

## ABSTRACT

Title of Thesis: PHOTOIRRADIATED CAFFEIC ACID AS AN ANTIMICROBIAL TREATMENT FOR FRESH PRODUCE

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The antimicrobial efficacy of 400 nm photoirradiated caffeic acid (CA, 5 mM) was evaluated against *Escherichia coli* O157:H7 and *Listeria innocua*. A stronger antimicrobial effect was observed on *E. coli* than on *L. innocua* where the combined treatment resulted in 4 and 1 log CFU/mL reductions, respectively. The treatment's effects on metabolism (resazurin assay), uptake of CA (fluorescence technique) and membrane damage (propidium iodide assay) were studied in both species. CA uptake increased in both species, but membrane damage was only observed in *E. coli*. The treatment had minimal impact on metabolic activity in both species. The treatment applied to the surface of spinach leaves was found to be effective against *E. coli*. A combined treatment of 400 nm light and plant extracts known to contain CA was also evaluated for antimicrobial activity against *E. coli* and found to be effective. The novel treatment proposed in this study has the potential to improve the microbial food safety of fresh produce.

PHOTOIRRADIATED CAFFEIC ACID AS AN ANTIMICROBIAL  
TREATMENT FOR FRESH PRODUCE

by

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## Dedication

This work is dedicated to my parents, Dr. Percy Gilbert and Mrs. Shireen Gilbert, who have shown me love and support throughout my academic and professional career.

## Acknowledgements

I would like to thank my advisor, Dr. Rohan Tikekar, for his guidance and encouragement. I would also like to thank Dr. Abani Pradhan and Dr. Wendy-Ann Peer for serving on my committee and providing me with valuable instruction during the pursuit of my degree. I would also like to thank my lab mates Dr. Solmaz Alborzi, Dr. Luis Bastarrachea, Dr. Hongchao Zhang, Qinyang Wang, Qiao Ding, Heather Dolan, and Robert Sherman-Wood for their help and support during the research process.

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## Chapter 1: Introduction

As consumer preferences and awareness change, the demand for fresh and minimally processed foods has increased in recent years. The consumption of fresh produce eaten raw has likely increased due to more widespread availability, taste, and perceived health benefits (Ragaert, Verbeke, Devlieghere, & Debevere, 2004; Siddiqui, n.d.; Yildiz, 2017). Unfortunately, consuming fruits and vegetables without an adequate “kill step”, that typically results in a 5-log reduction in bacteria (Breidt, Hayes, Osborne, & Mcfeeters, 2005), increases the risk of foodborne illness. From 1998 to 2008, 46% of all foodborne illnesses from the US were caused by fresh produce (Painter et al., 2013). Ready to eat fresh cut produce that is typically washed in a production facility has also been implicated in large foodborne illness outbreaks. Most recently Mann Packing recalled a number of minimally processed vegetable products due to possible *Listeria monocytogenes* contamination in products sold in US and Canadian markets in October 2017 (Affairs, n.d.). In the summer of 2017, several salmonellosis outbreaks caused by contaminated papaya led to 210 confirmed cases with one death, across 24 states (“Infections Linked to Yellow Maradol Papayas | July 2017 | Salmonella | CDC,” n.d.).

To combat pathogenic bacteria present while still preserving the texture, taste, and quality of the fresh produce, sanitizers are added to the wash water used to clean the produce post-harvest. A commonly used sanitizer in the United States is chlorine in the form of sodium hypochlorite, used at concentrations at or below 2000 ppm (Nutrition, n.d.). This concentration of chlorine results in a 2-log reduction in the

bacteria present, however, this level of inactivation may be too low to prevent illness outbreaks (Schmid-Hempel & Frank, 2007). Pathogens such as *Listeria monocytogenes* and some strains of Shiga Toxin Producing *Escherichia Coli* (STEC) only need to be present at levels of 1 to 2 log Colony forming units (CFU) per serving to cause illness. In addition to its low antimicrobial efficacy, chlorine treatment has several other drawbacks. Sodium hypochlorite tends to bind to organic matter, preventing it from inactivating the bacteria present, and when added to wash water at high enough volumes can lead to the formation of gaseous trihalomethanes that can be very hazardous to workers (Shen, Norris, Williams, Hagan, & Li, 2016; WHO, 1998). Due to safety concerns, chlorine treatments are not acceptable sanitizers in the European Union (Johnson, 2015).

To combat pathogens, alternative non-thermal chemical and physical antimicrobial treatments are being explored for use on fresh produce. Plant based phenolic compounds have been shown to be mild antimicrobials (Daglia, 2012) and certain compounds, such as benzoic acid, are already added to food products as preservatives. These compounds are typically already present in plants and may satisfy consumer desires for an all-natural product. Caffeic acid is one such phenolic compound commonly found in a number of foods (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004) and is present in post processing food waste items such as grape seeds and olive leaves (Ramos-Cormenzana, Juárez-Jiménez, & Garcia-Pareja, 1996; Shi, Yu, Pohorly, & Kakuda, 2003). Prior research has shown that it is a mild antimicrobial agent that may be suitable for use in a variety of food applications due to its emulsifying properties (Almajano, Carbó, Delgado, & Gordon, 2007). Almajano

and coworkers reported that caffeic acid had minimum inhibitory concentrations of 0.2% to 0.4% in nutrient broth against seven strains of bacteria and one strain of yeast (Almajano, Carbó, Delgado, & Gordon, 2007). Caffeic acid is also a more lipophilic compound, and this property may allow it to interfere with bacterial membranes. The lipophilic nature of the compound may be responsible for its antimicrobial properties (Vaquero, Alberto, & de Nadra, 2007). In addition to chemical treatments, light based physical treatments show promise as a more effective antimicrobial treatment for fresh produce. Both ultra violet and visible light have been shown to be effective at inactivating pathogenic bacteria. Visible light in the blue range of wavelengths has been shown to be highly effective at killing or suppressing the growth of *Listeria* species and through the generation of reactive oxygen species (O'Donoghue et al., 2016; Guffey, 2016).

Combining a chemical phenolic treatment with a physical light-based treatment is currently being explored as a non-thermal antimicrobial treatment for fresh produce and other minimally processed foods. Research has shown that certain phenolics can act as photosensitizers when combined with light (Canonica, Jans, Stemmler, & Hoigné, 1995; Nakamura et al., 2012). Upon excitation, the phenolics will generate reactive oxygen species (ROS) that can inactivate pathogenic bacteria through oxidative cell damage (Vatansever et al., 2013). In some cases, small phenolics are believed to be uptaken by pathogenic bacteria and release ROS in the bacteria cytosol, resulting in an effective treatment that will negatively impact the sensory attributes of the produce being washed (Nakamura et al., 2012). We are currently exploring the combination of caffeic acid and 400 nm light as an antimicrobial

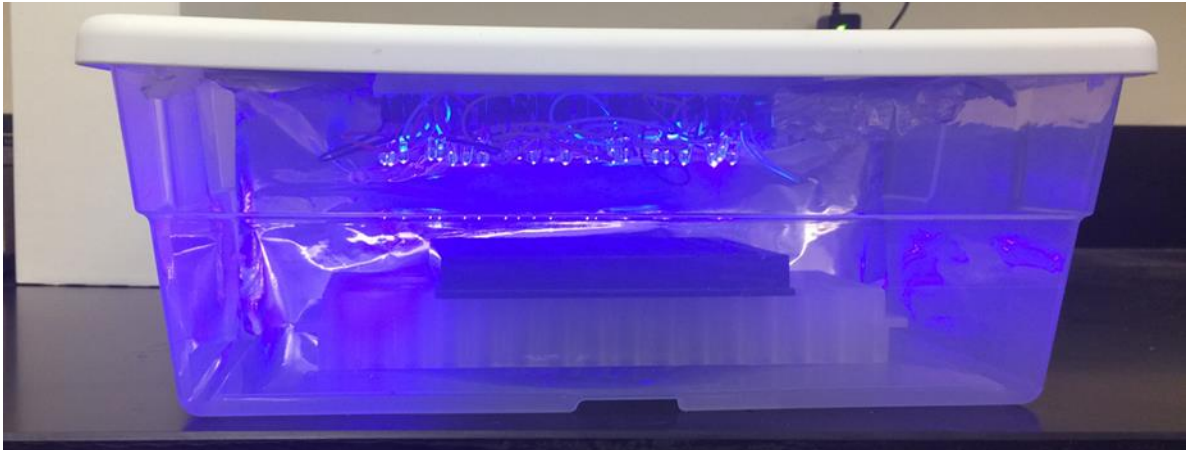
treatment for fresh produce. Prior research has shown that caffeic acid is likely to yield ROS in solution when treated with 400 nm light leading to pathogen inactivation. The combination of the two treatments is thought to have a synergistic effect with the simultaneous treatment leading to greater microbial inactivation than either of the two treatments applied alone. Light emitting diodes (LEDs) were used as the light source as they are less energy intensive as compared to other light sources and are also being explored as an indoor light source for sprouts and microgreens (D'Souza, Yuk, Khoo, & Zhou, 2015). Caffeic acid and blue light may also have a non-ROS mediated mechanism for microbial inactivation. When the ideal treatment combination is determined, caffeic acid can be a highly effective, non-thermal antimicrobial treatment for fresh produce and other foods commonly consumed without heat treatment. I hypothesize that caffeic acid combined with light will result in a synergistic antimicrobial effect against pathogenic bacteria. I also hypothesize that this combined treatment will be similarly effective on non-spore-forming species of gram-positive and gram-negative bacteria. Lastly, I hypothesize the mechanism of microbial inactivation is driven by oxidative stress from reactive oxygen species generated by the caffeic acid treated with 400 nm light.

