ABSTRACT

Title of Dissertation: NOVEL ANTIMICROBIAL TREATMENTS BASED ON THE INTERACTION OF GALLIC ACID AND UV LIGHT: CHARACTERIZATION, INVESTIGATION OF ANTIMICROBIAL MECHANISM AND APPLICATION ON FRESH PRODUCE

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Produce safety continues to be a challenge because produce undergoes minimal processing prior to consumption and existing sanitizers are not effective in inactivating pathogens. Novel decontamination technologies for produce are required as alternatives to traditional methods. In this project, two non-thermal process techniques were developed based on the interaction of UV light and gallic acid (GA) to enhance the safety of fresh produce.

The first technique is the simultaneous application of UV-A light and GA (UVA+GA). UVA+GA treatment was effective against *E. coli* O157:H7, and the mechanism behind the synergistic antimicrobial effect was associated with the cellular uptake of GA, generation of

reactive oxidative species (ROS), inactivation of enzymes superoxide dismutase, and damage to the bacterial membrane. In the second technique, the antimicrobial activity of GA was enhanced by its prior UV-C exposure (UVC-GA) against *E. coli* O157:H7 and was persistent for at least 4 weeks. The antimicrobial activity was affected by solution pH and the wavelength of UV-C exposure. The generation of ROS during UV light exposure and photo-oxidized compounds of GA such as quinone contributed to the antimicrobial activity of the UVC-GA solution. Both UVA+GA and UVC-GA treatments can enhance the inactivation of inoculated *E. coli* O157:H7 on produce such as spinach leaves and tomatoes without affecting the color and firmness. Common environmental stresses could confer complex cross-stress response in *E. coli* O157:H7 towards UVA+GA and UVC-GA treatments in that both resistance and sensitization can be induced depending on the stress applied and the technology studied. Repeated exposure to moderate UVA+GA or UVC-GA treatment can also select for sub-population that demonstrates higher resistance towards these treatments as well as cross-resistance to other lethal stress such as heat and acid. ROS scavenging enzymes and alternative sigma factor RpoS are highly likely to be associated with the adaptive response process.

In conclusion, both UVA+GA and UVC-GA treatments are promising novel non-thermal techniques that are potential alternative methods for fresh produce disinfection. For future work, a better understanding of the inactivation mechanisms, optimizing of processing parameters, and the development of adaptive response associated with the two treatments need to be explored.

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Chapter 1: Literature review and statement of problem

1.1 Current food safety issues

Foodborne diseases are increasingly serious public health problems all over the world with enormous associated costs. Some countries have made great progress in controlling foodborne diseases, but the number of those affected by foodborne disease is still growing globally (WHO, 2003). Center for Disease Control and Prevention (CDC) revealed that every year about 48 million people are sick, 128,000 are hospitalized, and 3,000 die from foodborne diseases in the United States, with approximately 90 percent of those being due to seven pathogens. *Salmonella*, norovirus, *Campylobacter*, *Toxoplasma*, *E. coli* O157, *Listeria*, and *Clostridium perfringens* are the pathogens that commonly associated to the reported foodborne diseases (CDC, 2010). Foodborne pathogens, which are present widely in nature, can readily contaminate food and food products at multiple points during manufacturing process and be widely distributed over great distances and resulting in widespread impact of foodborne diseases. Many opportunities exist for food to become contaminated as it is produced and prepared. For example, fresh fruits and vegetables can be contaminated if they are washed or irrigated with water that is contaminated with animal manure or human sewage (Steele, M., & Odumeru, 2004). Foodborne microorganisms can also be transferred by cross-contamination during food processing. For example, microorganisms can be introduced from one food to another food by using the same utensils used to prepare food without washing (CDC, 2011). Therefore, developing effective antimicrobial techniques for inactivating pathogens in agricultural and food products is one of the most significant steps for controlling foodborne disease (Rahman, Khan, & Oh, 2016). In order to inhibit bacterial growth, extend product shelf life, and ensure food safety, the food

industry has employed several decontamination techniques throughout the food chain. However, some of these techniques possess disadvantages such as high cost, chemical residues, low efficacy, and adverse effects on the quality of food products (Uthukumarappan, Ourke, & Ullen, 2009).

1.1.1 E. coli O157:H7

Escherichia coli is a species of Gram-negative bacteria found in the environment, foods and intestines of people and animals. Although the majority of *E. coli* are harmless, some strains can cause diseases such as diarrhea or urinary tract and bloodstream infections, especially in infants and elderly people (CDC, 2018). The strains of *E. coli* that can cause illness can be spread through contaminated water or food, or through contact with animal or people.

