium treated with 200 mM H_2O_2 for 30 min served as positive control while 10 mg of low a_w *S.* Typhimurium with no treatment (H_2O_2 and LED) served as untreated control. Ten milligrams of vacuum dried *S.* Typhimurium were also analyzed for membrane lipid oxidation caused due to drying. The cell suspensions were diluted to maintain the cell numbers per second in the range of 300 to 3000 events per second. Flow cytometry was performed using BD LSR Fotessa X-20 and data were analyzed using the FlowJo software as mentioned in section 5.2.2.

5.2.6 Weight loss and surface temperature increase due to LED treatments

The treatments with the light pulses emitted from the LEDs can produce weight loss and increase in the surface temperature of the samples. Therefore, the weight of the low a_w *S.* Typhimurium was monitored before and after the treatments with the light pulses of 365, 395 and 455 nm wavelengths emitted from the LEDs at 80% power level and at 4 cm from the LED head with treatment dose of ~217, ~217 and ~250 J/cm^2, respectively, using a weighing balance. Also, the surface temperature of the bacteria was monitored using a thermocouple connected with a digital thermometer (1507726, Fischer Scientific, Hampton, NH, USA) during the LED treatments.

5.2.7 Statistical analysis

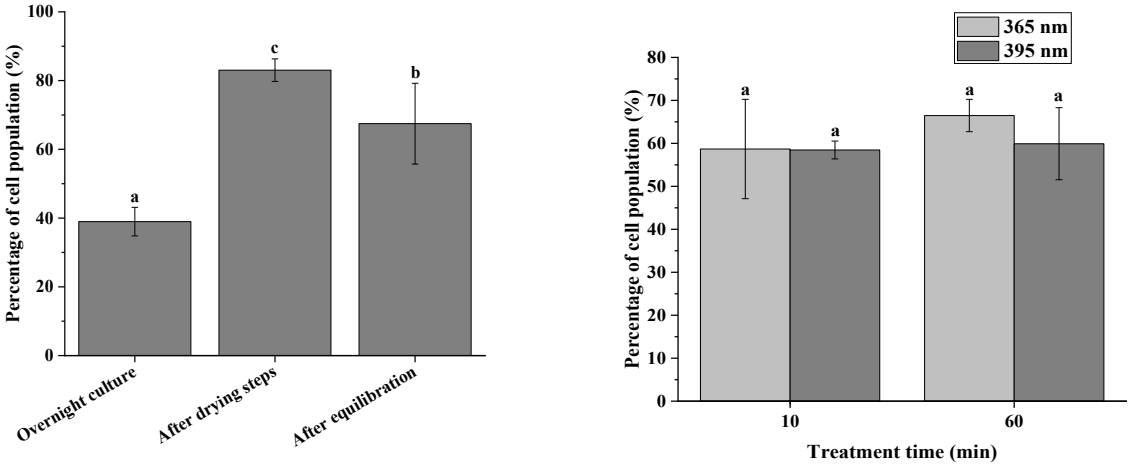
All the experiments were done in triplicate (n=3). SAS University edition (SAS studio 9.4) was used for the statistical analysis. The significant differences between means was performed by Tukey's LSD test with $p<0.05$. The evaluation of cell membrane damage due to the LED treatments were analysed by two-way ANOVA. The determination of log reduction, membrane lipid oxidation, intracellular ROS production, surface temperature increases, and weight loss was analyzed by one-way ANOVA.

5.3 Results

5.3.1 Cell membrane damage due to LED treatments

High intensity light pulses emitted from LEDs can induce cell membrane damage in the bacterial cells. Therefore, LIVE/DEAD assay was performed in low a_w *S.* Typhimurium in this study. Since the preparation of the low a_w *S.* Typhimurium involved a total drying period of 5-6 days followed by equilibration period of 7 days, LIVE/DEAD analysis was performed after the drying and equilibration steps during the preparation of the dry bacteria. Also, the cell membrane damage in the overnight culture of *S.* Typhimurium was performed, which showed a damage in 38.9% of the cells (Figure 5.1a). The drying step (air-drying and silica gel drying) produced significantly higher ($p=0.0008$) number of damaged cells labelled with PI. However, the percentage of cell population labelled with PI reduced ($p=0.0391$) after equilibration (Figure 5.1a). This could be due to the death or loss of the damaged cells after the drying step, as indicated by reduction in the cell counts from 10^{12} to 10^{9}-10^{10} after the drying and equilibration steps of *S.* Typhimurium in this study.

Chapter 3 in this research showed the inactivation efficacy of 365 and 395 nm LED treatments against low a_w *S.* Typhimurium prepared as mentioned in section 5.2.2. For instance, the LED treatment with 365 nm light pulses with 28.9 and 188.1 J/cm^2 dose showed a reduction of 0.4 and 1.41 log CFU/g in *S.* Typhimurium cell counts in low a_w conditions. Also, the 395 nm LED treatment with 138.8 and 834.4 J/cm^2 dose resulted in a reduction of 1.92 and 2.96 log CFU/g in the *S.* Typhimurium cell counts (Figure 3.1). The LIVE/DEAD assay of the LED treated low a_w *S.* Typhimurium did not show any cell membrane damage. Since, majority of the bacterial cells were damaged during the preparation of the dry bacteria, the LIVE/DEAD assay could not detect any significant cellular damage after the LED treatments. There was no effect of treatment time (or dose) due to the LED treatments. Moreover, there was no effect of the 365 and 395 nm LED treatments on the cellular membrane damage in bacterial cells (Figure 5.1b). Overall, the



(a) (b)

Figure 5.1: Effect of the vacuum drying and equilibration to 0.75 a_w (a); and 365 and 395 nm LED treatments (b) on the cell membrane of the *S.* Typhimurium cells by LIVE/DEAD Baclight assay. Here, the y-axis represents the percentage of cell population that showed cell membrane damage. The 365 and 395 nm LED treatments of low a_w *S.* Typhimurium cells were performed for 10 and 60 min corresponding to a treatment dose of 28.9 and 188.1 J/cm^2 for 365 nm, and 138.8 and 834.4 J/cm^2 for 395 nm LED treatments, respectively, at 60% power level and at 4 cm from the LED heads. Results are represented as means ± standard deviation of three independent replicates. Values with different superscripts differ significantly ($p<0.05$).

LIVE/DEAD bacterial viability study was not suitable to understand the effect of the 365 and 395 nm LED treatments on the cell membrane of the low a_w *S.* Typhimurium due to long drying and equilibration periods involved in the preparation of dry bacteria.

5.3.2 Inactivation effect of 365 and 395 nm LED treatment

The inactivation efficacy of 365 and 395 nm LEDs was evaluated by treatment of 10 mg of low a_w *S.* Typhimurium with ~217 J/cm^2 dose at 4 cm from the LED head and 80% power level. The drying (18-20 h) and equilibration (20-24 h) steps were shorter as compared to that used in section 5.3.1 and the cell counts were not significantly reduced during the preparation of the dry bacteria (Figure 5.2). Overall, the efficacy of the 365 nm LED treatments against low a_w *S.* Typhimurium was higher than 395 nm treatments with same dose ($p<0.0001$). For example, 365 nm LED produced a reduction of 2.95 log CFU/g as compared to 1.08 log CFU/g by 395 nm light pulses in low a_w *S.* Typhimurium cell counts with ~217 J/cm^2 dose treatment (Figure 5.2).

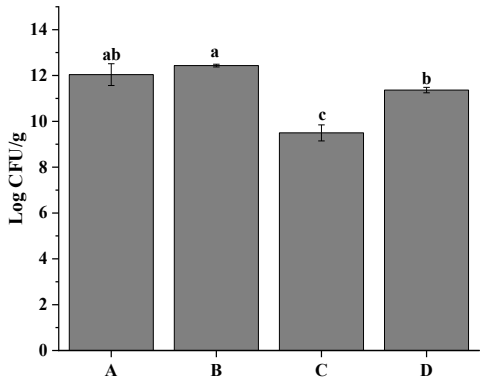


Figure 5.2: Effects of vacuum drying for 18-20 h (A), followed by equilibration to 0.75 a_w for 24 h (B), and 365 nm (C), and 395 nm (D) LED treatments of dried and equilibrated *S.* Typhimurium ATCC13311, on the survival of *S.* Typhimurium cells. The 365 and 395 nm LED treatments of low a_w *S.* Typhimurium ATCC13311 were performed at 80% power level and at 4 cm from the LED head with ~217 J/cm^2 dose corresponding to treatment times of 60 and 12 min, respectively. Results are represented as means ± standard deviation of three independent replicates. Values with different superscripts differ significantly ($p<0.05$).

The high intensity light pulses produced a significant increase in the surface temperature of the treated low a_w bacteria ($p=0.0008$). The 395 nm LED produced significantly higher increase in the surface temperature as compared to 365 nm LED treatments with the same dose ($p=0.0036$) (Table 5.1). Therefore, the surface temperature increase might play a role in the inactivation efficacy of the 395 nm LED. The LED treatments also produced a significant reduction in the weight of the treated low a_w *S.* Typhimurium ($p<0.0001$) and the weight loss could be attributed to the drying effect produced by the 365 and 395 nm LED treatments. Here, 365 nm LED produced significantly higher reduction in the weight compared to 395 nm LED treatments with same dose ($p=0.005$) (Table 5.1), indicating that drying is more pronounced in 365 nm LED which might influence its inactivation effect against *S.* Typhimurium in dried form.

Table 5.1: Effect of 365 and 395 nm LED treatment on the weight and surface temperature of low a_w *S.* Typhimurium. Intracellular ROS generation using carboxy-H2DCFDA dye in low a_w *S.* Typhimurium due to the 365 and 395 nm LED treatments are also shown. The 365 and 395 nm LED treatments were performed at 80% power level and at 4 cm from the LED head with ~217 J/cm^2 dose corresponding to treatment times of 60 and 12 min, respectively. Here, the arbitrary value $I = (I_t-I_o)/I_o$, where I_t and I_o are the mean fluorescent values of the treated and untreated low a_w *S.* Typhimurium, respectively and represents the intracellular ROS generation.

	Weight loss (%)	**Surface temperature (°C)**	**Arbitrary value (I) representing the intracellular ROS formation**
Control	0.00 ± 0.00^c	23.78 ± 0.69^c	0.00 ± 0.00^a
365 nm	15.51 ± 5.42^a	31.33 ± 2.31^b	0.97 ± 0.53^b
395 nm	10.33 ± 3.69^b	43.33 ± 4.93^a	0.81 ± 0.51^b

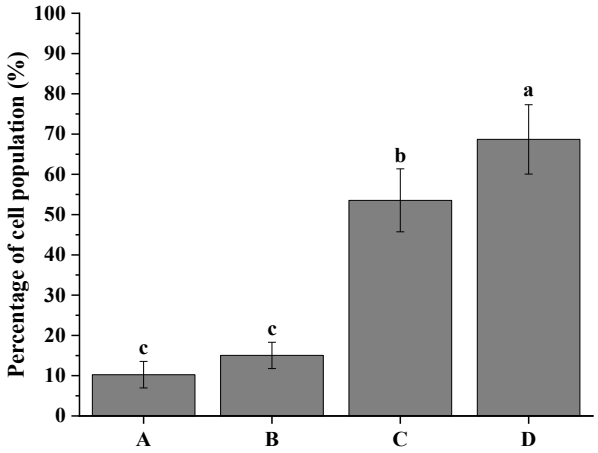
Results are represented as means ± standard deviation of three independent replicates. Values with different superscripts in each column differ significantly ($p<0.05$).

5.3.3 Oxidative stress in low a_w *S.* Typhimurium

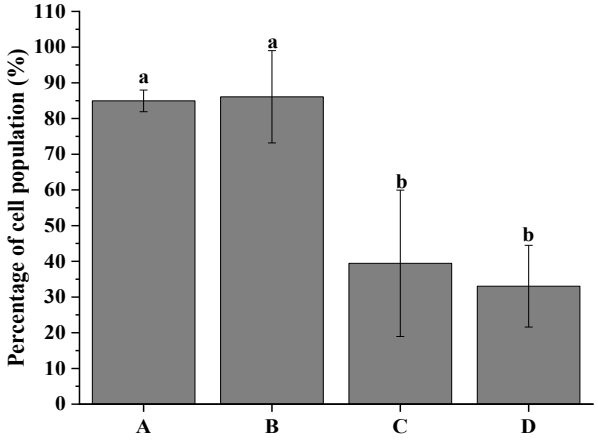
The LED technology produces antibacterial effect by photodynamic inactivation (PDI), which involves generation of ROS in the presence of oxygen in the bacteria. Therefore, the formation of

intracellular ROS due to the LED treatments was analyzed in this study. The vacuum drying involved in the preparation of dry *S.* Typhimurium produced significant intracellular ROS in the bacteria, but there was no significant difference after the equilibration step ($p=0.256$) (data not shown). Both 365 ($p=0.0123$) and 395 ($p=0.0288$) nm LED treatments with the same dose (~217 J/cm^2) resulted in the significant intracellular ROS production in the low a_w *S.* Typhimurium. However, there was no effect of changing the wavelength of light pulses from 365 to 395 nm ($p=0.5922$) on the ROS generation (Table 5.1).