## Chapter 2: Materials and Methods

### Evaluation of Antimicrobial Effect of Caffeic Acid and 400 nm Blue Light

#### **Construction of LED Light Array**

A bench-top, batch type light chamber was constructed to emit 400 nm light at the intensity of 1.3 million lumen per square meter as measured with a luminometer (LT3000 Extech Instruments, Waltham MA). A light array was constructed using a prototype board and single wavelength, through-hole LED bulbs with a 30-degree viewing angle (Super Bright LEDs, St Louis MO). The prototype board was connected to a DC power source using a power supply and plug purchased from an online retailer. The light array was placed inside a Sterilite brand container (United States Plastics Corp. Lima, Ohio) and lined with aluminum foil. For all experiments, the light source was 10 cm from the surface of the item treated. The light array is pictured below.



## **Evaluation of Hydrogen Peroxide Generation**

A ferrous oxidation-xylenol orange (FOX) assay was performed to evaluate generation of hydrogen peroxide in photoirradiated caffeic acid (Jiang, Hunt, & Wolff, 1992). Caffeic acid was solubilized in deionized water with 5 minute agitated heating and an additional 5 minute of stirring on a hot plate, then allowed to cool to room temperature. 10 mL of the caffeic acid solution was transferred to a six well plate (Costar, Corning NY) and placed inside of bench top batch type UV-cross linking chambers for UVA (365 nm), UVB (312 nm), and UVC (254 nm) light as well as the self-constructed light array. After treatment, 300  $\mu$ L of the solution was incubated for 15 min with the FOX assay reagent. Then 100  $\mu$ L of the solution was transferred to a 96-well plate and measured for absorbance at 560 nm using a Spectramax M5e plate reader (Molecular Devices LLC, Sunnyvale CA). The absorbance spectra of Caffeic acid was also evaluated using the cuvette reader of the aforementioned plate reader.

## **Antimicrobial Assay**

The antimicrobial efficacy of photoirradiated caffeic acid was evaluated using *Escherichia coli* O157:H7 and *Listeria innocua*. Bacteria strains were incubated in tryptic soy broth overnight at 37°C for 20 h reaching a concentration on roughly  $10^9$  log CFU/mL. Experiments were carried out with *E. coli* after the 20-hour incubation, with the pathogen in the mid-stationary phase of growth. With *L. innocua*, 200  $\mu$ L of the overnight culture was re-inoculated in tryptic soy broth for an additional five h

prior to performing the microbial inactivation experiments. Both strains of bacteria were diluted in deionized water to reach an initial concentration of  $10^4$  to  $10^5$  log CFU/mL. Experiments were carried out using 5mM caffeic acid solution, pH 3.55, prepared in deionized water. This concentration was chosen since it is close to the saturation point for the compound in room temperature water of 0.98g/L (Mota, Queimada, Pinho, & Macedo, 2008). Caffeic acid was solubilized with agitated heating for 5 minute and an additional 5 minute of stirring on a hot plate, then allowed to cool to room temperature and filter sterilized with a 25 mm diameter 0.2 micron filter (Fisher Scientific, Pittsburgh PA). 0.2 % buffered peptone water was used as a control. 5 mL of solution with initial microbial loads of  $10^4$  to  $10^5$  CFU/mL were used for the initial microbiological assay. Samples were placed in a 6-well plate (Costar, Corning NY) under the light array. 100  $\mu$ L aliquots were collected at 15-minute intervals over a 2-hour period. Samples were then diluted in 0.2% buffered peptone water and plated on tryptic soy agar. The combined treatment was evaluated on *E. coli* after a 30 minute treatment time. Bacteria were enumerated by plating on tryptic soy agar.

### *Examination of Antimicrobial Mechanism*

#### **Evaluation of Antimicrobial Activity in Presence of Dimethyl Sulfoxide**

To examine the role of reactive oxygen species (ROS) on antimicrobial activity, the antimicrobial experiments were performed in the presence of dimethyl sulfoxide (DMSO), a well-known ROS quencher (Jacob & Herschler, 1986). Experiments were performed on *E. coli* following the antimicrobial efficacy procedure described above

with all solutions containing 1% DMSO. Five mL of each solution was transferred to a six-well plate and treated with blue light for 60 minute. Bacteria were enumerated on tryptic soy agar.

### **Evaluation of Antimicrobial Activity at Elevated pH**

To examine the effect of pH on antimicrobial activity, the antimicrobial experiments were performed in the presence of 0.1 M phosphate buffered saline, pH 6.31. Experiments were performed on *E. coli* following the antimicrobial efficacy procedure described above with all solutions containing 0.1 M phosphate buffered saline. Five mL of each solution was transferred to a six-well plate and treated with blue light for 60 minute. Bacteria were enumerated on tryptic soy agar.

### **Measurement of Metabolic Activity within Bacteria**

A resazurin assay was performed on both *E. coli* and *L. innocua* to assess the treatment's effect on cell metabolism after 15 minute of treatment. Metabolic activity was observed at this treatment time as no microbial inactivation was observed at 15 minute in previous experiments. The treatment procedure was modeled after the method used by Cossu and coworkers (Cossu et al., 2016). Overnight cultures of *E. coli* and *L. innocua* were pelletized by centrifuging 1 mL of the culture at 10,000 RCF for 2 minute. The pelletized cells were suspended in 5mM caffeic acid solution and treated under 400 nm light for 15 minutes. Post treatment, the cells were pelletized, washed, and re-suspended in tryptic soy broth with 50 µm resazurin dye. 100 µL of each sample was transferred to a 96- well plate and fluorescence readings



(emission/excitation: 560/590nm) were then taken using a Spectramax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) while incubating at 37°C over 4 h.

### **Evaluation of Bacterial Membrane Damage**

Membrane damage caused by the combination of caffeic acid and 400 nm light was evaluated using propidium iodide (PI). The propidium iodide was used in a spectrofluorometric assay following a procedure similar to that of Wang and coworkers (Wang, de Oliveira, Alborzi, Bastarrachea, & Tikekar, 2017). Pelletized overnight cultures of *E. coli* and *L. innocua* were suspended in 5 mM caffeic acid solution and treated under blue light for 30, 60 and 90 minute. After treatment, the cells were re-pelletized, washed twice in DI water, and incubated with 50 µL of propidium iodide for 15 minute. The pellet was the suspended in 500µL 1× phosphate buffered saline. Fluorescence readings were taken at excitation and emission wavelengths of 490/635 nm.

### **Evaluation of Caffeic Acid Uptake**

Caffeic acid uptake was evaluated using a diphenylboric acid 2-aminoethyl ester (DPBA) as an indicator following a procedure similar to that of Wang and coworkers (Wang et al., 2017). Pelletized overnight cultures of *E. coli* and *L. innocua* were suspended in 5 mM caffeic acid solution and treated under blue light for 30 mintue. After the treatment, the cells were re-pelletized, washed twice in DI water, and suspended in 450 µL of DPBA solution (2% v in DMSO). 100 µL of each sample

was then transferred to a 96 well plate and evaluated for fluorescence using the Spectramax 5Me plate reader with excitation/emission wavelengths of 405/465 nm.

*Applications of Caffeic Acid and 400 nm Light as Antimicrobial Treatment in Sanitation of Fresh Produce*

**Evaluating Bacterial Inactivation on the Surface of Fresh Spinach**

The inactivation of *E. coli* using 5mM caffeic acid and 400 nm light was evaluated on baby spinach. Conventionally grown spinach was purchased from a local retailer, and washed with 2% bleach solution, rinsed twice with DI water and air dried in a biosafety cabinet for 30 min. An overnight culture of *E. coli* was diluted to  $10^5$  to  $10^6$  CFU/mL. After drying, 100  $\mu$ L of the bacterial suspension was spot inoculated onto the adaxial side of the leaf and allowed to dry for 10 minutes. The leaves were then flooded with 5mM caffeic acid solution treated under the LED light array for 30, 60 and 90 minutes. After treatment, the leaves were placed in a whirl-pak bag with 9 mL of 0.2% buffered peptone water and rubbed and agitated to remove bacteria from the surface. Bacteria were enumerated by plating on Luria-Bertani agar supplemented with 50ug/L rifampicin. Folin and Ciocalteu's reagent was also used to evaluate the spinach leaves for the presence of phenolic compounds on the leaf surface (Ainsworth & Gillespie, 2007). Leaves were placed in whirl-pak bags with 1mL of DI water and massaged for 30 s. 100  $\mu$ L of the water was removed and incubated with 200  $\mu$ L Folin and Ciocalteu's reagent for 2 h. 100  $\mu$ L was transferred to a 96 well plate and evaluated for absorbance at 765 nm with a Spectramax M5e plate reader (Molecular

Devices LLC, Sunnyvale CA). Concentration was evaluated using a Caffeic acid standard curve.