Escherichia coli that cause disease when they have acquired certain virulence genes. For example the pathogenic Shiga toxin-producing *E. coli* have acquired the gene(s) for Shiga toxin formation and related virulence characteristics. This group of bacteria are a significant food safety concern and cause human illness outbreaks worldwide (Gutiérrez-Larraínzar et al., 2012). According to CDC, the illnesses caused by STEC infect 265,000 per year in the United States. This includes 3,600 hospitalizations and 30 deaths (CDC, 2018). Among all the Shiga toxin-producing *E. coli*, *E. coli* O157:H7 is the serotype most commonly linked with foodborne outbreaks. It can cause hemorrhagic colitis, hemolytic uremic syndrome, and kidney failure (Farrokh et al., 2013). The association of *E. coli* O157:H7 with human disease was first described during two foodborne outbreaks in the United States in 1982, and since then, over 200 outbreaks have been reported in at least 30 countries, including both developing and developed countries (Pablos et al., 2018).

The mode of *E. coli* O157:H7 transmission and epidemiological investigations of related human infections imply that the infectious dose of this pathogen is very low (may be as low as 10 cells) (Jinneman, K.C., Trost, P.A., Hill,W.E.,Weagant, S.D., Bryant, J.L., Kaisers, C.A., & Wekell, M.M., 1995). In addition, it is highly tolerant to environmental stress such as acid and heat. Acid tolerance enables this pathogen to survive in highly acidic foods such as apple cider, mayonnaise, fermented sausage and dry salami, and to survive human stomach acidity (G. Wang & M.P. Doyle, 1998). *E. coli* O157:H7 has also been shown to survive at temperatures that are lower than those suitable for growth. For example, it can survive for several weeks or months in meat, fruits, ice cream and yogurt when frozen at -18 to -20 °C, and outbreaks have been linked to frozen ground beef patties that contained viable *E. coli* O157:H7 (Strawn & Danyluk, 2010).

1.1.1.1 Outer membrane protects E. coli O157:H7 from antimicrobials

One reason Gram-negative pathogens such as *E. coli* O157: H7 are especially problematic is that these bacteria possess an outer membrane, which provides them inherent resistance to some important classes of antimicrobials (Zgurskaya, H. I., Lopez, C. A., & Gnanakaran, 2015). The comparison of cell wall structure between Gram-negative and Gram-positive bacteria is shown in **Figure 1-1**. The outer membrane is an asymmetric bilayer of phospholipid and lipopolysaccharides (Lambert, 2002). It plays an important role in providing an extra layer of protection to the bacteria, while the exchange of material required through the membrane is not compromised (Delcour, 2009a). Antimicrobials must overcome this formidable barrier to inactivate the Gram-negative bacteria (Helander & Mattila-Sandholm, 2000). There are essentially two pathways that antibiotics can take to permeate and pass through the outer membrane: one is a lipid-mediated pathway for hydrophobic antimicrobials, and another is

through general diffusion porins for hydrophilic antimicrobials. The lipid and protein compositions of the outer

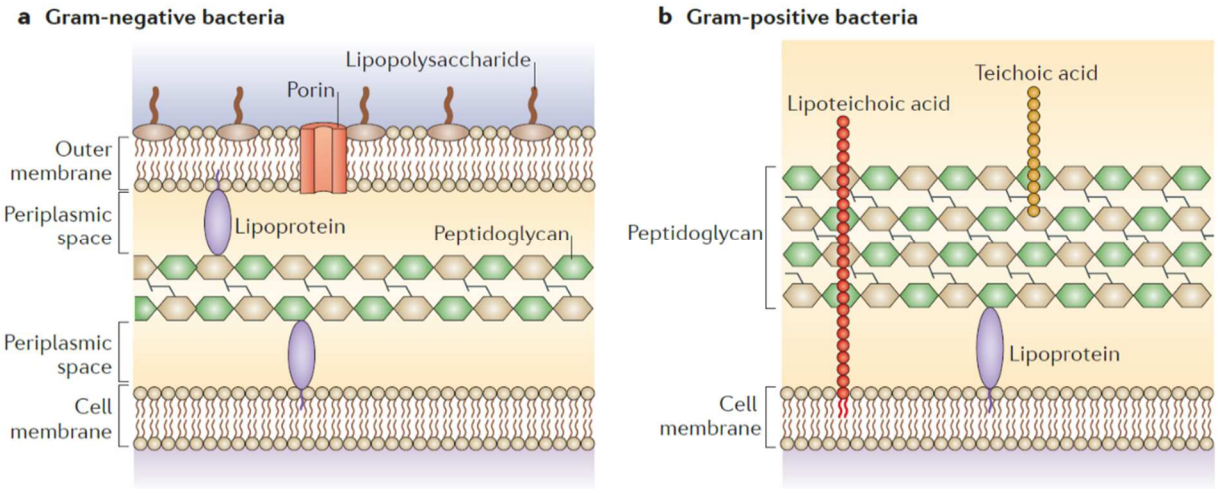

Figure 1-1. Cell wall structure of Gram,-negative and Gram-positive bacteria (Brown, Wolf, Prados-Rosales, & Casadevall, 2015).