After observing the production of intracellular ROS in low a_w *S.* Typhimurium due to the 365 and 395 nm LED treatments, their influence on the membrane lipid oxidation in *S.* Typhimurium was explored in this study. The drying step of 18-20 h produced membrane lipid oxidation in 10.3% of the cell population, while ~85% of the cell population remained unoxidized (Figure 5.3a & b). There was no effect of equilibration period on the membrane lipid oxidation of the dried *S.* Typhimurium ($p=0.3772$). A significant reduction in the percentage of unoxidized cells was observed after the LED treatments, with no significant difference among the 365 and 395 nm LED treated low a_w *S.* Typhimurium with the same dose (Figure 5.3b). The LED treatments with both 365 and 395 nm of low a_w *S.* Typhimurium resulted in a significant increase in the percentage of membrane lipid oxidation ($p<0.0001$) (Figure 5.3a). This membrane lipid oxidation of *S.* Typhimurium cells was significantly higher ($p=0.0182$) in the case of 395 nm LED treatment as compared to the 365 nm LED treatment with the same dose. Therefore, the generation of intracellular ROS and membrane lipid oxidation play an important role in the antibacterial efficacy of both the 365 and 395 nm LED treatments against low a_w *S.* Typhimurium. However, the membrane lipid oxidation in bacterial cells were more pronounced in the case of 395 nm LED treatment in this study.



(a)



(b)

Figure 5.3: Flow cytometric quantification of membrane lipid oxidation in *S.* Typhimurium ATCC13311 due to vacuum drying for 18-20 h (A), followed by equilibration to 0.75 a_w for 24 h (B); and treatment of dried and equilibrated *S.* Typhimurium with 365 nm (C) and 395 nm (D) LEDs using C11-BODIPY$^{581/591}$ dye as a fluorescent indicator. The stained and oxidized (a); and stained and unoxidized (b) cells are shown as the percentage of total cell populations. The 365 and 395 nm LED treatments of low a_w *S.* Typhimurium ATCC13311 were performed at 80% power level and at 4 cm from the LED head with ~217 J/cm^2 dose corresponding to treatment times of 60 and 12 min, respectively. Results are represented as means ± standard deviation of three independent replicates. Values with different superscripts differ significantly ($p<0.05$).

5.3.4 Effect of 455 nm LED treatments on low a_w *S.* Typhimurium

To understand the inactivation effect and the underlying antibacterial mechanism of 455 nm LED pulses against low a_w *S.* Typhimurium, a dose of ~250 J/cm^2 (12 min) at 80% power level and at 4 cm distance from the LED head was selected in this study. The 455 nm pulses produced a significant reduction ($p=0.0014$) in the cell counts of low a_w *S.* Typhimurium by 0.859 log CFU/mL (Table 5.2). This inactivation efficacy could be attributed to the generation of intracellular ROS as a significant increase ($p=0.0072$) in the fluorescence values after treatment with the 455 nm LED was observed (Table 5.2). A significant increase ($p<0.0001$) in the percentage of cell population of *S.* Typhimurium oxidized due to the 455 nm LED treatment by C11 BODIPY$^{581/591}$ assay shows that the membrane lipid oxidation plays a role in the antibacterial effect of the 455 nm LED in this study. Also, the 455 nm LED treatment resulted in a significant ($p<0.0001$) reduction in the weight of the treated low a_w *S.* Typhimurium with a weight loss of 9.66% and a significant increase ($p<0.0001$) in the surface temperature of the bacteria during the treatment (Table 5.2). This indicates that the drying and temperature increase might play a role in the antibacterial efficacy of the 455 nm LED treatments against low a_w *S.* Typhimurium.

Table 5.2: Effect of the 455 nm LED treatment on the survival, weight and surface temperature of low a_w *S.* Typhimurium ATCC13311. Flow cytometric quantification of membrane lipid oxidation using C11-BODIPY$^{581/591}$ dye, and intracellular ROS generation using carboxy-H2DCFDA dye in low a_w *S.* Typhimurium due to the 455 nm LED treatments are also shown. The 455 nm LED treatment was performed at 80% power level and at 4 cm from the LED head with ~250 J/cm^2 dose corresponding to a treatment time of 12 min. Here, the arbitrary value $I = (I_t-I_o)/I_o$, where I_t and I_o are the mean fluorescent values of the treated and untreated low a_w *S.* Typhimurium, respectively and represents the intracellular ROS generation.

	Log CFU/g	**Weight loss (%)**	**Surface Temperature (°C)**
Control	12.43 ± 0.06^a	0.00 ± 0.00^b	23.78 ± 0.69^b
455 nm treated	11.57 ± 0.18^b	9.66 ± 1.57^a	52.33 ± 2.52^a
	Unoxidized percentage of cell population as per C11 BODIPY assay (%)	**Oxidized percentage of cell population as per C11 BODIPY assay (%)**	**Arbitrary value (I) representing the intracellular ROS formation**
Control	86.09 ± 12.93^a	15.03 ± 3.28^b	0.00 ± 0.00^a
455 nm treated	20.29 ± 14.72^b	75.94 ± 12.91^a	1.19 ± 0.45^b

Results are represented as means ± standard deviation of three independent replicates. Values with different superscripts in each column corresponding to a specific assay differ significantly ($p<0.05$).

5.4. Discussion

The LED technology is an emerging decontamination method, which has shown promising inactivation efficacy against foodborne pathogens in high moisture foods like fresh-cut fruit surfaces, juices, etc., (Ghate et al., 2017; Kim, Bang, & Yuk, 2017a; Kim et al., 2017c). This technology has also shown its potential application as an antibacterial technology in low a_w foods (Du et al., 2019; Subedi et al., 2020). This study focusses on further understanding the underlying mode of action of the light pulses from the LEDs emitting light of different wavelengths against *S.* Typhimurium in low a_w conditions.

In this study, the long drying (4-5 days) and equilibration (7 days) steps involved during the preparation of low a_w *S.* Typhimurium for LIVE/DEAD assay analysis resulted in the majority of cell membrane damage in the bacteria (Figure 5.1a). Hence, the percentage of undamaged *S.* Typhimurium cells were not sufficient to evaluate the cell membrane damage produced by the 365 and 395 nm LED treatments. The reduction in the cell counts was observed during these preparation methods in Chapters 3 and 4 in this research. Therefore, the preparation of low a_w *S.* Typhimurium was modified to limit the effect of drying and equilibration periods in the subsequent studies. Low a_w *S.* Typhimurium showed susceptibility to the treatments with the 365 and 395 nm light pulses emitted from the LEDs with ~217 J/cm^2 dose at 80% power level and 4 cm distance from the LED head (Figure 5.2). Also, low a_w *S.* Typhimurium showed more sensitivity towards 365 nm LED treatment as compared to the 395 nm LED treatment with the same dose, power level and distance from the LED head, which was also reported in Chapter 3. This inactivation efficacy is dependent upon several factors like, illumination temperature, relative humidity, treatment time, treatment dose, power level, strain of bacteria used, etc. (Chapter 3; Subedi et al., 2020).

Understanding the underlying mechanism in the antibacterial effect of treatments with the 365 and 395 nm light pulses from the LEDs against low a_w *S.* Typhimurium would help in developing these LED light pulses as a potential decontamination method against foodborne pathogens in low a_w food systems. Carboxy-H2DCFDA was used as a fluorescent indicator of oxidative stress to understand the role of ROS production in the inactivation efficacy of the LED treatments in this study. The intracellular esterases in the live cells cleave the diacetate group of the agent and in the presence of ROS, the non-fluorescent compound converts to fluorescent form and emits fluorescence (George et al., 2019; Kim, & Kang, 2018a), which was detected by a spectrophotometer in this study, as an indicator for intracellular ROS production. The LED

treatments with 365 and 395 nm light pulses produced a significant intracellular ROS generation in low a_w *S.* Typhimurium cells (Table 5.1). Previously, the generation of ROS due to the 365 nm LED treatment in high moisture conditions has been reported (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Hamamoto et al., 2007; Li et al., 2010). Similarly, 405 nm LED treatment showed a significant increase in the intracellular ROS generation in *Staphylococcus epidermis* by using carboxy-H2DCFDA dye as the fluorescent indicator (Ramakrishnan et al., 2016). In high a_w foods like fresh-cut papaya inoculated with *Salmonella*, the 405 nm LED treatments showed oxidation of bacterial cells (Kim, Bang, & Yuk, 2017a). Increase in the oxidative stress might affect the antioxidant defence system of the cells. The generation of intracellular ROS can also lead to oxidation of cellular components like lipids, proteins, and might inhibit enzyme activity inside the cell, eventually leading to cell death (Meriga et al., 2004; Ramakrishnan et al., 2016; Shah et al., 2001; Sharma, & Dubey, 2005).

Since a significant oxidative stress of bacterial cells due to the 365 and 395 nm LED treatments was observed, further evaluation of the membrane lipid oxidation was performed. C11-BODIPY$^{581/591}$ dye was used to detect the membrane lipid oxidation in low a_w *S.* Typhimurium, which is oxidized by hydrogen peroxide (Drummen et al., 2002; Fang et al., 2020). Flow cytometry analysis facilitated in the quantification of oxidized and unoxidized cells. Hamamoto et al. (2007) showed that the inactivation efficacy of 365 nm LED treatment against *E. coli* DH5α was suppressed by the presence of catalase (a scavenger of hydrogen peroxide), indicating the presence of peroxide radical in the ROS produced due to the 365 nm LED treatments. Similarly, the 365 nm LED treatment produced a significant membrane lipid oxidation in low a_w *S.* Typhimurium in this study. Also, the 395 nm LED treatment with ~217 J/cm^2 dose produced membrane lipid oxidation in higher percentage of cell population of low a_w *S.* Typhimurium as compared to 365 nm LED

treatment with the same dose as opposed to the log reductions observed in this study (Figures 5.2 & 5.3a). This would indicate that there might be some additional mode of action involved in the inactivation efficacy of 365 nm LED treatments in low a_w conditions. Previously, 405 ± 5 nm LED treatment of *S.* Typhimurium suspension did not show significant membrane lipid peroxidation with thiobarbituric acid reaction substance (TBARS) assay (Kim, Bang, & Yuk, 2017a). Therefore, the generation of membrane lipid oxidation due to the LED treatments would depend upon the sample type, strain used, wavelength, treatment dose, etc.

This study showed a significant inactivation efficacy of 455 nm LED treatments with 250 J/cm^2 dose against low a_w *S.* Typhimurium, which is also supported by Subedi et al. (2020) and Chapter 4 of this research. Exogenous photosensitizers like curcumin when combined with blue (462 ± 3 nm) LED enhances its inactivation efficacy against *E. coli* and *S. aureus* suspensions and the generation of intracellular ROS in the cells (Bhavya, & Hebbar, 2019). In this study, a significant generation of intracellular ROS in low a_w *S.* Typhimurium with the 455 nm LED treatment alone was observed. This could be attributed to the photosensitization of endogenous photosensitizers like porphyrin compounds in *S.* Typhimurium cells by the 455 nm LED treatment, which leads to the generation of ROS in the presence of oxygen (Luksiene, & Zukauskas, 2009; Plavskii et al., 2018). On the contrary, the 462 nm LED treatment of *E. coli* and *S. aureus* suspensions alone did not produce any significant intracellular ROS (Bhavya, & Hebbar, 2019), indicating that the generation of intracellular ROS in a bacterial cell by the blue LED treatment was influenced by the wavelength, sample type and strain used. A significant membrane lipid oxidation of low a_w *S.* Typhimurium cells was observed with the 455 nm LED treatment in this study, indicating the presence of the peroxide in the ROS generated by the LED light pulses. This was supported by Orlandi et al. (2018) in their study, where the overexpression of catalase A

enzyme reduced the sensitivity of *Pseudomonas aeruginosa* to the 464 nm LED treatments, indicating that the blue LED produces the peroxide radical as a major ROS during the treatments.