### **Evaluation of the Antimicrobial Activity of Selected Plant Extracts**

Antimicrobial assays were performed using grape seed extract and olive leaf extract in combination with 400 nm light. Food grade plant extracts were purchased from a bulk supplement supplier (BulkSupplements.com, Henderson NV) dissolved in DI water and filter sterilized. The efficacy of the antimicrobial treatment was evaluated at various concentrations on an overnight culture of *E. coli* diluted to  $10^4$  to  $10^5$  CFU/mL. Five mL of the bacterial suspensions were then transferred to a 6 well plate (Corning, NY) and treated for 60 minute under 400 nm light. 100  $\mu$ L aliquots were removed and plated on tryptic soy agar. The absorbance spectra of the plant extracts were also evaluated using the cuvette reader of the Spectramax M5e plate reader (Molecular Devices LLC, Sunnyvale CA).

### *Data Analysis and Statistics*

All experiments were performed in triplicate and statistically evaluated using a paired *t*-test ( $\alpha = 0.05$ ) in Microsoft Excel.

## Chapter 3: Results and Discussion

### Evaluation of Antimicrobial Effect of Caffeic Acid and 400 nm Blue Light

#### **Evaluation of Hydrogen Peroxide Generation**

Figure 1 shows the concentration of hydrogen peroxide generated by exposure of 5 mM caffeic acid to UVA, UVB, UVC, and 400 nm blue light. Caffeic acid has been found to exhibit pro-oxidant behavior at concentrations above 5 mM in addition to generating reactive oxygen species when exposed to high intensity light (Maurya & Devasagayam, 2010; Nakamura et al., 2015). The measurements were performed using the FOX assay. The results of the FOX assay indicated that highest amount of hydrogen peroxide was generated when caffeic acid was treated with UVC light. UVA, and UVB both had significantly lower ROS generation over a 30 minute period. 400 nm light had lowest ROS generation, with a significantly lower amount of peroxide produced over the same treatment time period. ROS generation was wavelength dependent, and hydrogen peroxide generation was highest at 254nm and lowest at 400nm.

Initially UVC light was thought to be the best wavelength of light to use in combination with caffeic acid, as it induced higher ROS production. The antimicrobial efficacy of UVC light with caffeic acid was evaluated on *E. coli*, and caffeic acid was found to have attenuated the antimicrobial efficacy of UVC (Table 1). The absorbance spectrum of caffeic acid was then taken to determine the ideal wavelength of light to use in a combination treatment (Figure 2). Maximum absorbance occurred at wavelength 354 nm. Caffeic acid absorbance was also found to be high, greater than 3 Absorbance Units, for much of the ultraviolet spectrum. 400

nm light was chosen as the physical treatment as caffeic acid had low absorbance at that wavelength, reducing the possibility that the chemical treatment shielded bacteria from the antimicrobial effects of the light-based treatment.

**Table 1: Survival of *E. coli* treated with UVC light (254 nm) and 5 mM caffeic acid, 30 minute treatment**

	Caffeic Acid	Control
T0	6.29 Log(CFU/mL)	6.34 Log (CFU/mL)
T30, UVC	2.83 Log (CFU/mL)	ND, >1 Log (CFU/mL)
T30, no UVC	>4 Log (CFU/mL)	6.46 Log (CFU/mL)

**Figure 1: Hydrogen Peroxide Generation of Light Treated Caffeic Acid (5mM solution)**

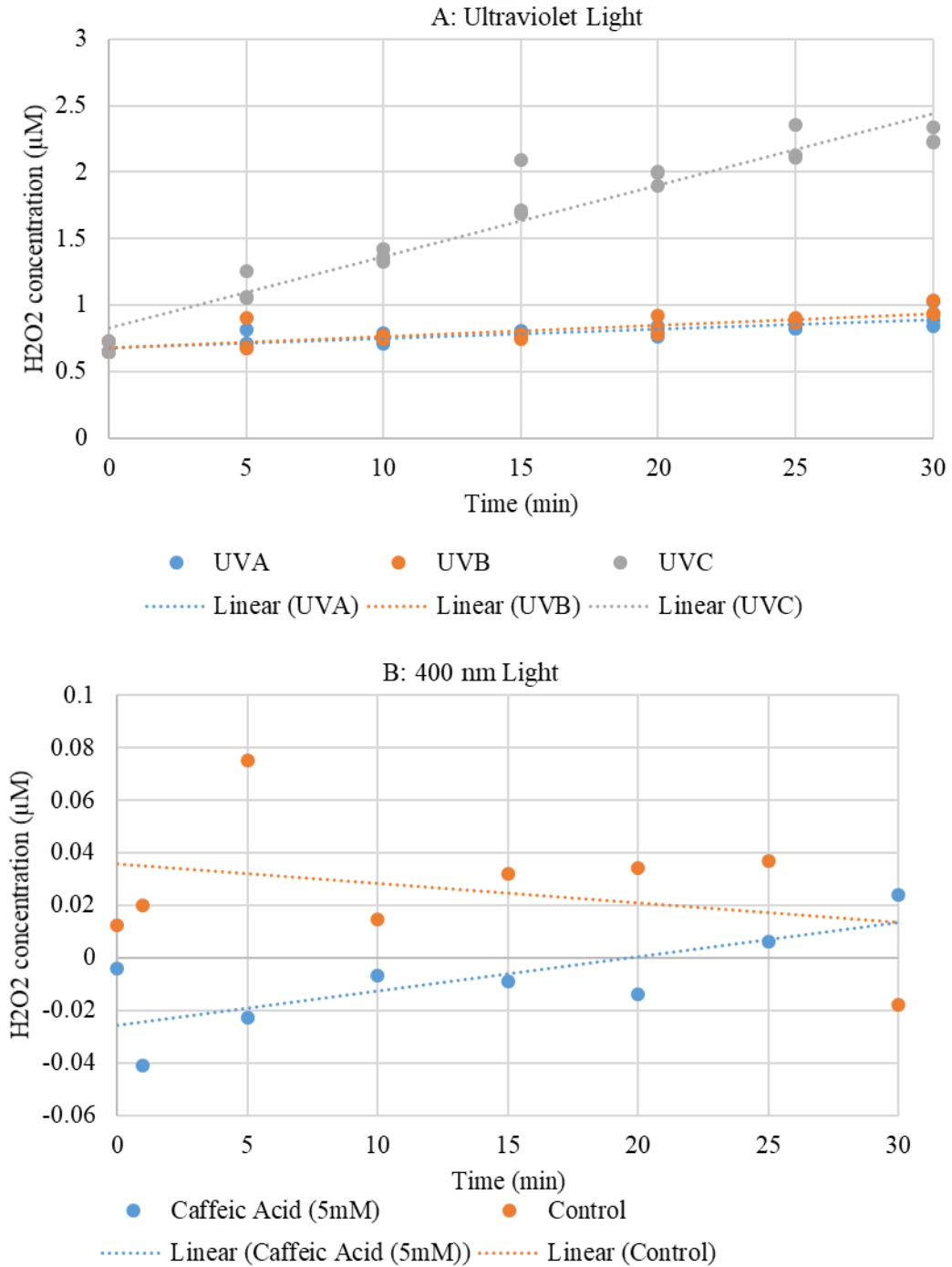
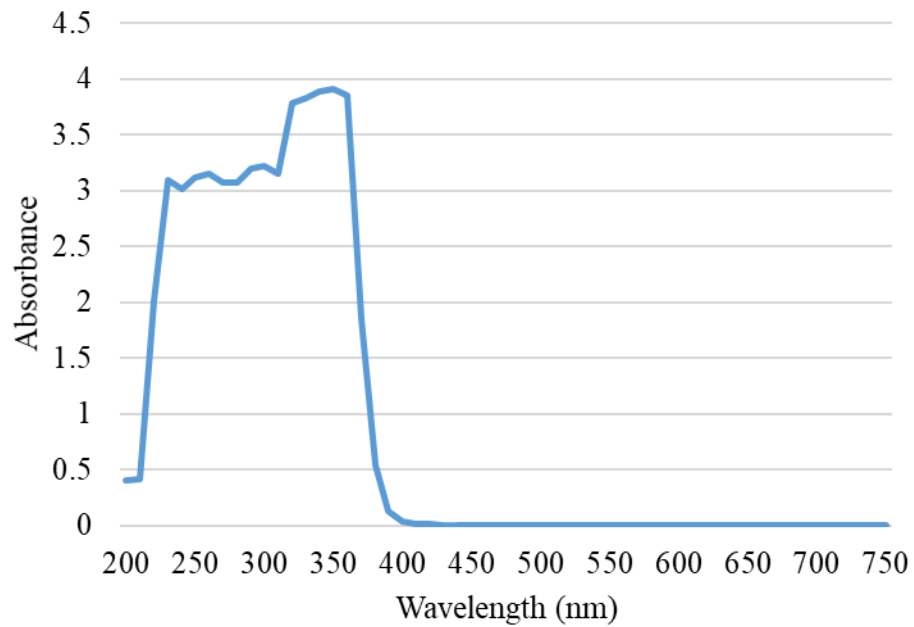


Figure 1a shows the hydrogen peroxide generation of 5mM caffeic Acid over 30 minutes when treated with UVA, UVB, and UVC light. Figure 1b shows hydrogen peroxide generation at 400 nm and 5mM caffeic acid treatment compared to deionized water control.