membrane have a strong impact on the sensitivity of bacteria to many types of antimicrobials (Delcour, 2009b). When the outer membrane is disrupted by permeabilizers such as chelating agent ethylene diamine tetra-acetate (EDTA), which acts by binding to Mg^{2+} ions in lipopolysaccharides, the permeability of the outer membrane is increased and the bacteria are rendered more sensitive to the antimicrobial agents (Driessen et al., 1995). To weaken the outer membrane of Gram-negative bacteria and increase the antimicrobial efficacy, potential synergistic effects can be applied where chemical antimicrobials are combined with other chemical or physical inactivation technologies such as high pressure, ultrasound, and ozone treatment (Ross, Griffiths, Mittal, & Deeth, 2003). As a consequence of applying these synergistic methods, bacterial cell membrane can be weakened or made susceptible to additional antimicrobial agents and finally lead to cell inactivation. The application of such non-thermal treatments in combination with antimicrobial agents to enhance their synergistic effectiveness in foods requires further investigation (Tiwari et al., 2009).

1.1.1.2 E. coli O157:H7 and fresh produce

E. coli O157:H7 has been associated in multiple outbreaks linked to the consumption of whole produce and fresh-cut leafy vegetables (CDC, 2006). A summary of the selected outbreaks can be found in **Table 1-1**. Consumption of leafy vegetables contaminated with *E. coli* O157:H7 poses an important risk for humans. Diverse opportunities for primary contamination and cross-contamination during pre-harvest phases and post-harvest handling are recognized. These include fecal contamination by animals or transmission by insects, use of untreated manure, application of contaminated irrigation water

Table 1-1: Selected outbreaks of *E. coli* O157:H7 linked to fresh produce

Product	Source of contamination	Year	Location	Number of infected	Number of deaths	Reference
Romaine lettuce	Romaine lettuce from the Yuma growing region	2018	USA	210	5	CDC, 2018
Leafy greens	Linked to the Canadian outbreak of romaine lettuce	2017	USA & Canada	25	1	CDC, 2018
Alfalfa sprouts	alfalfa sprouts produced by Jack & The Green Sprouts of River Falls, Wisconsin	2016	USA	11	0	CDC, 2016
Pre-packaged organic spinach and spring mix	Not identified	2012	USA	33	0	CDC, 2012
Romaine Lettuce	Romaine lettuce sold at several locations of a grocery store chain.	2011	USA	49	0	CDC, 2012
Strawberry	Deer feces contamination	2011	USA	15	2	Laidler, M., 2013
Lettuce	Not identified	2008	USA & Canada	134	0	Warriner and Namvar, 2010
Spinach	Packed fresh spinach supplied by a single manufacturing facility	2006	USA	199	3	CDC, 2006
Lettuce	Not identified	2006	USA	81	0	Olaimat & Holley, 2012
Lettuce	Contaminated irrigation water, probably due to cattle farms	2005	Sweden	135	0	Söderström, A., 2008
White radish sprouts	Four growers of radish sprouts in Japan	1996	Japan	6309	2	WHO, 1996

(Brandl, 2006). Surface contamination of the edible tissues of leafy vegetables is often due to pathogen transference from soil or water (Delaquis, Bach, & Dinu, 2007). *E. coli* O157:H7 can survive on plant surfaces for long periods of time, depending on factors such as nutrient availability, competition with indigenous microflora, relative humidity, and UV radiation (Brandl, 2006). One study showed that *E. coli* O157:H7 can persist for 150 to 217 days in soils with contaminated composts and then detected on lettuce and parsley for up to 77 and 177 days after planting (Solomon, Pang, & Matthews, 2003). Another study showed that *E. coli* O157:H7 spray-inoculated in a lettuce field was detected up to 28-35 days post-inoculation (Moyne et al., 2011). Post-harvest processing operations for fresh vegetables such as washing, cutting and shredding can increase the likelihood of cross-contamination of *E. coli* O157:H7 (Luo et al., 2011). Also, it has been shown that plant lesions can promote the rapid multiplication of *E. coli* O157:H7 over a short period of time on lettuce during post-harvest phases. The application of chemical sanitizers during the washing step can to some extent decontaminate the leafy vegetables and is one of the primary elements of a properly operated post-harvest sanitation program. However, the use of sanitizers does not guarantee the total inactivation of the pathogens (Artés, Gómez, Aguayo, Escalona, & Artés-Hernández, 2009).