In conclusion, the LEDs emitting light pulses of wavelengths 365, 395 and 455 nm showed antibacterial efficacy against *S.* Typhimurium under low a_w conditions. The 365 nm LED treatments showed better inactivation effect against low a_w *S.* Typhimurium than 395 nm LED treatment. Significant generation of intracellular ROS was observed with the LED treatments. Also, the membrane lipid oxidation of the *S.* Typhimurium cells was observed in the case of all the three LED treatments. Significant weight loss and increase in the surface temperature of the low a_w *S.* Typhimurium was observed with the 365, 395 and 455 nm LED treatments. Overall, this study presented the probable antimicrobial mode of action of the 365, 395 and 455 nm LEDs in the low a_w conditions, which would help in the development of the treatment with light pulses emitted from the LEDs as a potential decontamination method for low a_w food systems.

Chapter 6: Bacterial biofilm reduction by 275 nm and 455 nm light pulses emitted from LEDs

6.1 Introduction

Biofilm formation by foodborne pathogens like *Salmonella*, *Escherichia coli*, *Aeromonas sp.*, *Listeria monocytogenes*, etc., is a major concern. These microorganisms form the biofilms as a defence mechanism in response to stress conditions in food products like vegetables, meat, poultry, seafoods and in food contact surfaces like stainless steel equipment, plastics, and conveyor belts (Kumar, & Anand, 1998). The secretion of extracellular polymeric substances that include polysaccharides, proteins, lipids and extracellular DNA in the biofilms create a complex matrix encasing the bacterial cells, which protects them from the disinfectants like sanitizers and regular cleaning procedures in the food processing plants (Flemming et al., 2016; Visvalingam et al., 2019a); thus, increasing the risk of cross contamination of foods. This necessitates the need for exploring novel technologies for eradication of biofilms. Several studies focussed on understanding the antibiofilm activity of light based technologies like UV light, pulsed light and light emitting diode (LED) technology (Argyraki et al., 2017; Bumah, Masson-Meyers, & Enwemeka, 2020; Li et al., 2018a; Silva-Espinoza et al., 2020).

LED technology is being widely studied for biofilm inactivation in medical applications and has shown promising results (Angarano et al., 2020; Bak et al., 2010; Lacerda Rangel Esper et al., 2019). LEDs have several advantages like absence of warm-up time, monochromatic light emission and compact size, which makes it easy to incorporate into the existing processing lines (D'Souza et al., 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). LED

technology produces antimicrobial effect by means of photodynamic inactivation. This involves generation of reactive oxygen species (ROS) in the presence of oxygen, when light absorbing molecules like porphyrin compounds in the bacteria absorbs 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 focussed on understanding the antimicrobial efficacy of the LED system in high- and low-moisture foods (Du et al., 2020; Ghate et al., 2017; Ghate et al., 2016; Subedi et al., 2020). Also, there were 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 et al., 2019; Li et al., 2018a). There have been limited to no studies focussing on the antibiofilm efficacy of LEDs emitting light pulses of wavelength 275 (Ultraviolet-C, UV-C) and 455 nm against single and mixed species biofilms on stainless steel surfaces.

LED treatment can be used in food industries to inactivate pathogen biofilms attached to food contact surfaces such as stainless steel. Hence, understanding the antibiofilm activity of LEDs emitting light pulses of wavelengths 275 and 455 (Blue) nm during the incubation period of biofilm formation on stainless steel 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 activity against *Salmonella* Typhimurium and *Aeromonas australiensis* and to understand more details about their antibiofilm mechanism.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium ATCC 13311 and *Aeromonas australiensis* 03-09 were used in this study. Tryptic soy agar plates (TSA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 0.6% yeast extract (YE; Fischer Bioreagents) and Lennox broth with no salt agar plates (LB-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, USA) supplemented with 0.6% yeast extract or LB-NS broth and incubation at 37°C for 18-24 h, respectively.

6.2.2 Preparation of *S.* Typhimurium biofilms on stainless steel coupons

The polished stainless steel (SS) coupons (from local supplier) 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 ATCC 13311 were 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, USA) followed by static incubation at 37°C for 3 days. The inoculated SS coupons were washed with 3 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 et al., 2019b) 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 h. The final cell counts were recorded in CFU/cm^2.

6.2.3 Preparation of *A. australiensis* and mixed species biofilms on stainless steel coupons

Development of mixed species biofilms included both *S.* Typhimurium and *A. australiensis*. For the preparation of single species biofilm of *A. australiensis*, the overnight culture in LBNS 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 mixed species biofilms, the overnight cultures of both *S.* Typhimurium and *A. australiensis* prepared in LBNS broth were mixed in equal volume and diluted 100-fold in LBNS broth to get a final cell concentration of ~10^7 CFU/mL. The SS coupons were inoculated with the diluted single and mixed 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 6.2.2. 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 LBNS agar plates followed by incubation at 37°C for 24 h. In case of mixed species biofilm, the spread plated LBNS agar plates were incubated at: (1) 37°C for 24 h to get the cell counts for mixed species biofilms, (2) 42°C for 24 h to obtain the cell counts for *S.* Typhimurium cells in the mixed species biofilms, and (3) 18°C for 48 to 72 h to obtain the cell counts of *A. australiensis* in the mixed species biofilms. All the cell counts were reported in CFU/cm^2.

6.2.4 Crystal Violet (CV) staining assay

Single and mixed species cultures prepared as mentioned in section 6.2.3 in LBNS broth were used for the crystal violet staining assay. Two hundred microlitres of single and mixed species cultures were inoculated in a 96-well microplate in triplicates (Costar, Corning, NY, USA) and incubated at room temperature (~23°C) for 6 days. Similarly, 200 µL of sterile LBNS broth 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 with 200 µL of phosphate buffer saline of pH 7.4 (PBS; Gibco, Life Technologies, Waltham, MA, USA) 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 washed thrice with 200 µL of PBS. Two hundred microlitres 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, ON, Canada). The readings obtained for un-inoculated LBNS broth served as the background value and was subtracted from the sample readings. The CV samples were diluted 2 folds, when the OD_{595} value >1, and the reading was repeated again (Visvalingam et al., 2019a).

6.2.5 The Light Emitting Diode (LED) system

The LED system comprised of a controller unit (CF3000, Clearstone Technologies Inc., Hopkins, MN, USA) 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 at a frequency of 100 Hz as mentioned in Chapter 4. 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, Peabody, MA, USA) 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 (6.1) as follows (Subedi et al., 2020):

$$E = I \times t \quad (6.1)$$

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).

6.2.6 LED treatment of single and mixed species biofilms on SS coupons

The single and mixed 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 approximately 28.3 cm^2 and at 4 cm from the LED heads for the LED treatments and the biofilms formed on both the surfaces of the same 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 (1.8 J/cm^2 dose) min and with 455 nm light pulses for 5 (87.3 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 day incubation period of *S.* Typhimurium biofilm formation as mentioned in section 6.2.2. 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 LEDs; treatment times chosen were 5 min of 275 nm light pulses corresponding to an energy dose of 1.8 J/cm^2, and 2 and 5 min of 455 nm light pulses corresponding to dose of 34.92 and 87.3 J/cm^2, respectively, on each surface at 60% power level and 4 cm distance from the LED head. Similarly, the SS coupons with mixed species biofilm comprised 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 mixed species biofilms were performed 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 in section 6.2.3 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, USA) during the LED treatments on both the surfaces during the LED treatments. 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.

6.2.7 Confocal Laser Scanning Microscopy (CLSM) imaging

The biofilms on the SS coupons inoculated with mixed species of *S.* Typhimurium and *A. australiensis* were analysed with the CLSM microscope. For imaging, the mixed 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, USA) consisting of propidium iodide (PI) and SYTO9 dyes was used for the analysis of mixed 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 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 mixed species biofilms on SS coupons were considered as control.

6.2.8 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 followed by Tukey's LSD test ($p<0.05$).

6.3 Results

6.3.1 Determination of single and mixed 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 mixed species culture in 96-well microtiter plate. CV stains the biomass 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 mixed species cultures during the entire incubation period of 6 days ($p<0.0001$) (Figure 6.1). However, no significant difference in biomass production between *A. australiensis* and mixed species cultures were observed. The effect of incubation period on the biofilm formation by single and mixed 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 mixed species cultures was observed ($p<0.0001$). Biofilm production was observed to be more pronounced on Days 2 and 6. For example, the OD_{595nm} values for *A. australiensis* and mixed species culture were 1.267, 1.038 and 0.977, 1.259 on Days 2 and 6, respectively (Figure 6.1). Also, the lowest OD_{595nm} values were obtained on Day 1 of the incubation period for both single (*A. australiensis* alone) and mixed species cultures. Incubation period influenced the biofilm forming capacity of the single and mixed species cultures.

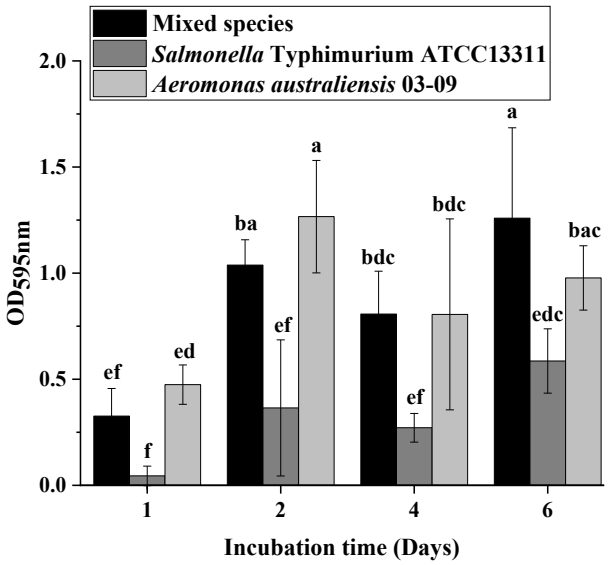


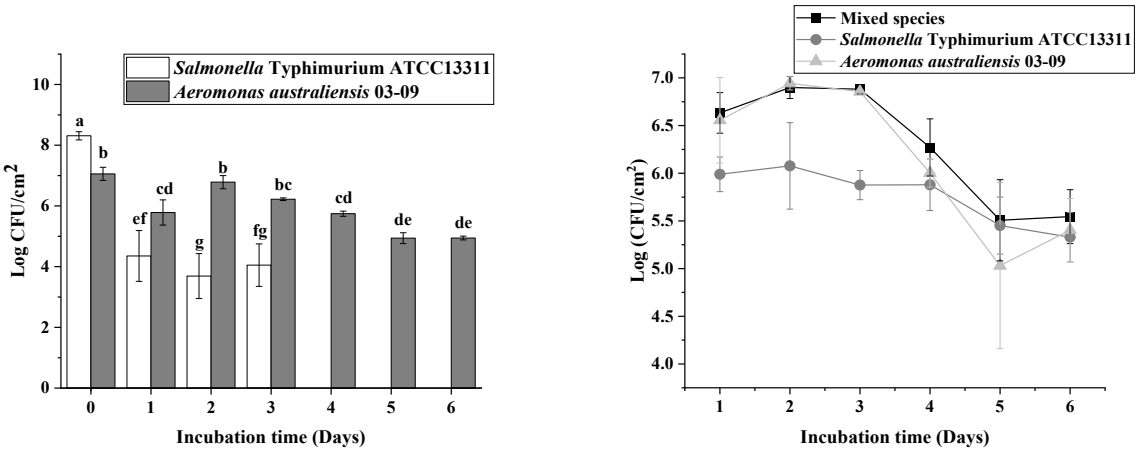
Figure 6.1: Biofilm formation by single and mixed species of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 on 96 well microtiter plate, incubated at ~23°C for 6 days. The quantification of the crystal violet (CV) stained biomass was done by their absorbance measurement at 595 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 ($p<0.05$).

6.3.2 Biofilm formation of single and mixed species on stainless steel coupons

The biofilm formation of single and mixed 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 ($p<0.0001$) (Figure 6.2a). For example, the highest cell attachment obtained in the case of *S.* Typhimurium was 4.35 log CFU/cm^2 on Day 1 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 6.2a). 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 ($p<0.0001$).