**Figure 2: UV-Vis Absorbance Spectrum of 5mM Caffeic Acid**



5mM caffeic acid exhibits low light absorbance in the visible light range with higher absorbance in the ultraviolet light range. Peak absorbance in the solution was observed at 354nm.

## Antimicrobial Assay

The inactivation of *E. coli* and *L. innocua* treated with 5mM caffeic acid and blue light was observed over a two-hour period (Figure 3a and 3b). Inactivation of *E. coli* (Figure 3a) fell below the limit of detection between 75 and 90 minute with the combined caffeic acid and light treatment. Inactivation was not as rapid as that reported by Nakamura and coworkers (2015) who showed greater than 5 log within 5 minutes of treatment with caffeic acid and blue light. The difference observed may have been due to the lower intensity of the light source, increased distance from the light source, and a larger volume tested in our experiment compared to their experimental set-up. Treatment with the caffeic acid alone resulted in approximately 2 log CFU/mL inactivation while 400 nm light alone caused less than 0.5 log CFU/mL reduction in *E. coli* after two hour treatment.

Results with *L. innocua* can be seen in figure 3b. The combined treatment resulted in slightly over 1 log CFU/mL reduction of *L. innocua* at 90 minutes, and then regrowth in microbial population was observed at subsequent time points. At the end of two hours, an approximately 1 log CFU/mL reduction was observed in *L. innocua* treated with blue light alone, likely due to the oxidative stress caused by the light treatment. Caffeic acid alone was ineffective in inactivating *L. innocua* and no noteworthy difference in population was observed compared to the control at any time point. Overall, the results with *L. innouca* indicate that the combined treatment is less effective on our model gram-positive bacteria than the gram-negative bacteria. The inactivation results also demonstrate that the chemical caffeic acid treatment is more effective on *E. coli*, while the physical light treatment is more effective on *L. innocua*.



**Figure 3: Survival of Bacteria in 5mM Caffeic Acid Solution Treated with 400 nm Blue Light**

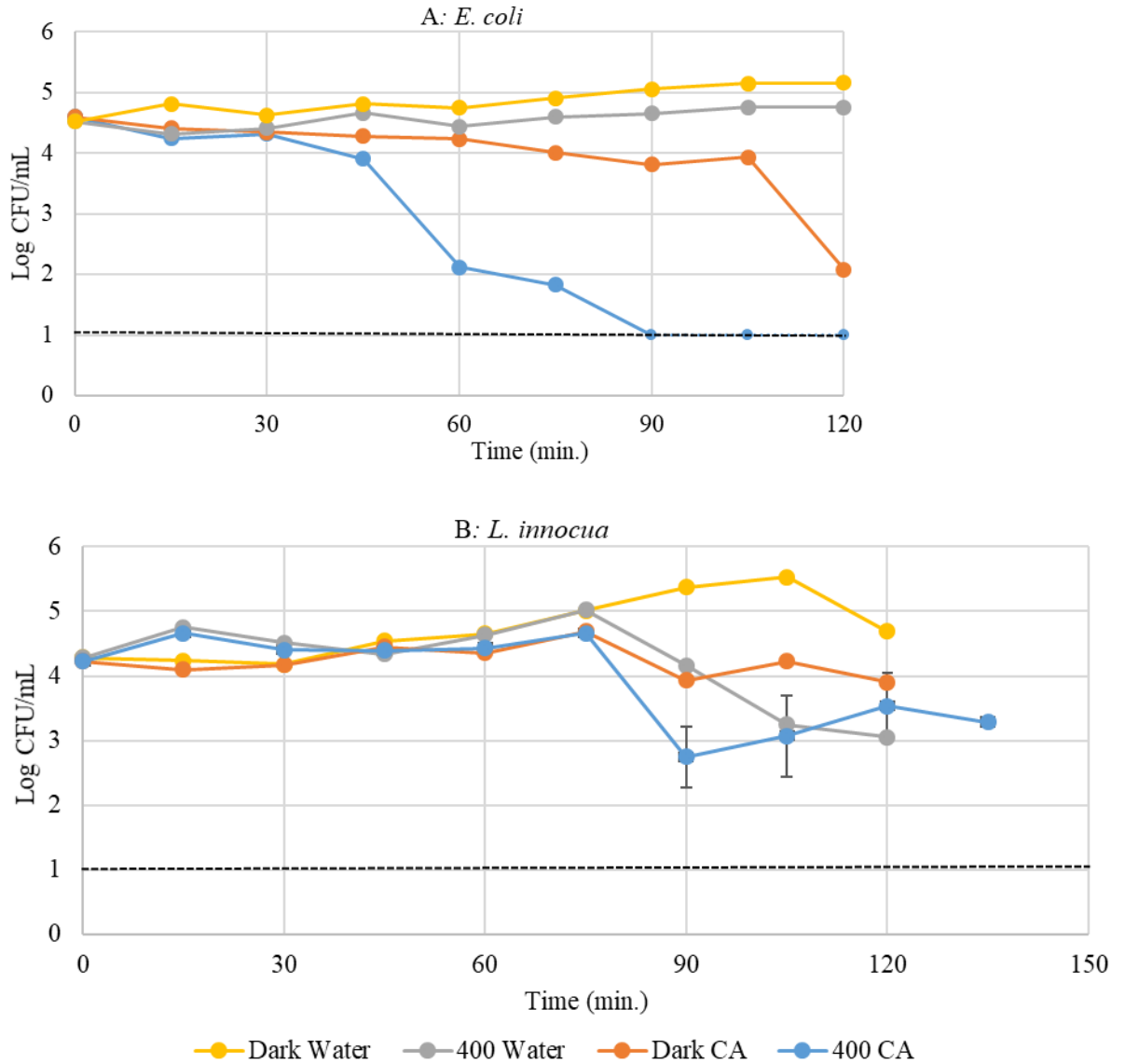


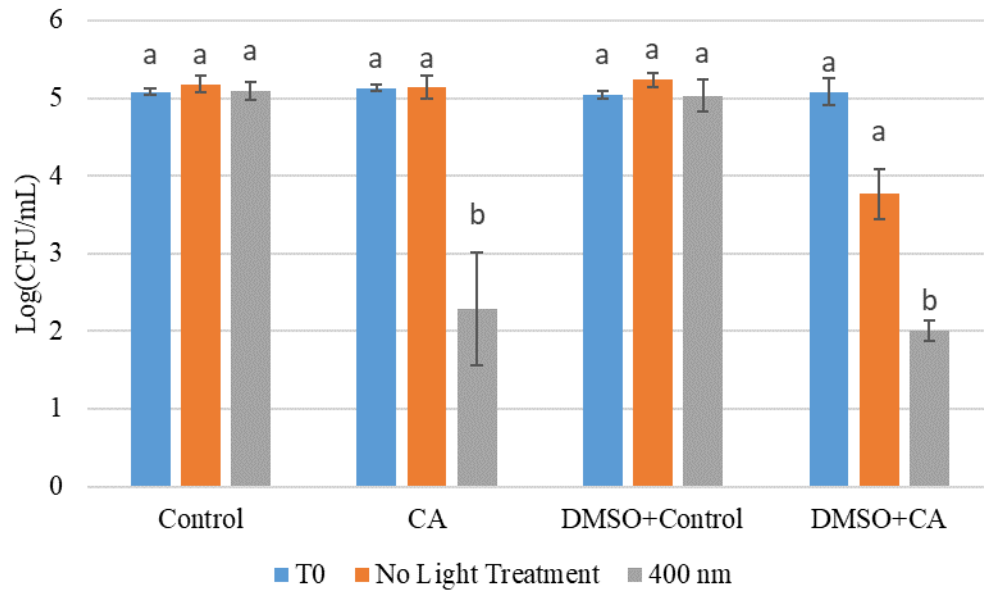
Figure 3a shows the reduction in *E. coli* population over time when treated with 5mM caffeic acid and 400 nm light. The combined treatment led to inactivation below the limit of detection and caffeic acid led to a 2-log CFU/mL reduction. In figure 3b, *L. innocua* levels increase after 90 min, and blue light had the highest level of inactivation after 2 h.