1.1.2 Post-harvest washing of fresh produce

Minimally processed foods such as fresh fruits and vegetables, are becoming increasingly popular due to their convenience and freshness (Pablos et al., 2018). However, they are also sources of foodborne diseases as these products are typically consumed raw (H. A. O. Pang, Lambertini, Buchanan, & Schaffner, 2017). In the fresh-cut vegetable processing, disinfection is one of the most important processing steps that affect the quality and safety of the end produce (Gonz, 2015). Since wash water retains soil, organic matter, viruses and bacteria, reusing

processing water may result in the build-up of microbial loads, including undesirable pathogens from the produce. Thus, wash water of poor sanitary quality can be a direct source for spreading bacterial contamination (FDA, 2008). In the absence of traditional processing technologies that provide a kill step for pathogens without significantly reducing produce quality, the presence of a sanitizing agent in the wash water is critical for preventing pathogen survival and transfer (Gil, Selma, López-Gálvez, & Allende, 2009).

1.1.2.1 Limitations of chlorine as a sanitizer

Chlorine has been used as a sanitizing agent in food processing for several decades and is probably the most widely used sanitizer in the food industry. Chlorine and chlorine-based chemicals are often used to sanitize produce and surfaces of processing facilities, as well as to reduce microbial load in washing or cleaning water used during processing. Currently, chlorinated water (50-200 ppm) is the most common decontaminant treatment for fresh produce washing (**Table 1-2**). The efficiency of chlorine and chlorine based derivatives have shown adequate water disinfecting capabilities (Gómez-López, Devlieghere, Ragaert, & Debevere, 2007). However, the use of chlorine has been criticized for its limited efficacy and the formation of potentially carcinogenic chlorine compounds (Manzocco et al., 2015; Mir, Morató, & Ribas, 1997; Singh, Singh, Bhunia, & Stroshine, 2002) For example, the efficacy of chlorine is pH dependent, but many users did not use it under optimum conditions and therefore did not achieve the maximum effectiveness of chlorine (Yildiz, F., & Wiley, 2007). In addition, chlorine-based disinfectants can react with organic matter in water to form carcinogenic by-products like trihalomethanes, haloacetic acids, haloketones, and chloropicrin, and the sanitizing efficacy can be compromised (COT, 2007). Due to the above limitations, the use of chlorine as

Table 1-2: Inactivation effectiveness of chlorine as a sanitizer on selected produce surfaces

Produce	Concentration of chlorine (ppm)	Temperature	Treatment duration (min)	Pathogen	Mean log reduction (CFU/mL)	Reference
Melon	200	22	2	*E. coli O157:H7*	0.6	Svoboda et al., 2016
Lettuce	100	4	3	*E. coli O157:H7*	1	Delaquis et al., 1999
Lettuce	200	22	10	*E. coli O157:H7*	2.48	WHO, 1998
Cabbage	200	22	10	*L. monocytogenes*	1.2	Zhang and Faber, 1996
Parsley	200	22	5	*E. coli O157:H7*	1.42	Lang, et al., 2004
Green pepper	200	4	1	*C. parvum*	0.45	Duhain, et al., 2012
Tomato	300	37	2	*Salmonella montevideo*	1.5	Zhuang, et al., 1995
Control (water)	200	4	5	*E. coli O157:H7*	5.7	Svoboda et al., 2016

sanitizers for minimal processed fresh produce could be reduced or even prohibited as it already is in some European countries such as Germany and Switzerland (Tirpanalan, Zunabovic, Domig, & Kneifel, 2011; Van Haute, Sampers, Holvoet, & Uyttendaelea, 2013). Hence, there is a need to develop alternative sanitizers to replace chlorinated water systems.

Extensive research has explored the potential of various antimicrobial agents in preserving produce safety and quality with less environmental and adverse health impacts (Pablos et al., 2018). Chemicals such as chlorine dioxide, ozone, peroxyacetic acid, acidified sodium chlorite and some essential oils have been already proposed as alternatives to chlorine. Each of the alternative has its own advantages and limitations compared with chlorine. For example, acidified sodium chlorite has shown strong antimicrobial activity against *E. coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes* and spoilage microorganisms on fresh-cut produce. However, its negative impact on organoleptic quality of some food products occurs when acidified sodium chlorite was used at the approved concentration range (Allende, McEvoy, Tao, & Luo, 2009). Peroxyacetic acid has also been shown to be effective against spoilage and pathogenic microorganisms, though it has relatively low antimicrobial efficacy at permitted levels for vegetables washing (Ramos, Miller, Brandão, Teixeira, & Silva, 2013). Chlorine dioxide is less pH dependent and generates less potential hazard compounds, however, it is an explosive and requires on-site generation. In addition, a final rinsing step is needed after the treatment for fresh produce (Mahmoud, Vaidya, Corvalan, & Linton, 2008). A summary of selected groups of alternative sanitizers on fresh produce are listed in **Table 1-3**. Although the proper use of these sanitizers increases safety of fresh produce, none of the sanitizing methods can control all the parameters that maintain the quality and safety of produce (Ramos et al.,

2013). Additional research to identify and evaluate potential new sanitizers or combined methods is needed to enhance the safety of produce.