For mixed species biofilm study on SS coupons, the cell counts of *S.* Typhimurium and *A. australiensis* were obtained by incubating the mixed culture at different incubation temperatures ideal for the faster growth of these individual bacteria. The mixed 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 mixed species culture ($p<0.0001$) and *A. australiensis* ($p=0.0004$) were significantly higher than *S.* Typhimurium (Figure 6.2b). Incubation period also produced a significant effect on the mixed species biofilm formation on SS coupons. A significant decrease in the biofilm formation was observed from Day 4 till Day 6 (Figure 6.2b). Also, a significant interaction of bacteria and incubation period was observed in the mixed species biofilm study ($p<0.0001$). *S.* Typhimurium showed improved biofilm formation when co-inoculated with *A. australiensis* on SS coupons as opposed to when used alone in this study. For example, the highest cell attachment of *S.* Typhimurium was 4.35 and 6.08 log CFU/cm^2 with initial inoculum of 8.31 and 7.19 log CFU/mL in single and mixed species biofilm study, respectively (Figure 6.2a & b).



(a) (b)

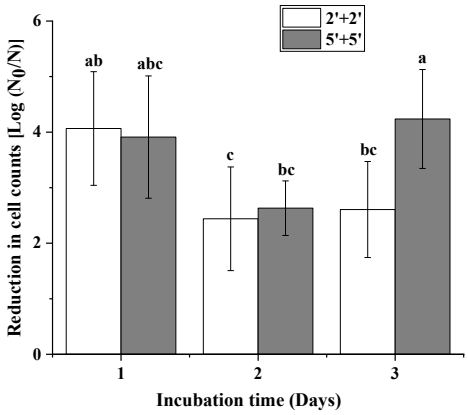
Figure 6.2: Biofilm formation by single species of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 on stainless steel coupons, incubated at 37°C and ~23°C for 3 and 6 days, respectively (a). Values for Day 0 corresponds to log CFU/mL of the inoculum used. Biofilm formation by *S.* Typhimurium and *A. australiensis* in mixed species cultures incubated at 23°C for 6 days (b). The quantification was performed by CFU enumeration every day during the incubation period and *S.* Typhimurium and *A. australiensis* were quantified individually in the mixed species biofilms. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p<0.05$).

6.3.3 Effect of 275 and 455 nm light pulses against single and mixed species biofilms

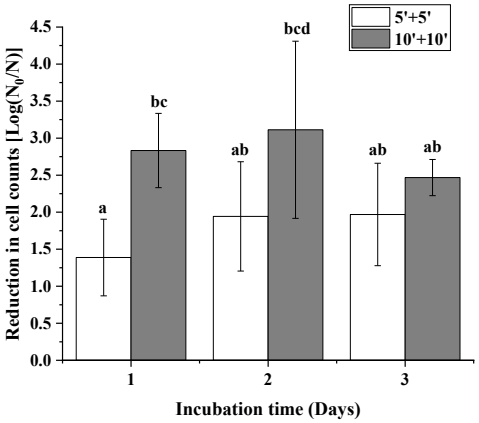
The efficacy of the 275 and 455 nm LED was tested against single and mixed species biofilms on SS coupons by treating both surfaces of the coupons at 4 cm distance from the LED head at 60% power level. The single species *S.* Typhimurium cell attachment and *A. australiensis* biofilms showed significant sensitivity to both 275 and 455 nm LED treatments. The incubation period produced a significant effect on the inactivation efficacy of the 275 nm LED treatment against *S.* Typhimurium ($p=0.0078$) while there was no effect ($p=0.065$) 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 J/cm^2 and 1.8 J/cm^2 on Day 2, produced significantly lesser log CFU/cm^2 reduction in *S.* Typhimurium cell counts as compared to that observed in Days 1 and 3, respectively (Figure 6.3a). Also, the

incubation period produced a significant effect on the sensitivity of *A. australiensis* biofilms towards 275 nm LED treatment for 5 min (~1.8 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 CFU/cm^2 on Days 2 and 4, respectively, to 4.45 log CFU/cm^2 on Day 6 (Figure 6.3a).

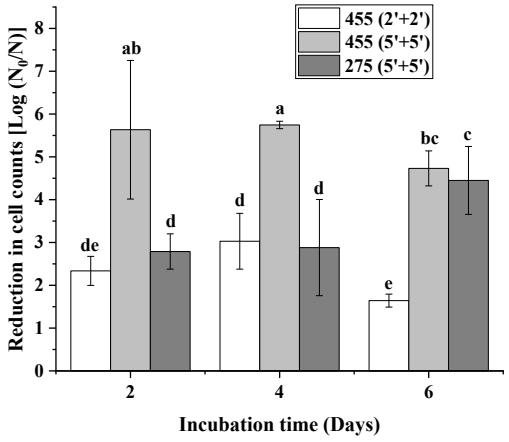
The 455 nm LED treatment for 10 min (174.6 J/cm^2 dose) on each surface of the SS coupons produced significantly higher ($p=0.0089$) reduction in cell counts of *S.* Typhimurium as compared to the 5 min (87.3 J/cm^2 dose) treatment on each surface on Day 1 (Figure 6.3b). However, the same trend was not 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 6.3b). On the contrary, the sensitivity of *A. australiensis* towards the 455 nm LED treatments improved significantly ($p<0.0001$) on increasing treatment time from 2 min (34.92 J/cm^2 dose) on each surface of the SS coupons to 5 min (87.3 J/cm^2 dose) on Days 2, 4 and 6 of the incubation period (Figure 6.3c). Overall, the efficacy of the 455 nm LED decreased significantly on Day 6 compared to Day 4 of the incubation period.



(a)



(b)



(c)

Figure 6.3: Log reduction in cell counts of single species biofilms of *Salmonella* Typhimurium ATCC13311 on stainless steel (SS) coupons after treatment of both the SS coupon surfaces with 275 (a) and 455 nm LED (b). The treatment of single species biofilms of *S.* Typhimurium with 275 nm light pulses was done for 2 (0.72 J/cm^2 dose) and 5 (1.8 J/cm^2 dose) min with 60% power level and at 4 cm distance from the LED head on each surface on Days 1, 2 and 3. The treatment of single species biofilms of *S.* Typhimurium with 455 nm light pulses was performed for 5 (87.3 J/cm^2 dose) and 10 (174.6 J/cm^2 dose) min with 60% power level and at 4 cm distance from the LED head on each surface on Days 1, 2 and 3. Log reduction in cell counts of single species biofilms of *A. australiensis* 03-09 after treatment of both SS coupon surfaces 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 (1.8 J/cm^2 dose) min with 60% power level and at 4 cm distance from the LED head on each surface on Days 2, 4 and 6. The treatment of single species biofilms of *A. australiensis* with 455 nm light pulses was performed for 2 (34.92 J/cm^2 dose) and 5 (87.3 J/cm^2 dose) min with 60% power level and at 4 cm distance from the LED head on each surface on Days 2, 4 and 6. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p<0.05$).

For mixed species biofilm study, the 275 nm LED treatment for 5 min (1.8 J/cm^2 dose) on each surface of the SS coupons resulted in significantly ($p<0.0001$) 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 mixed species biofilms (Figure 6.4a). 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 the case of *S.* Typhimurium and *A. australiensis* individually in the mixed species biofilms (Figure 6.4a). A significant interaction between the 275 nm LED treatment and the incubation period was observed ($p<0.0001$).

The 455 nm LED treatment showed significant inactivation efficacy against mixed species biofilms (also *S.* Typhimurium and *A. australiensis* individually in the mixed species culture (Figures 6.4b & c). The 455 nm LED treatments were also effective in reducing the mixed species biofilms on SS coupons, with the maximum reduction of ~5 log CFU/cm^2 obtained after treatment of the SS coupons for 5 min (87.3 J/cm^2 dose) on each surface. In this study, the 455 nm LED treatment with 5 min (87.3 J/cm^2 dose) on each surface of the coupons produced significantly higher reduction in *A. australiensis* cell counts as compared to *S.* Typhimurium ($p<0.0001$) and mixed species ($p=0.0015$) (Figure 6.4c), indicating that the inactivation efficacy of the 455 nm LED treatment was strain dependent. Overall, there was no significant difference in the inactivation efficacy observed on increasing treatment time (or dose) of the 455 nm LED from 2 min (34.92 J/cm^2 dose) to 5 min (87.3 J/cm^2 dose). Also, there was no influence of the incubation period observed on the inactivation efficacy of the 455 nm LED against mixed species biofilms on SS coupons.

The antibiofilm activity of the 275 and 455 nm LED treatments 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 had no effect in case of mixed species biofilms.

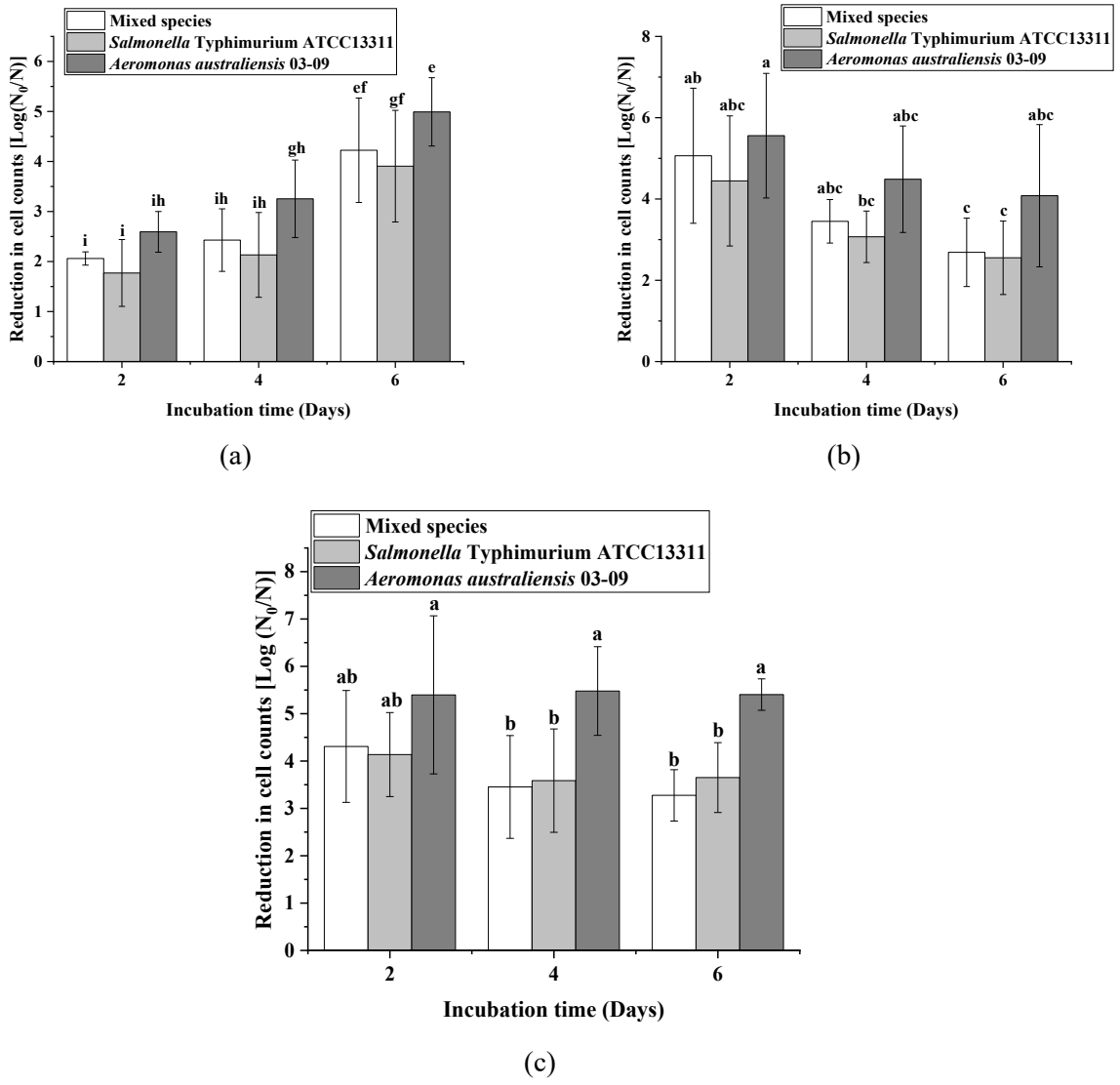
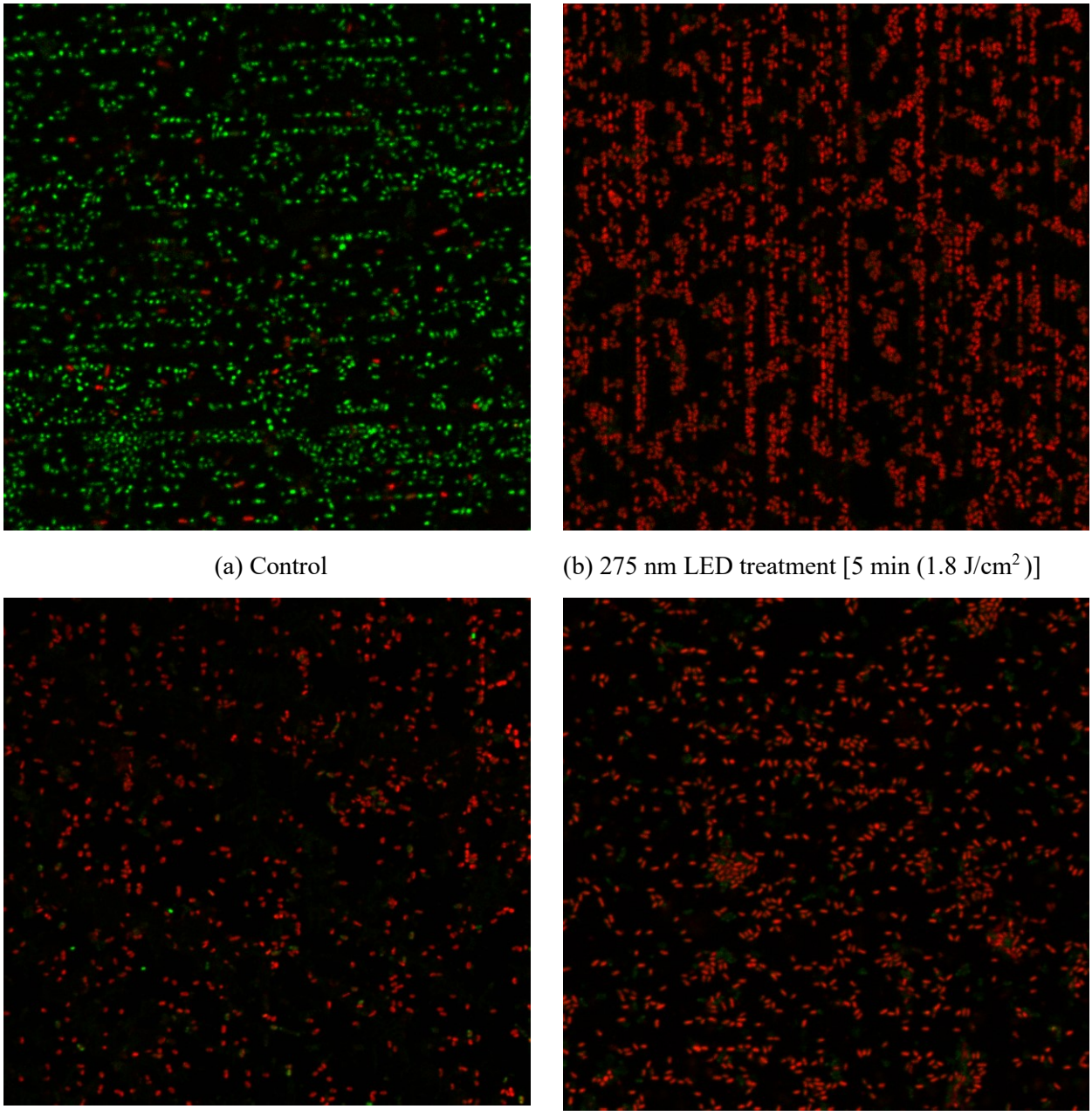