## Examination of Antimicrobial Mechanism

### **Evaluation of Antimicrobial Activity in Presence of Dimethyl Sulfoxide**

Experiments performed with 1% dimethyl sulfoxide showed no significant difference in antimicrobial efficacy of the treatment on *E. coli* as compared to the caffeic acid/deionized water solution after 60 minute treatment (Figure 4). Addition of DMSO also increased the efficacy of the caffeic acid treatment alone, with 1 Log CFU/mL reduction observed in *E. coli* treated with only caffeic acid and DMSO. In addition to being a reactive oxygen species quencher, dimethyl sulfoxide is a membrane penetration enhancer (Greve, Andersen, & Nielsen, 2008). The improved antimicrobial effect of caffeic acid and 1% DMSO without 400 nm light suggested that the antimicrobial mechanism of this treatment may be influenced by cellular uptake of caffeic acid.

**Figure 4: Effect of 1%DMSO on the inactivation of *E. coli* O157:H7 in 5mM Caffeic acid and 400 nm Light, 60 min treatment**

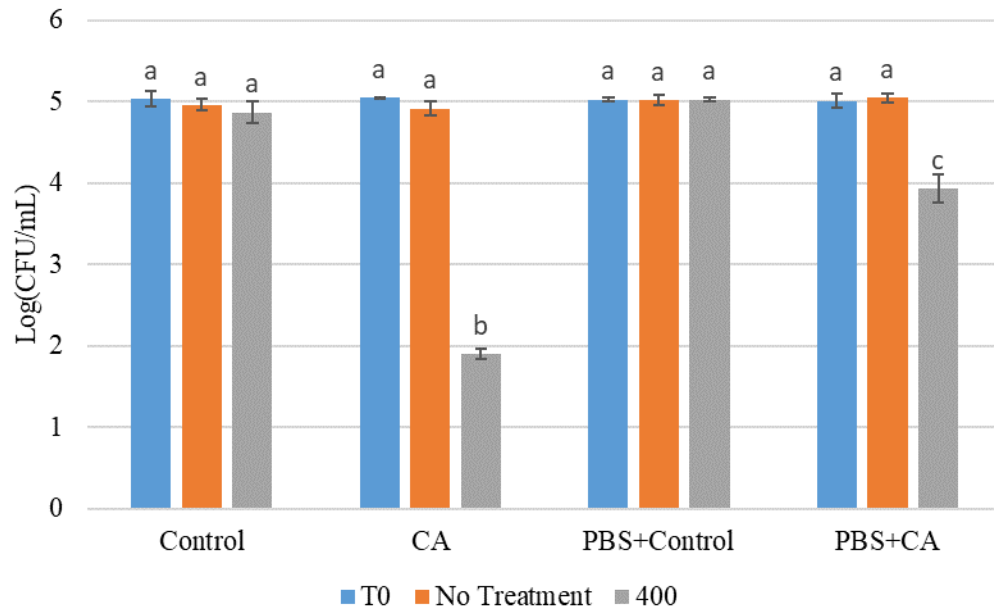


DMSO did not attenuate the antimicrobial effect of caffeic acid and blue light and did enhance the antimicrobial effect of caffeic acid alone. Experiments were performed in triplicate ( $\alpha=0.05$ )

### **Evaluation of Antimicrobial Activity at Elevated pH**

The experiments were performed with 5mM caffeic acid, 400 nm light, and 0.1M phosphate buffered saline to determine the effect of pH on the efficacy of the antimicrobial treatment (Figure 5). The solution containing 5mM caffeic acid and 0.1M PBS had a pH of 6.3, which is higher than the pH of a 5mM caffeic acid solution (pH 3.55). The addition of PBS raised the pH to 4.62, above the pKa of caffeic acid, (Parker, 1965), decreasing the antimicrobial activity of the compound as well as reducing the amount of acid stress on the *E. coli* tested. Antimicrobial activity of blue light and caffeic acid with buffer was significantly lower than that of caffeic acid and blue light alone. The addition of buffer did not significantly alter the antimicrobial effect of caffeic acid alone ( $p=0.81$ ). However, the combined treatment with buffer still led to a 1 Log CFU/mL reduction in *E. coli* after 60 minute treatment. This reduction was greater than the reduction observed in *E. coli* treated with blue light alone. The results of this experiment suggest that while the treatment efficacy is pH dependent, other factors may also contribute to the treatments mechanism of action.

**Figure 5: Effect of 0.1 M PBS on the inactivation of *E. coli* O157:H7 in 5mM Caffeic acid and 400 nm Light, 60 min treatment**



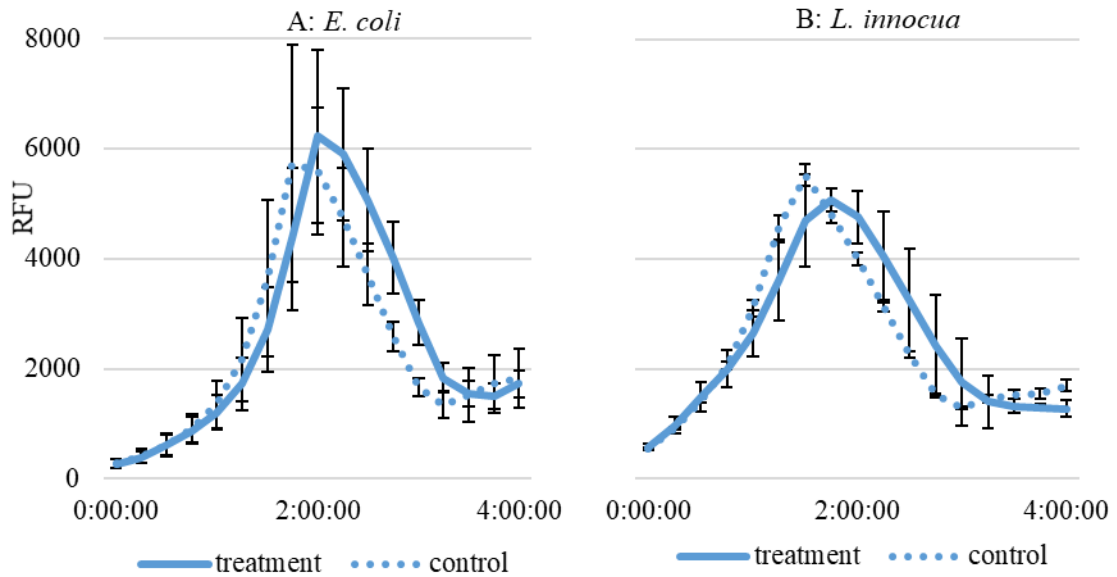
The addition of PBS attenuated the antimicrobial effect of caffeic acid and blue light but did not completely halt inactivation. Experiments were performed in triplicate ( $\alpha=0.05$ )



## Measurement of Metabolic Activity in Bacteria

Metabolic activity was evaluated using the resazurin assay, a fluorescent dye that oxidizes inside of viable cells. Bacteria metabolism was measured spectrofluorometrically after 15 minute treatment with caffeic acid and 400 nm light (Figure 6). With the combined treatment, peak metabolic activity was delayed less than 15 minute compared to the control for both *E. coli* and *L. innocua*. No noticeable delays were observed for the light treatments alone or the caffeic acid treatment alone for either species tested. Peak metabolic activity in *L. innocua* under the combined treatment was lower than that observed in the control. Some antimicrobial agents, especially antibiotics, have a strong impact on bacterial metabolism, slowing the occurrence of peak metabolism and suppressing metabolic activity (Duarte et al., 2009; Vidal-Aroca, Meng, Minz, Page, & Dreier, 2009). The resazurin results showed that the combined caffeic acid and blue light treatment had little impact on pathogenic metabolic activity at sub-lethal levels of treatment. For both *E. coli* and *Listeria innocua* a shoulder was observed in the inactivation experiments with little inactivation observed within the first 45 minute of treatment. This indicated that the treatment does not have a strong, immediate antimicrobial effect on the bacteria tested. The results of the metabolic assay provide insight into the lag in antimicrobial activity of the treatment.

**Figure 6: Effect of Combined 5mM Caffeic Acid and 400 nm Blue Light on Bacteria Metabolism**



Metabolic activity was evaluated using the resazurin assay after a 15 minute treatment. Peak metabolic activity was delayed in both *E. coli* and *L. innocua* after treatment. Lower overall metabolic activity was also observed in *L. innocua*.