Table 1-3: Selected sanitizers for the minimally processed fresh produce

Sanitizer	Advantages	Limitations	Reference
Chlorine	Low cost; Easily available; Long history of use	Formation of chlorinated by-products with potential adverse health effects; Efficacy is affected by the presence of organic matter; Corrosive; Sensitive to pH and temperature	Daglia, 2012
Chlorine dioxide	Less pH dependent compared with chlorine; Fewer potentially hazard formation; Less corrosive than chlorine and ozone	Not efficient at permitted levels for fresh produce; Requires on-site generation; Explosive; Required final water rising after treatment	Goodburn & Wallace, 2013
Acidified sodium chlorine	Greater efficacy than chlorine use to low pH	Limited research conducted	Meireles, Giaouris, & Simões, 2016
Organic acid	Low cost and easy to use; No toxicity; Allowed for organic products	Interferes with the sensory quality; Relatively low antimicrobial efficacy; Low pH condition only; Antimicrobial effect dependent on type of acid and strain of microorganism	Ramos et al., 2013
Hydrogen peroxide	No residue; Not corrosive at permitted levels; No harmful by-product formation	Negative impact on overall quality; Less effective against yeast, fungi, and viruses	Silva & Fernandes Júnior, 2010
Peroxyacetic acid	No harmful by-product formation; Efficacy is not affected by organic load and temperature	Low antimicrobial efficacy at permitted levels for vegetables	Sapers & Jones, 2006
Ozone	High antimicrobial activity; Good penetration ability; GRAS; No harmful by-product formation; Effective at low concentrations and short contact time	Possible deterioration of produce flavor and color; Unstable, highly reactive; Corrosive to equipment	Alexandre et al., 2011a

1.2 Introduction to emerging food antimicrobials agents and sanitizers

Food antimicrobials are chemical compounds used to inhibit or inactivate pathogenic or spoilage microorganisms, and thus prolong the shelf life of foods. They may be added to foods directly, or applied to food packaging, food contact surfaces, or food-processing environments (Sharif & Mustapha, 2017). Food antimicrobials may be classified into two groups: synthetic and naturally occurring. The former compounds are regulatorily approved and include compounds such as benzoic acid and benzoates, alkyl esters of parabens, nitrites and nitrates, sorbic acid and sorbates, sulfur dioxide and sulfites. The latter includes compounds from microbial, plant or animal sources that are, for the most part, only proposed for use in foods as antimicrobials (Hintz, Matthews, & Di, 2015). A few of them are approved in the United States and certain other countries for use in selected foods.

Sanitizers are antimicrobial products used to reduce, but not necessarily eliminate, microorganisms from the inanimate equipment and the environment to levels considered as safe as determined by public health codes or regulations (U.S. Environmental Protenction Agency, 2012). Two common types of sanitizers used in food industry include natural organic acids (acetic, citric, lactic, malic, and propionic) and oxidation-reduction potential sanitizers (sodium hypochlorite, chlorine dioxide, hydrogen peroxide, peroxyacetic acid, and ozone) (Ho, Luzuriaga, Rodde, & Tang, 2011). Although traditionally sanitizers are used to inactivate target microorganisms on the food contact surfaces, they have been applied for the inactivation of microorganisms on raw, unprocessed food products, such as meat carcasses and fresh produce or to prevent cross contamination (Mcdonnell & Russell, 1999). Sanitation of raw foodstuffs is an important intervention for reducing the occurrence of foodborne outbreaks. Direct application of antimicrobial agents to food is a widely used method and is carried out traditionally by spraying

or dipping using aqueous sanitizers (Oh, Gray, Dougherty, & Kang, 2005). Investigation of the inactivation mechanisms of different antimicrobial agents may help optimize application of antimicrobials, and identify potential synergies of activity, matching the activity of the compounds to the properties of the food (Tiwari et al., 2009).