Figure 6.4: Reduction in cell counts [log (N_0/N)] of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 in mixed species biofilms on stainless steel (SS) coupons after treatment of both the SS coupon surfaces with 275 nm LED for 5 min (1.8 J/cm^2 dose) (a); and with 455 nm LED for 2 min (34.92 J/cm^2 dose) (b) and 5 min (87.3 J/cm^2 dose) (c) on each surface. The treatment with 455 and 275 nm light pulses were performed with 60% power level and at 4 cm distance from the LED head on each surface on Days 2, 4 and 6. Results are represented as means ± standard deviation of three independent replicates. Values that carry different superscripts are significantly different from each other ($p<0.05$).

6.3.4 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 mixed 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 6.5. The control has few damaged cells, labelled with PI while the LED treatment with 275 and 455 nm wavelengths light pulses produced significant damage in the bacterial cell membrane (Figures 6.5 b, c & 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 (~87.3 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 6.6 shows the percentage of cell population labelled 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 effect of the wavelength or treatment time on this cell membrane damage.



(a) Control

(b) 275 nm LED treatment [5 min (1.8 J/cm^2)]

(c) 455 nm LED treatment [2 min (34.92 J/cm^2)]

(d) 455 nm LED treatment [5 min (87.3 J/cm^2)]

Figure 6.5: Confocal laser scanning microscopy images of mixed 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 mixed 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 min (1.8 J/cm^2 dose), (c) 455 nm LED treatment with 2 min (34.92 J/cm^2 dose), and (d) 455 nm LED treatment with 5 min (87.3 J/cm^2 dose). The treatments with 275 and 455 nm light pulses were performed with 60% power level and at 4 cm distance from the LED head on Day 2.

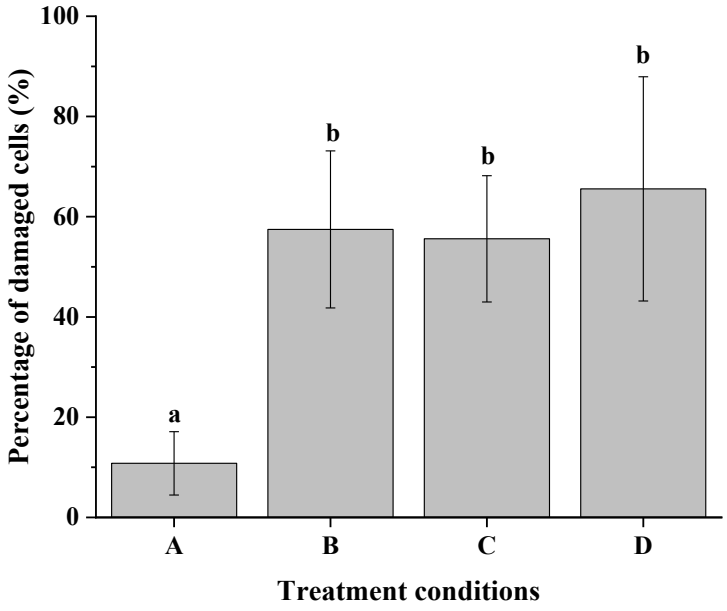


Figure 6.6: Percentage of damaged cells (%) in mixed species biofilms of *Salmonella* Typhimurium ATCC13311 and *Aeromonas australiensis* 03-09 stained with SYTO9 and Propidium iodide (PI) on a surface of stainless steel coupon. The quantification of PI and SYTO9 labelled cells was performed by analysing the z-stack images obtained from the confocal laser scanning microscope. Here, the x-axis includes (A) Control, (B) 275 nm LED treatment for 5 min (1.8 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 (87.3 J/cm^2 dose). The treatments with 275 and 455 nm light pulses were performed with 60% power level and at 4 cm distance from the LED head on Day 2 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 ($p<0.05$).

6.3.5 Temperature increase

LED treatment with 455 nm light pulses can produce surface temperature increase in foods like pet foods (Chapter 4). Therefore, the surface temperature of both surfaces of the SS coupons were monitored during the 275 and 455 nm LED treatments in this study (Table 6.1). 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 of surface 1. The 275 nm LED treatments produced small but

significantly higher surface temperature of SS coupons (Table 6.1). 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.

Table 6.1: Increase in the surface temperature of the stainless steel coupons treated with the LEDs emitting light 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^{dc}
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	1.8	27.33 ± 0.58^{i}	27.67 ± 0.58^{i}

Values are shown as means ± standard deviation of three independent replicates.
Values with same superscripts for the same LED treatments do not differ significantly ($p<0.05$).
*Both the surfaces of the same SS coupons were treated with the light pulses, with surface 1 being treated first, followed by treatment of the surface 2.

6.4 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; Subedi et al., 2020). 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 focussed 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 mixed species on stainless steel 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 mixed species culture (Figure 6.1), indicating that *S.* Typhimurium is a weak biofilm former and *A. australiensis* is a strong biofilm former. This was supported by the viable cell counts obtained from the biofilms formed on SS coupons (Figures 6.2a & b). However, the CV staining can stain the biomass produced by both viable and dead cells (Merino et al., 2019; Pitts et al., 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 mixed species culture in CV staining assay with the highest values obtained at Days 2 and 6 (Figure 6.1), while the CFU enumeration showed that their biofilm formation reduced significantly after Day 3 (Figure 6.2). 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 et al., 2019a).

In contrast, *S.* Typhimurium in single and mixed species biofilm study did not show an 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, & 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. *S.* Typhimurium showed improved attachment on the SS coupons in mixed species biofilm with *A. australiensis* compared to single species biofilm, indicating that the presence of a strong biofilm former (*A. australiensis*) improved the biofilm formation by a weak biofilm former (*S.* Typhimurium) with no effect on the counts of *A. australiensis* in single and mixed species biofilms. This synergistic effect of bacteria in multi-species biofilms on the biofilm formation of *S.* Typhimurium has been previously reported by Visvalingam et al. (2019a). 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 favourable micro-environment that facilitated the biofilm formation of *S.* Typhimurium in mixed species biofilms (Visvalingam et al., 2019a).

The biofilms formed by *S.* Typhimurium and *A. australiensis* in single and mixed species showed significant sensitivity towards treatments with LEDs emitting light pulses of wavelengths 275 (UV-C) and 455 (Blue) nm. Previously, *S.* Typhimurium ATCC14028 biofilms formed on stainless steel 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 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, cell attachment on the food contact surface, distance of the sample from the light source, sample type, power level, treatment time, dose, illumination temperature, relative humidity, etc. (Chapters 3 & 4). In this study, the inactivation efficacy of the

275 nm LED against *A. australiensis* in single and mixed species biofilms was lesser on Days 2 and 4 and was maximum on Day 6 of the incubation period for treatment of 5 min (~1.8 J/cm^2) on each surface (Figures 6.3c & 6.4a). The cell counts of biofilms formed by *A. australiensis* alone and mixed species were higher on Days 2 or 4 and it reduced significantly for Day 6 of the incubation period (Figure 6.2a & 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 towards the UV-C LED treatment (Flemming et al., 2016; Silva-Espinoza et al., 2020).

Significant dose requirements with 87.3 (5 min) and 174.6 J/cm^2 (10 min) dose on each surface were required 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 6.3b). The biofilm attachment of mixed species and *A. australiensis* in single and mixed species biofilms reduced after Day 3 of the incubation period (Figure 6.2a & b). Also, the inactivation efficacy of the 455 nm LED treatments reduced after Day 4 against the single species biofilms of *A. australiensis* (Figure 6.3c). 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 (Valérie, & Brigitte, 1995). However, this trend was not observed in the case of *A. australiensis* in mixed species biofilms, indicating that the presence of *S.* Typhimurium in the mixed species biofilm might have influenced the efficacy of the 455 nm LED treatments against *A. australiensis* in mixed species biofilm. Also, *A. australiensis* in single species biofilms was more resistant to the 455 nm LED treatment of 2 min (~34.92 J/cm^2 dose) on each surface than for the mixed species biofilms (Figures 6.3c & 6.4b). 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 et al., 2019a).

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 et al., 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 emitted with 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, the green fluorescence corresponding to the SYTO9 labelling reduced with the LED treatment using 275 and 455 nm light pulses in this study (Figure 6.5). Also, the treated SS coupon surfaces showed majority of PI labelled red fluorescence (Figure 6.5b, c & 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 showed cells labelled entirely with PI, which can be correlated with the high log reductions obtained with the LED treatments of mixed species biofilms. In the case of 455 nm LED treatments, the surface temperature increase could have also contributed to the increased cell membrane damage (Chapter 4). Since LED treatment is a surface decontamination technology, the confocal 2D images in this study, represent 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 6.6). However, more studies focussing on understanding the inactivation mechanisms of LED technology against biofilms is important.

In conclusion, the 275 and 455 nm light pulses emitted from the LEDs showed significant inactivation efficacy against single and mixed 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 mixed 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 the 455 nm LED treatment. The treatment of the mixed 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 showed the potential application of LED treatment using 275 and 455 nm light pulses for the inactivation of biofilms formed on food contact surfaces, like stainless steel surface.

Chapter 7: Conclusions and recommendations

7.1 Overall conclusions

In this research, the inactivation efficacies of 365 (UV-A), 395 (NUV-Vis) and 455 (Blue) nm light pulses emitted from LEDs at low water activity (a_w) conditions were determined, and the inactivation efficacies were tested in low a_w pet food pellets as a model food system. The drying potential of these high intensity light pulses was also explored. This research also showed the probable inactivation mechanisms of the LEDs emitting light pulses with wavelengths of 365, 395 and 455 nm under low a_w conditions.