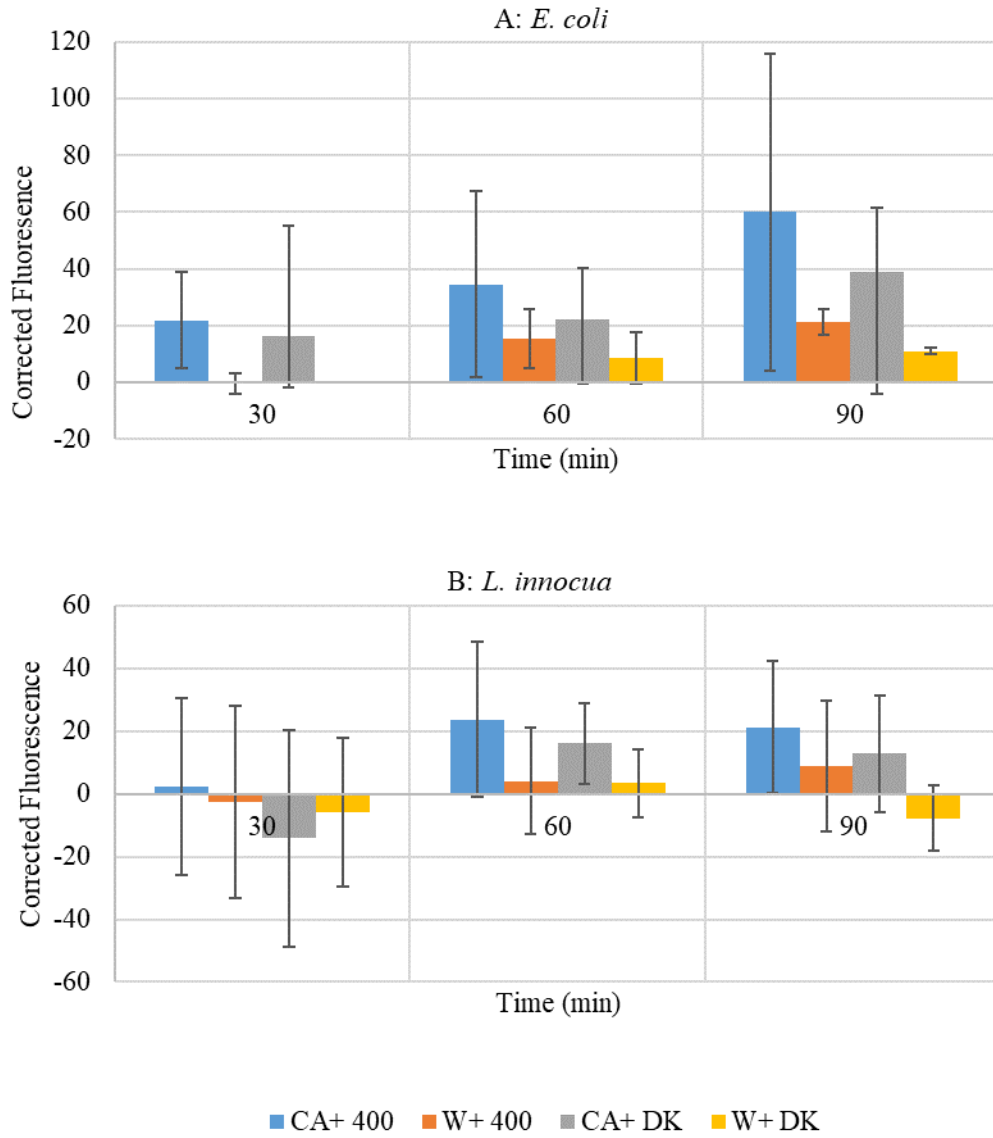


## Evaluation of Bacterial Membrane Damage

Membrane damage was evaluated using the propidium iodide fluorometric assay (Figure 7). Propidium iodide binds to cells with damaged membranes and increased fluorescence is indicative of higher levels of membrane damage (Wang, de Oliveira, Alborzi, Bastarrachea, & Tikekar, 2017). Fluorescence readings were taken after 30, 60, and 90 minutes of treatment time. Fluorescence readings were corrected against the fluorescence values of the no-light treatment, buffered peptone water reading at 30 minutes for both species evaluated. The assay results indicate higher levels of membrane damage resulting from the treatment in *E. coli* than in *L. innocua*. *E. coli* under the combined treatment had the highest level of membrane damage with a clear proportional relationship between time and membrane damage. Significant increases in membrane damage were not observed, likely due to the high level of variation in the fluorescence values. Caffeic acid alone also had the same proportional relationship between time and membrane damage, however fluorescence values were lower than those from the combined treatment. Some membrane damage was observed after 90 minutes of 400 nm light treatment alone as well. Less discernable trends were observed for *L. innocua*. All corrected fluorescence values for the combined treatment were lower than those observed in *E. coli* at all time points. For the combined treatment, caffeic acid treatment, and control, membrane damage

values appeared to plateau between 60 and 90 minute of treatment. For the light treatment alone, membrane damage increased with time; however, the increase was substantially lower than that observed in *E. coli*. The membrane damage results for *L. innocua* appear consistent with the inactivation data. In the antimicrobial experiments, *L. innocua* inactivation with the combined treatment stopped after 90 minutes, but continued for the light treatment after 90 minutes. The lower corrected fluorescence values for *L. innocua* may be due to the differences in cell wall structure between gram-positive and gram-negative bacteria.

**Figure 7: Membrane Permeability of Bacteria Treated with 5mM Caffeic Acid and 400 nm Blue Light**

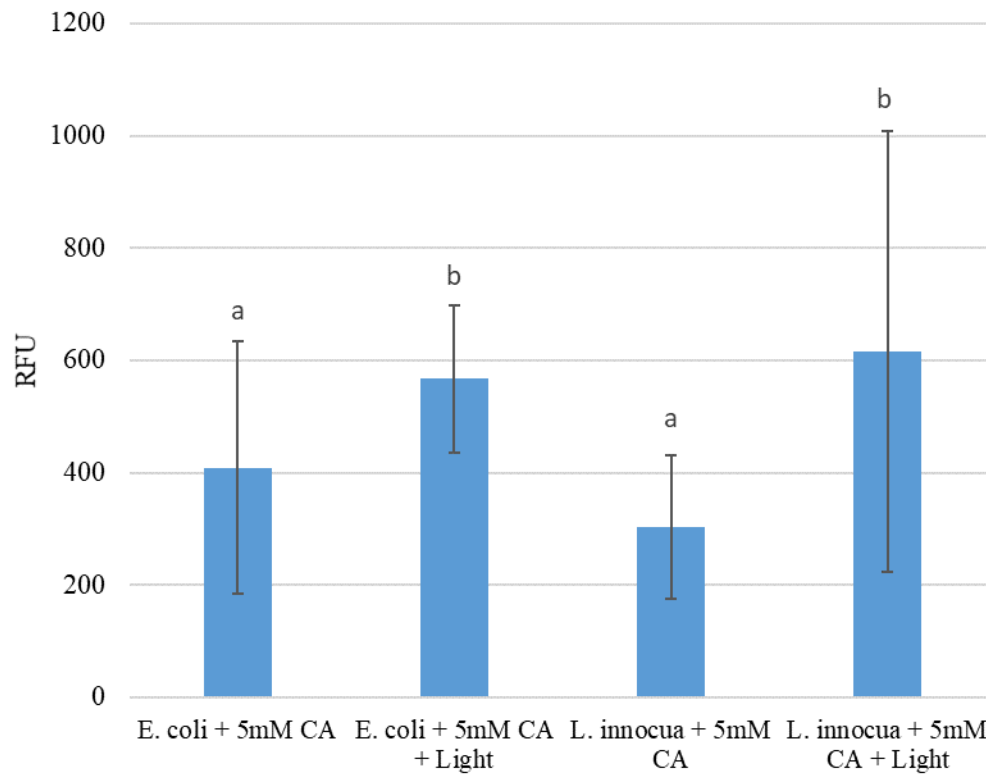


High levels of membrane damage were only observed in *E. coli* with the combined treatment resulting in the highest damage. Caffeic acid alone, also caused damage in *E. coli*. All treatments of *L. innocua* had lower damage compared to *E. coli* and damage did not increase with time.