1.2.1 Natural antimicrobial agents and sanitizers

The investigation of natural antimicrobial agents and sanitizers for food preservation has received increased attention due to consumer demand for natural, nutritious, and fresh-looking food products (Rasooli, 2007), and a growing concern of microbial resistance towards conventional food processing and preservation methods (Holley & Patel, 2005). The demand has led to exploration of antimicrobials derived from a variety of natural sources (Cowan, 1999). Many of these naturally occurring compounds have evaluated have been widely studied in regard to their antimicrobial properties. Animal-derived antimicrobials except chitosan used in food, are mostly polypeptides in nature (Landers, Cohen, Wittum, & Larson, 2012). These antimicrobials exert activities through multiple mechanisms, including electrostatic destabilization of outer membrane components of bacteria, hydrolysis of peptidoglycan, sequestration of essential microbial nutrients, and the enzymatic formation of antimicrobial products from substance in raw foods (Timofeeva & Kleshcheva, 2011). These antimicrobials tend to have different levels of observed antimicrobial activity against bacteria and fungi, but limited activity against food-borne viruses (M. R. E. Santos et al., 2016). Antimicrobials from microbial source are primarily the bacteriocins of gram-positive bacteria, which are polypeptides with low molecular weights that are produced by various fermentative and respiring microorganisms during their growth. Examples of such antimicrobials are nisin and natamycin (Yang, Lin, Sung, & Fang, 2014). In addition to bacteriocins, some bacteriophages, which are viruses that infect bacteria, have been

given approval for use in food system for control of pathogens in recent years (Jassim & Limoges, 2014).

The application of natural antimicrobial agents is likely to grow steadily in the future because of greater consumer demands for minimally processed foods and those containing naturally derived preservation ingredients (Lucera, Costa, Conte, & Del Nobile, 2012). Investigation on the combinations of different types of natural antimicrobials is also increasing, particularly with the respect to optimization for practical applications. For the future, intelligent selection of appropriate systems based on detailed and quantitative studies to evaluate the efficiency of antimicrobial is necessary. The impact of product formulation, extrinsic storage parameters, and intrinsic product parameters on the efficacy of novel applications of combined antimicrobial systems requires further study (Tiwari et al., 2009).

Plants are rich sources of natural antimicrobials. The most important phytochemicals used as food preservatives are essential oils, which have been used by humans across the continents since ancient times. The use of plant extracts with known antimicrobial properties have exhibited inhibition or inactivation of bacterial and fungal species in the food system (Ehsani, Alizadeh, Hashemi, Afshari, & Aminzare, 2017). Chemical substances that produce a definite action on the microbiological of foods have been grouped in several categories including polyphenols, flavonoids, tannins, alkaloids, polypeptides, or other oxygen substituted derivatives. Many of these compounds have been found are phenolic compounds in essential oils of leaves, seeds, flowers, and bulbs (Negi, 2012). It is thought that these compounds are the secondary metabolites that allow plants to resist pathogens and insects (Lattanzio, Lattanzio, Cardinali, & Amendola, 2006).

1.2.1.1 Phenolic compounds

Phenolic compounds are one of the most diverse groups of secondary metabolites found in a wide variety of fruits, vegetables, nuts, seeds, stems and flowers as well as tea, wine, and honey (Lin et al., 2016). Based on their chemical structures, they may be divided into different categories including simple phenolic compounds, flavonoids, quinones, tannins, and coumarins (Lin et al., 2016). The categories of phenolic compounds were summarized in **Table 1-4**. The use of phenolic compounds as antimicrobial agents could potentially provide additional benefits, including dual-function effects of both preservation and delivery of health benefits.

Simple phenolic compounds are simple bioactive phytochemical comprised of a single substituted phenolic ring (Galal, 2006). Some evidence suggests that degree of toxicity to microorganisms are associated with the sites and number of hydroxyl groups on the phenolic ring. Higher hydroxylation resulting in increased toxicity, of which the mechanism is the inhibition of enzyme activity by the oxidized compounds (Juneja, V. K., Dwivedi, H. P., & Sofos, 2018). Phenolic compounds are known to alter microbial cellular permeability, resulting in loss of macromolecules, and interact with membrane proteins, causing structure changes (Cowan, M. M. 1999).

Flavonols are another groups of phenolic compounds that have a carbonyl and a 3-hydroxyl group, while flavonoids are hydroxylated phenolic compounds with a C3-C6 aromatic ring linkage. They are effective against many microorganisms because of their ability to bind to and inactivate proteins and to complex with bacterial cell walls (J. Pang, Zhao, Wang, Ma, & Xiao, 2014). Unlike simple phenolic compounds, the degree of hydroxylation does not predict the level of toxicity to microorganisms (Cetin-Karaca & Newman, 2015).