In Chapter 3, the antibacterial efficacy of 365 and 395 nm LEDs against *S.* Typhimurium and *E. coli* AW1.7 were observed in different sample types (high a_w and low a_w dried bacteria). The 365 and 395 nm LED treatments showed promising antibacterial effect against highly resistant *Salmonella* cocktail in low a_w pet foods, which was improved by intermediate rehydration of the pet foods. The 365 nm LED treatments showed better inactivation efficacy against *Salmonella* in comparison to 395 nm LED treatments with the same dose (J/cm^2). A significant effect of irradiances (or power levels) was observed on the inactivation efficacy of 365 and 395 nm LED treatments. The high intensity LED treatments produced a significant increase in the surface temperature of the low a_w bacteria and pet foods, which was more pronounced in the case of 395 nm LED treatments. Significant drying due to the LED treatments was mapped using the Guggenheim-Anderson-de Boer (GAB) model and significant reductions in the a_w and moisture content (dry basis) were observed due to both LED treatments.

Since significant drying of treated low a_w samples was observed, the simultaneous drying and antibacterial potential of the 455 nm LED treatments against *Salmonella* at low a_w conditions

and in pet food pellets (food matrix) was explored in Chapter 4. *S.* Typhimurium in dry powdered form showed less sensitivity towards 455 nm LED treatment as compared to that in low a_w pet food pellets. The surface temperature increase due to the LED treatments was more pronounced in pet foods than in dry powdered bacteria, establishing the inactivation efficacy and drying potential of high intensity 455 nm light pulses. This inactivation efficacy was significantly influenced by the illumination temperature and power levels. Introducing a pre-treatment of low intensity 275 nm LED treatment of *Salmonella* inoculated low a_w pet foods improved the inactivation efficacy of the 455 nm LED treatments, while reducing the surface temperature increase. The 455 nm LED treatments showed significant lipid oxidation in low a_w pet food pellets, indicating the generation of reactive oxygen species (ROS). Also, the post-treatment storage showed recovery of *Salmonella* cells in low a_w pet foods.

Chapter 5 focussed on understanding the antibacterial mode of action of the 365, 395 and 455 nm LED treatments. A significant generation of intracellular ROS in the bacterial cells was observed, hence establishing the oxidative stress produced by the light pulses in bacteria as their probable inactivation mechanism in low a_w *S.* Typhimurium. Also, a significant membrane lipid oxidation of *S.* Typhimurium cells was observed due to the 365, 395 and 455 nm LED treatments.

Chapter 6 showed the anti-biofilm potential of 275 and 455 nm LED treatments in single and mixed species biofilms formed by *S.* Typhimurium and *Aeromonas australiensis* on stainless steel coupons. The cell membrane damage observed in CLSM images due to the LED treatments can be one of the mechanisms of LED treatment against biofilms.

Even though the light pulses of specific wavelengths emitted from the LEDs showed promising antibacterial efficacy in high a_w bacteria and against biofilms on stainless steel coupons,

their inactivation effect in low a_w foods showed certain limitations like limited penetration in food matrix, surface temperature increase, and high treatment dose requirements against highly resistant microorganisms in low a_w food, as observed in this research. For instance, the maximum reduction in *S.* Typhimurium cell counts in low a_w pet food pellets equilibrated to 0.75 a_w was by 3 log CFU/g after the treatment with light pulses emitted from the 455 nm LEDs with 784.7 J/cm^2 (~6 kJ/g) dose (corresponding to a treatment time of 45 min) (Figure 4.1, Chapter 4). This treatment also increased the surface temperature of the low a_w pet food pellets from 24 to 74.6°C and produced a significant lipid oxidation of the pellets (Figures 4.1 & 4.9, Chapter 4). Additionally, high doses of 658, 658, and 1614.6 J/cm^2 of the 365, 395 and 455 nm LED treatments reduced the *Salmonella* (5 strain cocktail) cell counts in low a_w pet foods by 0.54, 0.77 and 1.22 log CFU/g (Figure 3.3 & Table 4.2). This bacterial reduction is less than the 4 log reductions in *Salmonella* cell counts in almonds required by FDA (7 CFR 981.442(b)). On the contrary, the treatment of low a_w foods like almonds and pistachio with superheated steam at 200°C for 15 and 30 s produced a reduction of ~6.5 and >5.5 log reductions in *S.* Typhimurium cell counts (Ban, & Kang, 2016). Also, microwave heat treatment of peanut butter with 0.5 a_w using 6 kW power for 5 min reduced the cell counts of *E. coli* O157:H7 and *S.* Typhimurium by 4.03 and >5.17 log CFU/g (Song, & Kang, 2016). Although the other heating technologies require high power and high temperatures in the low a_w conditions, they could reduce the cell counts of foodborne pathogens to desirable levels, as opposed to the low reduction of *Salmonella* obtained with the treatment of low a_w pet foods with the high dose of light pulses emitted from the LEDs in this research.

Food irradiation, which involves ionization of gamma rays, X-rays, and electron beam, is another decontamination technology which has been studied extensively for their antimicrobial efficacy in low a_w foods. For example, the X-rays irradiation of eggshells with 0.5 kGy dose

reduced the *Salmonella* cell counts by 5 log CFU/g (Mahmoud et al., 2015). Similarly, gamma irradiation of cookie dough with 2.5 kGy produced a reduction of 5.34 log CFU/g in *S.* Typhimurium cell counts (Jeong, & Kong, 2017). This shows that these radiations produced better inactivation efficacy against *Salmonella* in low a_w conditions in comparison with the light pulses emitted from LEDs in this research. Additionally, several studies have reported the promising antibacterial efficacy of combining different thermal and non-thermal technologies against foodborne microorganisms in low a_w systems like spices. For instance, the combination of superheated steam treatment and infrared treatment at 90°C for 70 s produced a reduction of 5.73 log CFU/g in *Salmonella* population on grated almonds (Bari et al., 2009). Similarly, combining the UV-C light treatment with 2.04 J/cm^2 dose with a mild heat treatment at 65°C reduced the *E. coli* O157:H7 and *S.* Typhimurium cell counts by 2.88 and 3.06 log CFU/g in powdered red pepper, respectively, with minimal effect on the food quality (Cheon et al., 2015).

Therefore, improving the inactivation efficacy and reducing the high dose requirement of the high intensity light pulses emitted from the LEDs in low a_w foods, would require certain intervention strategies like combining the technology with other thermal or non-thermal technologies. Also, in this research, the 365 nm LED light pulses produced better inactivation efficacy against *S.* Typhimurium in low a_w conditions as compared to the high intensity 395 and 455 nm LED light pulses with minimal increase in the surface temperature, indicating the better germicidal efficacy of 365 nm wavelength light pulses. Therefore, combining the 365 nm LED light pulses with mild heat treatment or other non-thermal technologies like irradiation or high-pressure CO_2, could help in the improvement of the inactivation efficacy of the light pulses emitted from the LEDs in low a_w system with limited dose requirement and minimal effect on the quality of the food. This strategy could also be explored for the high intensity light pulses emitted from

the 395 and 455 nm LEDs in low a_w food matrices. Additionally, understanding the inactivation efficacy and the underlying mechanisms of the light pulses emitted from the LEDs against multilayered biofilms of pathogens is important.

7.2 Recommendations

This research has demonstrated the antibacterial efficacy of the LED technology emitting light pulses of specific wavelengths in the low a_w food systems with an insight into their antibacterial mechanisms in such foods. Also, this study has established the antibiofilm activity of UV-C and blue light pulses. The following aspects of the LED treatments can be further explored:

1) The potential application of the combination of LEDs emitting lights with different wavelengths was observed in Chapter 4. More detailed research in understanding the antibacterial efficacy of LEDs when combined with other non-thermal technologies like high pressure CO_2 or with mild thermal treatments, and their underlying mode of action can be explored.

2) The production of ROS was observed in both 365 and 395 nm LED treatments. However, the 365 nm LED produced more inactivation as compared to 395 nm LED treatments with the same dose. Future research directions could include understanding the effect of light pulses on the DNA and protein of the bacteria or understanding its effect on the proliferation of bacterial cells.

3) The detection of the porphyrin compounds (light absorbing molecules) in specific bacteria and determination of their light absorption range could be performed for understanding the role of endogenous photosensitizers in the antibacterial activity of the LED treatments.

4) Since a recovery of *Salmonella* during the post-treatment storage of low a_w pet food pellets were observed in this research, the effect of LEDs emitting light pulses of 275, 365, 395 and 455 nm on the defence mechanism of *Salmonella* could be evaluated in such foods.

5) Since a significant lipid oxidation of the low a_w pet food pellets was observed in this research, more research on understanding the effect of the light pulses of different wavelengths on the quality of the food product is required. Future research should explore ways to reduce lipid oxidation or other quality changes in foods during LED treatment.

6) The effect of light pulses emitted from the LEDs on other low a_w foods like powdered spices, nuts and others could be studied. Future research can explore ways to improve the drying and inactivation efficacy of the LED treatment. For instance, conducting the LED treatments under vacuum condition can possibly improve the drying potential of the LED technology, while reducing the lipid oxidation of food products.

7) The LEDs emitting light pulses of different wavelengths can be evaluated for their antibiofilm activity, as the mode of action of light pulses might vary depending on the wavelength used. Detailed study on understanding the antibiofilm mechanisms of the LED treatments on different food contact surfaces can be explored.

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Appendix 1: Supplementary figure for Chapter 3

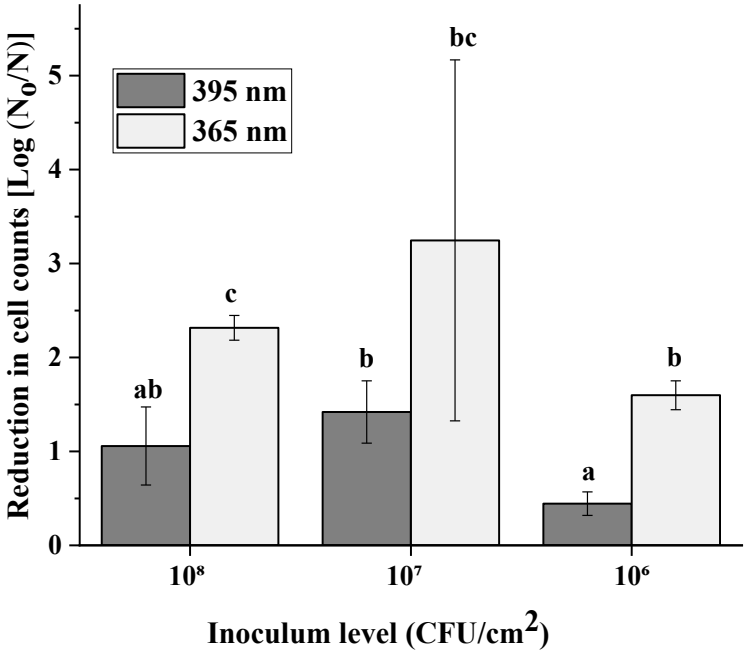


Figure A1: Efficacy of 365 and 395 nm against different inoculum levels of *Salmonella enterica* Typhimurium ATCC13311 equilibrated to 0.75 a_w. The treatments of low a_w *S.* Typhimurium with 365 and 395 nm light pulses were performed with similar dose of 139 J/cm^2, corresponding to treatment times of 45 and 10 min, respectively. The treatments were performed at 60% power level and at 4 cm from the LED heads. Bars with same letter are not significantly different ($p<0.05$). Error bars indicate the standard deviation (n=3).