## Evaluation of Caffeic Acid Uptake

Diphenylboric acid 2-aminoethyl ester (DPBA) binds with caffeic acid and other phenolic compounds. After treatment with caffeic acid, and caffeic acid and blue light, the uptaken caffeic acid contained in the cell lysate is labeled with DPBA and analyzed fluorimetrically. For both strains, the DPBA assay showed a significant increase in the amount of caffeic acid uptaken by both *E. coli* ( $p=.001$ ) and *L. innocua* ( $p=.006$ ) when treated with 400 nm light. This result suggests that the light treatment may increase the membrane permeability of bacteria. While *L. innocua* did not show increased inactivation when treated with caffeic acid and 400 nm light, caffeic acid uptake did increase in the presence of light. Prior research has shown that blue light causes oxidative damage in *L. innocua* (O'Donoghue et al., 2016), and the blue light may increase membrane permeability. However, caffeic acid has been shown to exhibit both antioxidant and pro-oxidant behavior (Maurya & Devasagayam, 2010), and the internalized caffeic acid may attenuate the damaging effects of the 400 nm light treatment. The high level of caffeic acid uptake combined with low levels of membrane damage in *L. innocua* suggest that that simultaneous application of the treatments may not be ideal for some strains of bacteria.

**Figure 8: Caffeic Acid Uptake, after 30 minute Treatment with 5mM Caffeic Acid and 400 nm Light**



The DPBA assay showed a significant ( $p < 0.05$ ) increase in the amount of caffeic acid uptake by both *E. coli* and *L. innocua* when treated with 400 nm light. This suggests that the light treatment may increase the membrane permeability of bacteria. Experiments were performed in triplicate ( $\alpha=0.05$ )

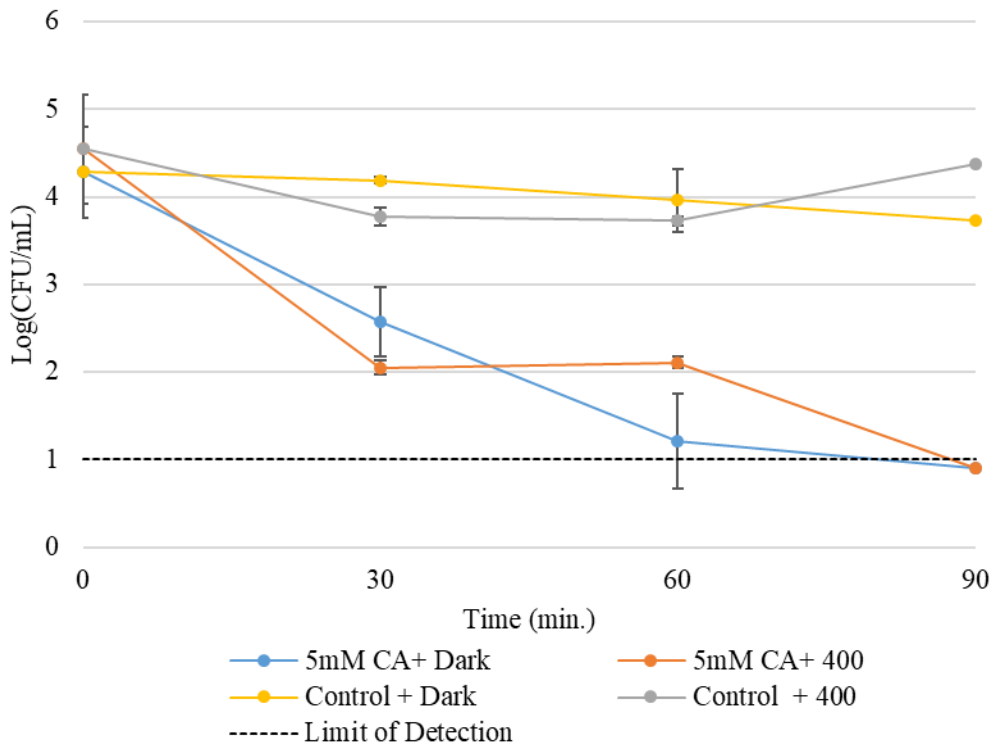
Applications of Caffeic Acid and 400 nm Blue light as an Antimicrobial Treatment in Sanitation of Fresh Produce

**Evaluating Bacterial Inactivation on the Surface of Fresh Spinach**

The application on fresh produce was only tested with *E. coli*, as *L. innocua* had much lower levels of microbial inactivation in solution. Application of the treatment on spinach indicates that inactivation of *E. coli* on whole leaf spinach occurred more rapidly than in solution alone with inactivation below the limit of detection observed at 90 minute (Figure 9). The addition of blue light did not result in an increase in antimicrobial efficacy as on spinach leaves as it did in solution, and inactivation below the limit of detection was observed in one replicant at 60 minute. The results indicate that the treatment efficacy may depend on the substrate on which the treatment is applied. The caffeic acid treatment may have had greater efficacy due to other compounds present on the leaf surface. Spinach leaves treated with blue light alone showed no substantial reduction in *E. coli*. This indicates that the increased treatment efficacy may not have been caused by the reduced volume caffeic acid and *E. coli* solution being tested (1 mL solution on spinach compared to 5 mL used in antimicrobial experiments). Further analysis could examine the effect of sequential chemical and physical treatment applied on fresh produce. Spinach surface phenolic concentration was evaluated using Folin & Ciocalteu's reagent (Table 2). The surface of the spinach leaves tested had an average of  $0.5 \pm 0.14$  mM of phenolic compounds present. The increased antimicrobial efficacy of caffeic acid on spinach leaves may have been in part due to the presence of other phenolic compounds, however, the concentration on the leaf surface was very low compared to the concentration of the

solution. The improved antimicrobial efficacy may also be due to other factors, including the presence of non-phenolic compounds on the leaf surface.

**Fig. 9: Survival of *E. Coli* on Whole Spinach Leaves Treated with Caffeic Acid and 400 nm Blue Light**



Inactivation of *E. coli* on whole leaf spinach occurred more rapidly than in solution alone with treatment times of 60 and 90 minute resulting in inactivation below the limit of detection. The addition of blue light did not result in as great of an increase in antimicrobial efficacy as on spinach leaves as it did in solution.

**Table 2: Spinach surface phenolic determination using Folin& Ciocalteau's reagent**

Leaf	Absorbance	Phenolic Concentration (Caffeic acid equivalent, mM)
A	0.146	0.35
B	0.162	0.39
C	0.133	0.32
D	0.143	0.35
E	0.275	0.67
F	0.282	0.68
G	0.248	0.60
I	0.181	0.44
J	0.213	0.52
K	0.180	0.44
L	0.279	0.68
Average	0.204 ( $\pm$ 0.06)	0.50 ( $\pm$ 0.14)

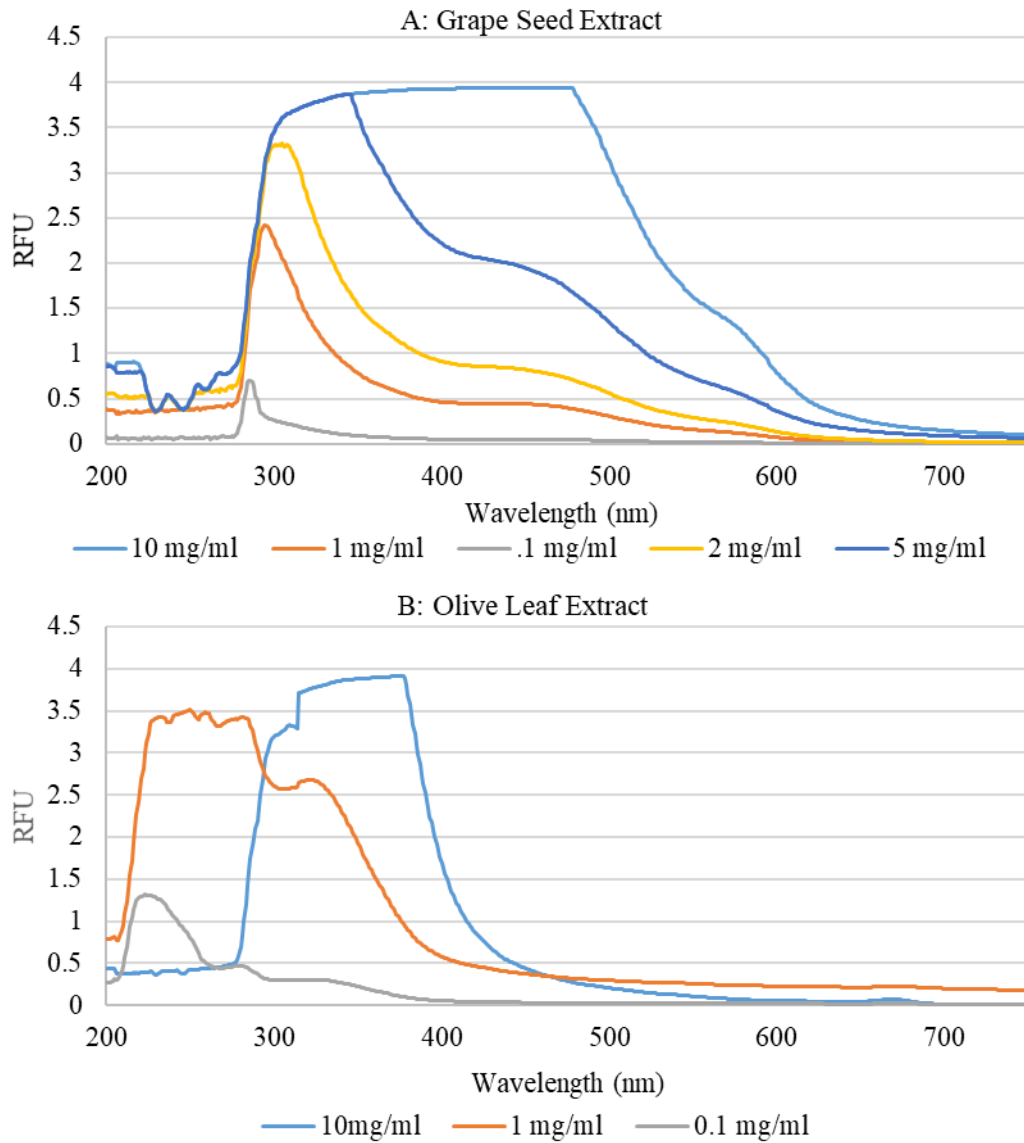
Experiments with Folin and Ciocalteau's reagent did indicate the presence of some surface phenolic compounds. Average phenolic surface concentration of the leaves tested was 0.50 mM.