Table 1-4: Main groups of plant derived compounds with antimicrobial activity

Plant-derived antimicrobial category	Examples	Selected antimicrobial spectrum	Antimicrobial activity properties	Reference
Flavonoids	Falvones (rutin, apigenin) Flavanones (naringenin, fisetin) Catechins (catechin, epicatechin) Anthocyanins (cyanidin, petunidin)	*Listeria monocytogenes; Staphylococcus aureus; Escherichia coli O157:H7; Salmonella enterica; Vibrio cholera; Pseudomonas aeruginosa; Pseudomonas aeruginosa; Klebsiella pneumonia; Aspergillus flavus; Penicillium sp.; Cladosporium sp.*, etc.	Membrane disruption; Complex with cell wall; Enzyme inactivation Substrate deprivation; Highly hydrophobicity (capable of penetrating cell phospholipid membranes)	Daglia, 2012
Quinones	Anthraquinone, Benzoquinone	*Staphylococcus aureus; Pseudomonas aeruginosa; Bacillus subtilis; Cryptococcus neoformans*, etc.	Adhesion binding; Complex with cell wall; Enzyme inactivation	Hammer, Carson, & Riley, 1999
Tannins	Tannic acid, Gallic acid, Proanthocyanidin	*Staphylococcus aureus; Bacillus subtilis; Listeria monocytogenes; Salmonella enterica; Campylobacter jejuni,* etc.	Protein binding; Adhesion binding; Enzyme inhibition; Substrate deprivation; Complex with cell wall Strong affinity for iron; Membrane disruption;	Orhan, Ozçelik, Ozgen, & Ergun, 2010
Coumarins	Ammoresinol, Ostruthin, Anthogenol	*Staphylococcus aureus; Listeria monocytogenes; Salmonella Typhimurium; Escherichia coli O157:H7; Salmonella Enteritidis; Salmonella Enteritidis*, etc.	Interaction with eucaryotic DNA (antiviral activity)	Savoia, 2012
Terpenoids (isoprenoids)	Carotenoids, Terpinene, Isopentenyl	*Staphylococcus aureus; Pseudomonas aeruginosa; Vibrio cholera; Salmonella typhi*, etc.	Membrane disruption	Toudert, Djilani, & Djilani, 2009
Lectins and polypeptides	Concanavalin A, Wheat germ agglutinin, Aleuria aurantia lectin	*Staphylococcus aureus; Bacillus subtilis; Escherichia coli; Pseudomonas aeruginosa; Candida albicans,* etc.	Disulfide bridge formation; Block of viral fusion or adsorption	Daglia, 2012

The possible modes of action for phenolic compounds as antimicrobial agents have been previously reviewed. However, the exact mechanism of action is not clear (Murphy Cowan, 1999). At low concentrations, phenols affect enzyme activity, particularly those associated with energy production, while at high concentrations, they cause protein denaturation (Rai, 2011). The antimicrobial activity of phenolic compounds may be explained by their ability to damaging microbial cell membrane, changing the permeability, and thus leading to the loss of macromolecules from the cell. They can also interfere with membrane proteins, causing deformation in structure and functionality. The high antibacterial activity of phenolic components can be further explained in terms of alkyl substitution into the phenol nucleus (Dorman & Deans, 2000). The composition, structure as well as functional groups of the compounds play an important role in determining their antimicrobial activity, and usually compounds with phenolic groups are the most effective (Deans, S. G., Noble, R. C., Hiltunen, R., Wuryani, W., & Penzes, 1995). Most studies related to the antimicrobial efficacy of those plant-based antimicrobials have been conducted in vitro using microbiological media. Therefore, information related to their antimicrobial efficacy when applied to real food systems with complex structure is limited. Further study for the appropriate choice and optimized application of natural antimicrobials in food during processing and storage conditions are needed (Nychas, E., & Skandamis, 2003). The combinations of natural antimicrobials with other non-thermal processing technologies a promising for minimizing deleterious sensory or textual effects on food products as well as optimizing microbial inactivation (Ross et al., 2003).

1.2.1.1.1 Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid; GA) is a naturally abundant, low molecular weight plant phenolic compound and it is well known as a component of hydrolysable tannins. The

structure of GA is shown in **Figure 1-2**. It can be found in free form or as a derivative in different food sources such as nuts, tea, grapes, and sumac. Other sources include gallnuts, oak bark, honey, different berries, pomegranate, mango, and other fruits, vegetables, and beverages (Badhani, Sharma, & Kakkar, 2015a). Gallic acid is found in plant tissues as esters, with diverse esters with sugars glycosides, polyols and phenols identified. Apart from the plant species involved, the amount of GA in plant tissues may be affected by external stimuli such as UV irradiation, chemical stressors, and microbial infections (Mierziak, Kostyn, & Kulma, 2014).