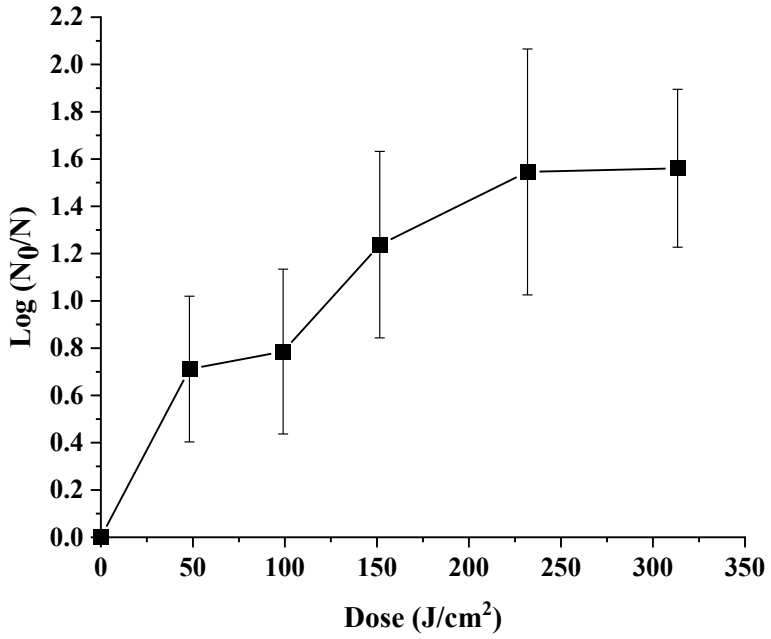
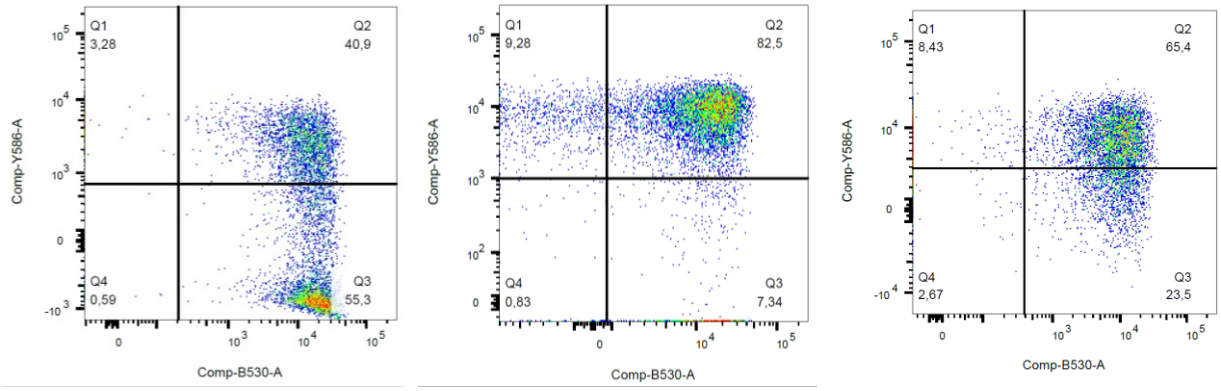


Figure A2: The efficacy of 365 nm LED against dried *Salmonella enterica* for different dose treatments at 100% power level with 4 cm height between the sample and the LED head. The dose used were 48.1 J/cm^2 (10 min), 99.0 J/cm^2 (20 min), 151.5 J/cm^2 (30 min), 231.8 J/cm^2 (45 min) and 313.4 (60 min) J/cm^2. Here, N_0 represents the CFU/g in control and N represents the CFU/g in the treated samples. Error bars indicate the standard deviation (n = 3).

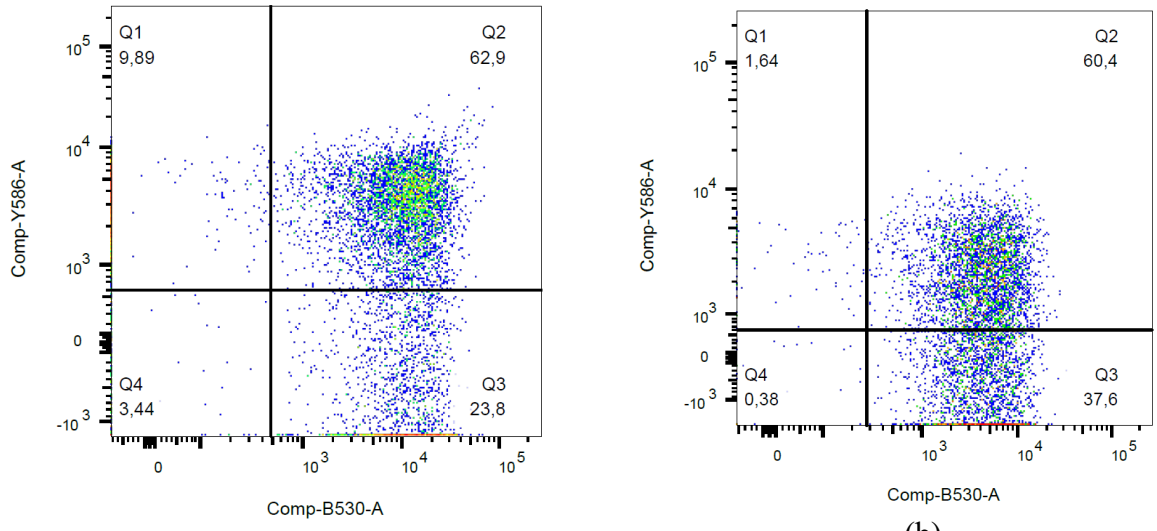
Appendix 2: Flow cytometry layouts for Chapter 5

LIVE/DEAD Baclight assay



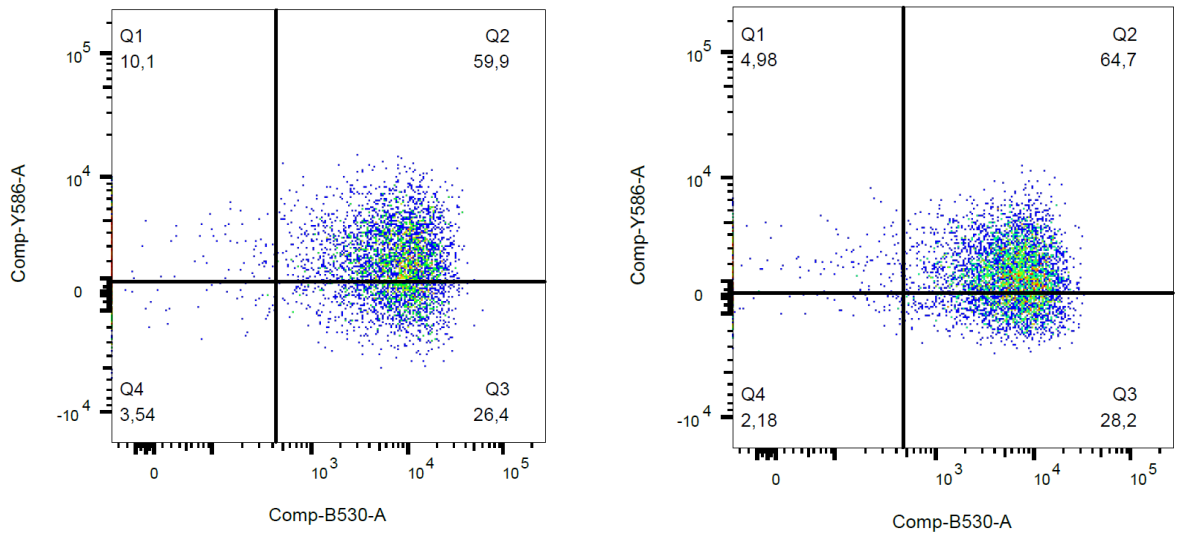
(a) (b) (c)

Figure A3: Flow cytometry layouts for the LIVE/DEAD Baclight assay of *Salmonella* Typhimurium ATCC13311 in overnight culture (a), after drying (b), and after equilibration to 0.75 a_w (c).



(a) (b)

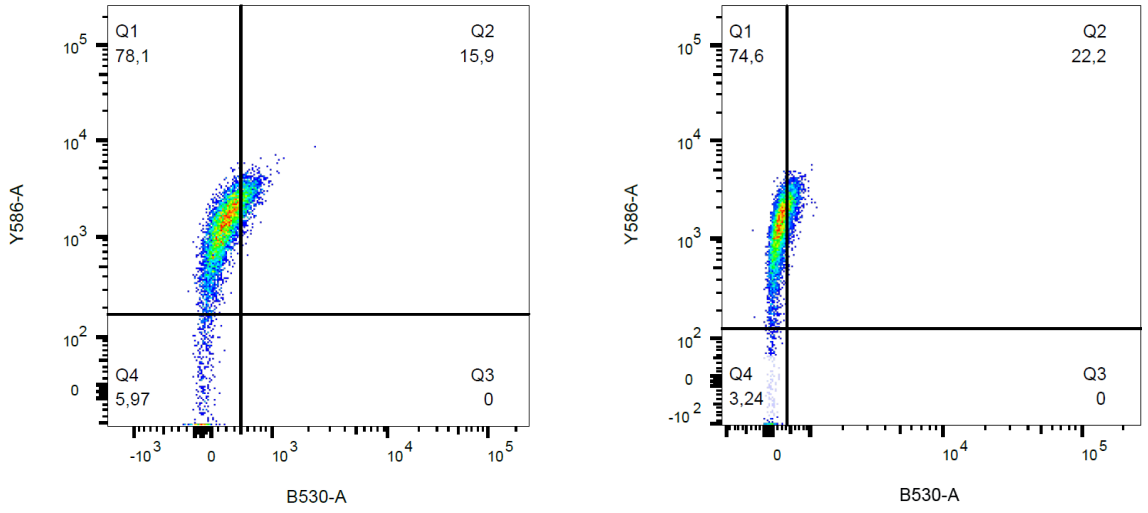
Figure A4: Flow cytometry layouts for the LIVE/DEAD Baclight assay of *Salmonella* Typhimurium ATCC13311 equilibrated to 0.75 a_w after treatment for 10 min (28.9 J/cm^2 dose) (a), and 60 min (188.1 J/cm^2 dose), with 365 nm light pulses at 60% power level and at 4 cm from the LED head.



(a) (b)

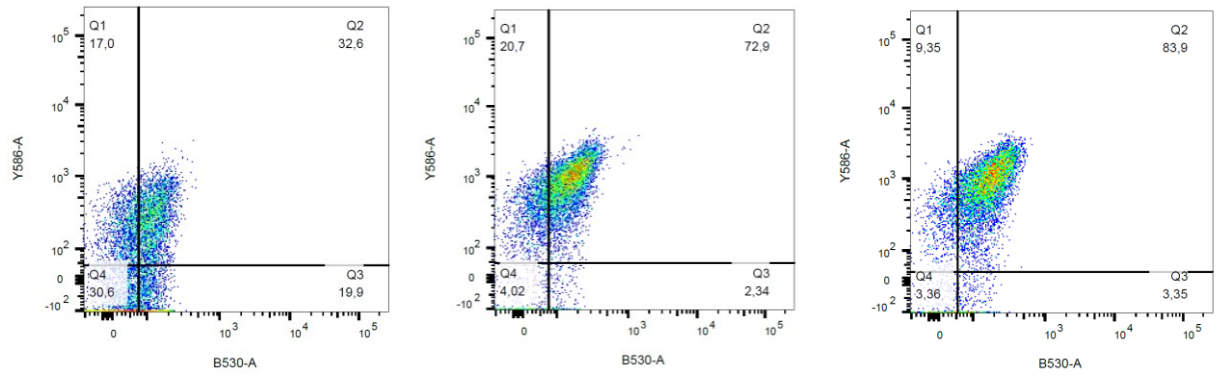
Figure A5: Flow cytometry layouts for the LIVE/DEAD Baclight assay of *Salmonella* Typhimurium ATCC13311 equilibrated to 0.75 a_w after treatment for 10 min (138.8 J/cm^2 dose) (a), and 60 min (834.4 J/cm^2 dose), with 395 nm light pulses at 60% power level and at 4 cm from the LED head.

C11-BODIPY$^{581/591}$ assay



(a) (b)

Figure A6: Flow cytometry layouts for the membrane lipid oxidation of *Salmonella* Typhimurium ATCC13311 cells after vacuum drying (for 18-20 h) (a) and equilibration to 0.75 a_w (20-24 h), using C11-BODIPY$^{581/591}$ dye as a fluorescent indicator.



(a) (b) (c)

Figure A7: Flow cytometry layouts for the membrane lipid oxidation of *Salmonella* Typhimurium ATCC13311 cells equilibrated to 0.75 a_w due to the treatments with 365 nm (217 J/cm^2 dose) (a), 395 nm (217 J/cm^2 dose) (b) and 455 nm (250 J/cm^2 dose) (c) light pulses at 80% power level and at 4 cm from the LED head, using C11-BODIPY$^{581/591}$ dye as a fluorescent indicator.