### **Evaluation of the Antimicrobial Activity of Selected Plant Extracts**

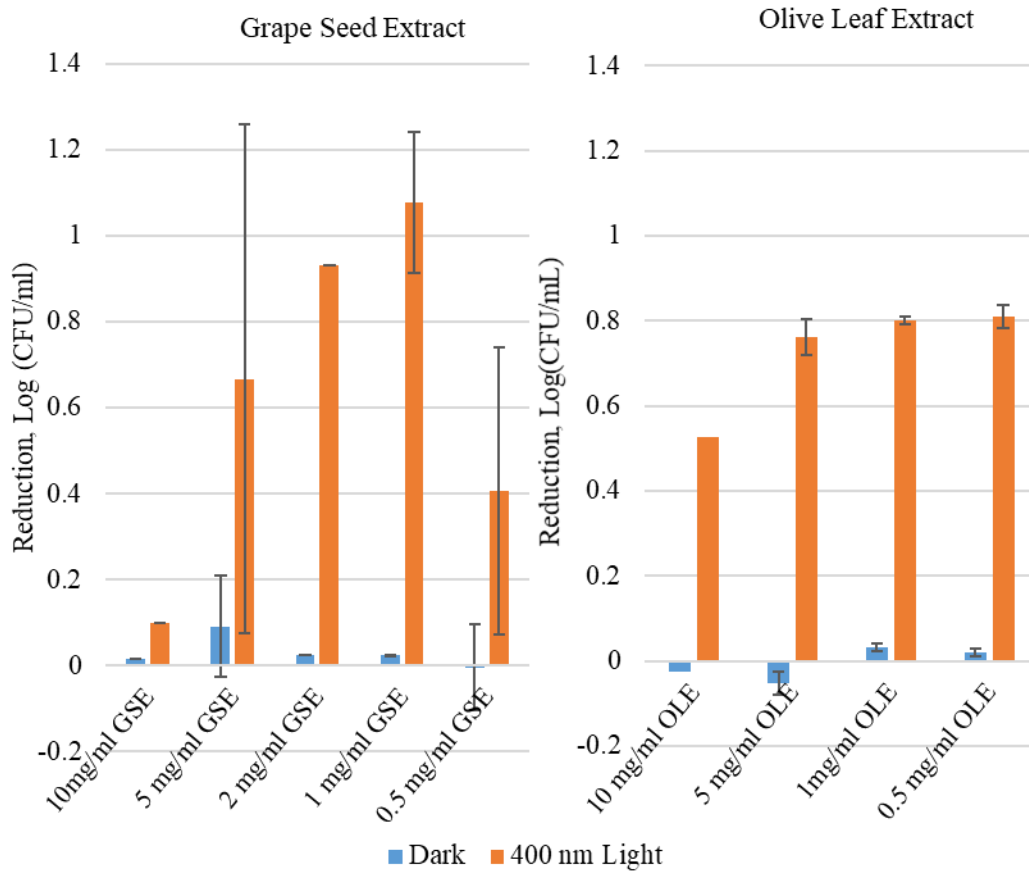
Both grape seed extract and olive leaf extract were found to have lower antimicrobial efficacy than pure caffeic acid at all concentrations tested (Figures 10 and 11). Grape seed extract combined with blue light had the highest antimicrobial activity at the 1mg/mL concentration, with 1 log CFU/mL reduction observed after 60 minute treatment (Figure 11). The higher concentrations of 2, 5, and 10 mg/mL showed reduced efficacy with increased concentration, and the lower concentration of 0.5mg/mL also had a lower log reduction than both 2 and 5 mg/mL. The UV-visible absorbance spectra for the varying concentrations of grape seed extract suggest that the higher concentrations of this extract have too high absorbance at 400 nm for the combined light and chemical treatments to be effective (Figure 10). Similar to the experiments with caffeic acid and UVC light, the compounds high absorbance at these wavelengths may reduce the efficacy of the light treatment in impacting membrane permeability and any photoexcitation of phenolic compounds. Olive leaf extract had maximum antimicrobial efficacy at both concentrations of 0.5 and 1 mg/mL, with 0.8 CFU/mL reduction in *E. coli* observed after 60 minute treatment. Like grape seed extract, antimicrobial efficacy was lower at the concentrations of 10 and 5 mg/mL. Olive leaf extract at 1mg/mL was less effective than grape seed extract. Both grape seed extract and olive leaf extract had similar absorbance at 400 nm, the slight difference in efficacy may have been due to differences in overall phenolic content.

**Figure 10: UV-Visible Absorbance Spectra of Selected Plant Extracts**



Both selected plant extracts indicated higher absorbance at 400 nm than caffeic acid, which may explain the treatments reduced efficacy at higher concentrations.

**Figure 11: Inactivation of *E. coli* treated with Selected Plant Extracts and 400 nm Blue Light, 60 Minute Treatment Time**



Caffeic acid containing plant extracts were less effective at inactivating *E. coli* than pure caffeic acid. Olive leaf extract led to a 0.8 Log (CFU/mL) reduction after 60 minutes, while grape seed extract led to a 1 Log (CFU/mL) reduction.

## Chapter 4: Conclusion

A combined caffeic acid with 400 nm blue light treatment has the potential to be an easy-to-implement, low-cost alternative to chlorine antimicrobial treatments in fresh produce. The combined treatment exhibited a synergistic antimicrobial effect on *E. coli*, although this effect was not seen in *L. innocua*. Experiments thus far have indicated that the combined treatment has differing mechanisms on gram-positive and gram-negative bacteria, with caffeic acid possibly protecting *Listeria* from the damaging effects of blue light. The chemical treatment appears to have more damaging effects on *E. coli* and likely other gram-negative pathogens. On gram-positive bacteria such as *L. innocua*, the light treatment appears to have the greater damaging impact compared to *E. coli*. While the treatment appears to have limited efficacy on *Listeria innocua*, modifications to the treatment design and application could increase the efficacy of caffeic acid and blue light on *Listeria* species and other gram-positive bacteria. Increased light intensity combined with sequential rather than simultaneous treatment with caffeic acid and blue light may improve the antimicrobial effect on these food pathogens. Experiments with fresh produce and plant extracts indicate that the efficacy of the combined treatment is influenced by the presence of other compounds in the fresh produce. With greater understanding of the antimicrobial mechanism of phenolics, the treatment could be highly effective to ensure the safety of fresh produce and other ready-to-eat foods.

Currently, the treatment outcomes and parameters do not make caffeic acid and blue light readily adaptable as an antimicrobial treatment for produce washing. However, the treatment could be an effective, low-cost antimicrobial treatment if

applied at an alternative point in the production chain. LED lights are a highly energy efficient source of light that could be incorporated into the transport operations for minimally processed and ready-to-eat foods. This treatment may also be an effective antimicrobial treatment for gram negative spoilage organisms such as *Pseudomonas aeruginosa* and could potentially extend the shelf life of some highly perishable ready-to-eat produce items. Photoirradiated caffeic acid also has potential as a pre-harvest antimicrobial treatment. Since 400 nm blue light is in the visible range of the electromagnetic spectrum, the compound could be applied to crops with sunlight acting as the light source. Since treatment efficacy was influenced by, but not wholly dependent on, solution pH, buffers could be added to preserve crop quality when applying the treatment in a field. While reduced efficacy was observed when using grape seed and olive leaf extract instead of purified caffeic acid as the chemical antimicrobial treatment, they do show promise as an antimicrobial treatment as well. Since these plant extracts are derived from agricultural waste, the use of these products as antimicrobial treatments may become an attractive and sustainable alternative to the sanitization options available today.

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