Appendix 3: Supplementary figures for Chapter 6

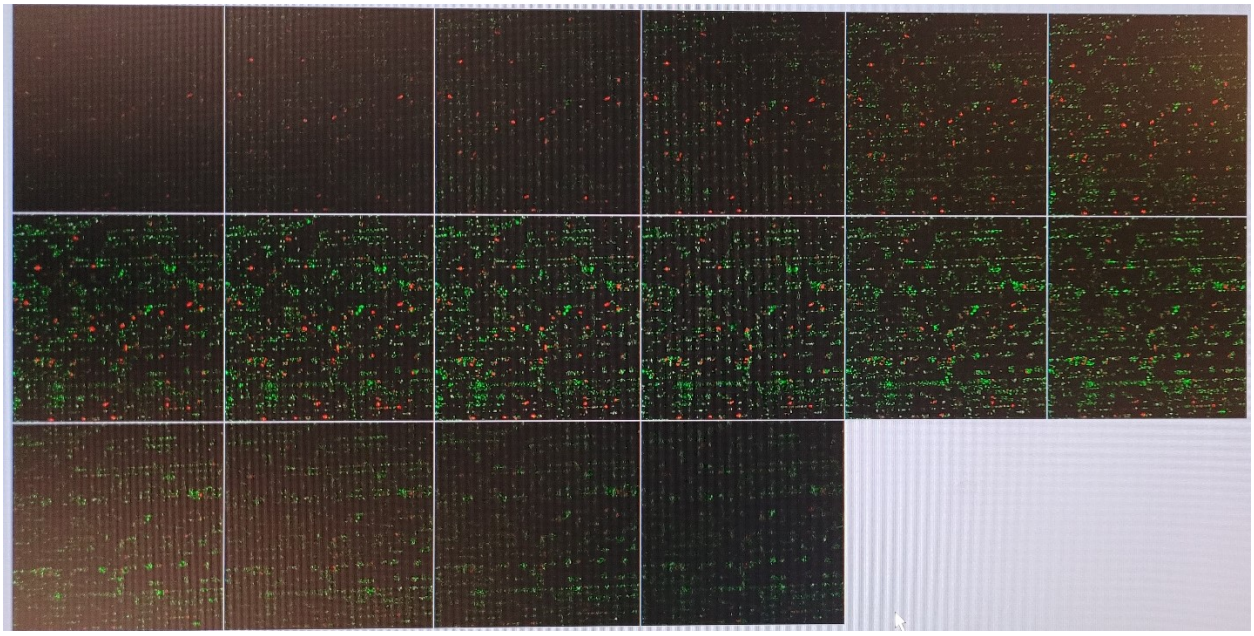


Figure A8: Confocal laser scanning microscopy image of untreated mixed species biofilms of *S.* Typhimurium and *A. australiensis* (Control) attached on stainless steel coupons stained with SYTO9 and Propidium iodide (PI). Here, the images of the layers scanned in the z-stack image are shown.

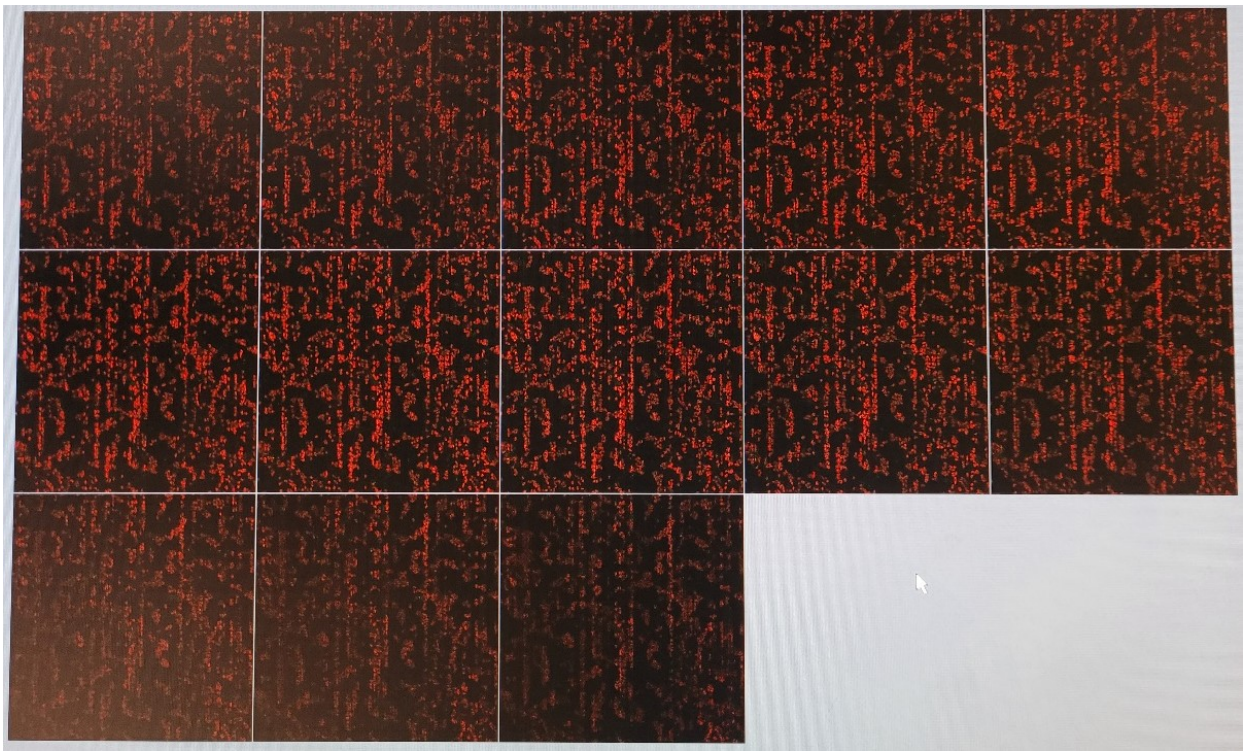


Figure A9: Confocal laser scanning microscopy image of mixed species biofilms of *S.* Typhimurium and *A. australiensis* attached on stainless steel coupons after treatment with LEDs emitting light pulses of 275 nm, followed by staining with SYTO9 and Propidium iodide (PI). Here, the images of the layers scanned in the z-stack image are shown. The 275 nm LED treatment was performed for 5 min (~1.8 J/cm^2 dose) at 60% power level and at 4 cm from the LED head.

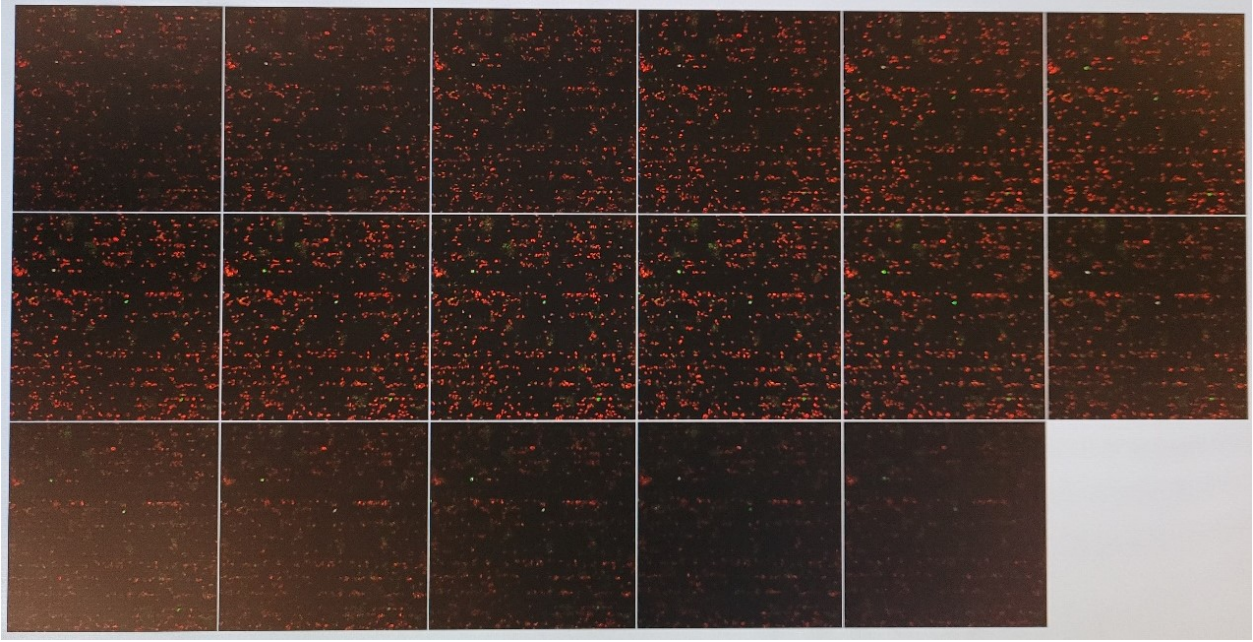


Figure A10: Confocal laser scanning microscopy image of mixed species biofilms of *S.* Typhimurium and *A. australiensis* attached on stainless steel coupons after treatment with LEDs emitting light pulses of 455 nm, followed by staining with SYTO9 and Propidium iodide (PI). Here, the images of the layers scanned in the z-stack image are shown. The 455 nm LED treatment was performed for 2 min (~34.92 J/cm^2 dose) at 60% power level and at 4 cm from the LED head.

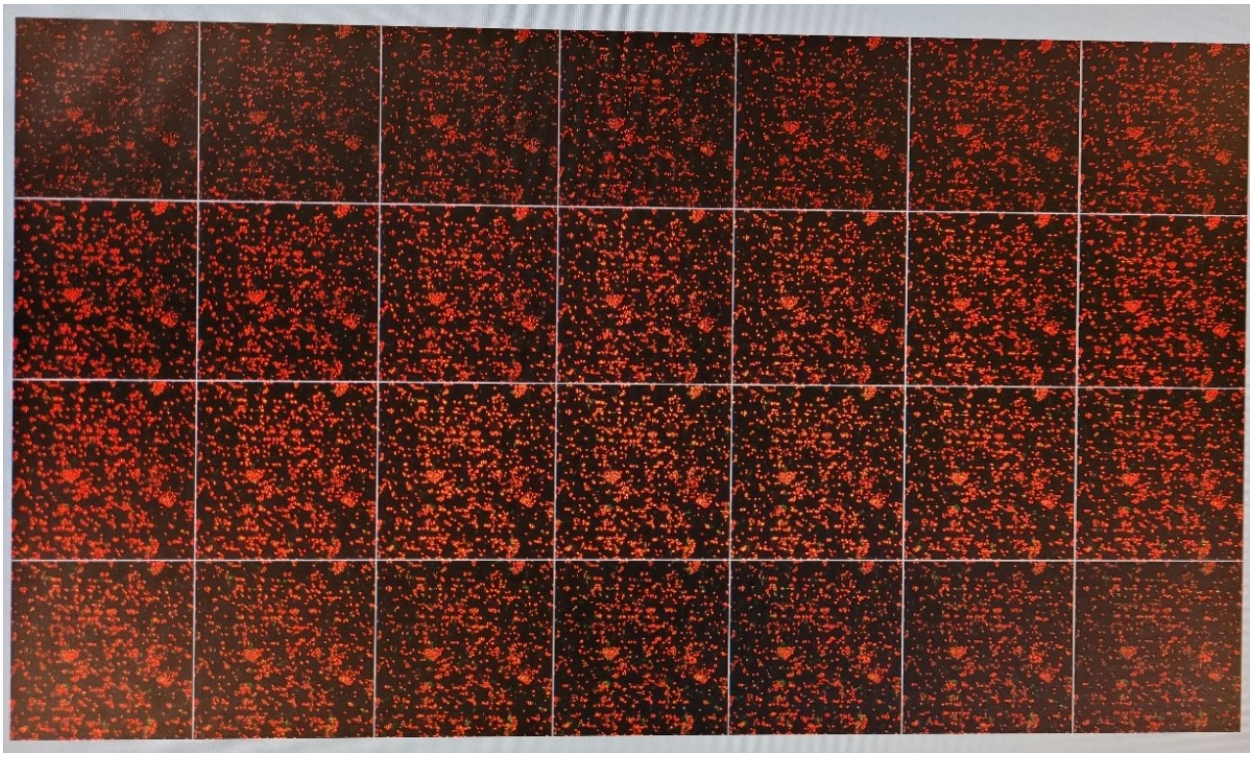


Figure A11: Confocal laser scanning microscopy image of mixed species biofilms of *S.* Typhimurium and *A. australiensis* attached on stainless steel coupons after treatment with LEDs emitting light pulses of 455 nm, followed by staining with SYTO9 and Propidium iodide (PI). Here, the images of the layers scanned in the z-stack image are shown. The 455 nm LED treatment was performed for 5 min (~87.3 J/cm^2 dose) at 60% power level and at 4 cm from the LED head.