The structure of GA consists of an aromatic ring, three phenolic hydroxyl groups and a carboxyl acid group. The three hydroxyl groups are bonded to the aromatic ring in an ortho position with respect to each other. It is this order of arrangement which is the chief determinant for the strong antioxidant capacity of phenolic compounds (Sroka & Cisowski, 2003). Accordingly, several factors such as the number and the position of hydroxyl group, the presence of other functional groups and their position with respect to hydroxyl groups have been shown to affect the antioxidant and antiradical activity. The antioxidant activity of a molecule increases with increase in the number of hydroxyl groups attached to the aromatic ring (G. Cirillo*, O. I. Parisi & Picci, 2012). Thus, GA was found to exhibit the highest antioxidant capacity among various polyphenols. The nature and the position of the substituents with respect to the hydroxyl group also affect the activity of phenolic compounds. The easily ionizable carboxylic group contributes to the efficient hydrogen donation tendency of phenolic acids (Leopoldini, Marino, Russo, & Toscano, 2004). Gallic acid showed a higher antioxidant activity

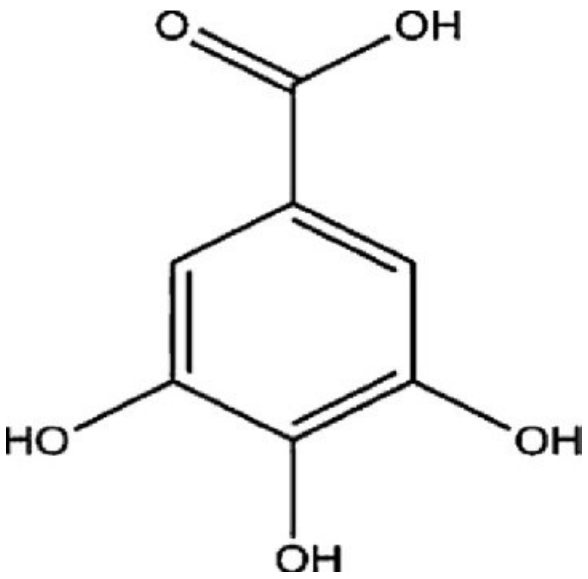

Figure 1-2. Structure of gallic acid (Pachauri, V., & Flora, S. J. S., 2015)

than pyrogallol, thus proving a beneficial influence of carboxylate on antioxidant activity of phenolic acids.

GA possesses four acidic protons with pKa values of 4.0 (-COOH group), 8.7, 11,4, and >13 (-OH group). The first three pKa values for gallate free radical, derived from GA, are 4 (-COOH group), 5 and 10 (-OH groups). The three hydroxyl groups attached to the aromatic ring are prone to oxidation, resulting in the formation of hydrogen peroxide, quinones, and semiquinones (Ji, Zhang, & Shen, 2006; Jovanovic, Hara, Steenken, & Simic, 1995). In a spectrophotometric study, GA and its analogues were shown to be rapidly oxidized by atmospheric oxygen at pH values >7, forming intermediate semiquinones radicals (Friedman & Jürgens, 2000). At a pH range of 2-7, the oxidation is irreversible, and hypothesized to involve a two-electron oxidation scheme leading to the production of quinoid structures in acidic media (Abdel-Hamid & Newair, 2011; Friedman & Jürgens, 2000; Gunckel et al., 1998).

GA also has been demonstrated as one of the antioxidant components responsible for the efficient antiradical and anticancer properties of several plant extracts. In addition to its antioxidant property that provides efficient protection against oxidative damage caused by reactive species, numerous studies demonstrated the antimicrobial potential of GA. For instance, GA showed antifungal activity against *Fusarium fusiformis*, *F. semitectum* and *Alternaria altternata*. It also has antimicrobial effect on *Helicobacter pylori*, a Gram negative bacterium which is one of the leading causes of gastric cancer. Derivatives of GA have also been shown to display antimicrobial properties (Fujita & Kubo, 2002; Kubo, Xiao, & Fujita, 2002; Strippoli, D'Auria, Tecca, Callari, & Simonetti, 2000). The property of GA to exhibit both antioxidant and prooxidant characteristics, gives rise to a diverse range of biological activities, including antitumor, antimicrobial, antimelanogenic, and anticholesterol. The properties of induction of

apoptosis, is mainly related to its prooxidant instead of antioxidant behavior (Badhani, Sharma, & Kakkar, 2015b). Derivatives of GA have also been found in a number of phytomedicines with diverse biological and pharmacological activities, such as ROS scavenging, interfering the cell, interfering the cell signaling pathways, and apoptosis of cancer cells (Sakaguchi, Inoue, & Ogihara, 1998).

1.2.1.1.2 Prooxidant property of phenolic compounds

As stated above, phenolic compounds have both antioxidant and prooxidant potentials. They acts as prooxidants under the condition such as high doses or the presence of metal ions (Hayakawa et al., 1997). The consequences of prooxidant activity could be the damage to the biomolecules such as DNA, proteins and lipids, and eventual cellular death (Aruoma, 2003). For example, it was shown that an anticancer action of plant phenolics is executed by intracellular copper mobilization and ROS generation, which would be a feature of prooxidative properties of polyphenols, leading to cancer cell death. Another example is antibacterial activity of catechins. It was reported that catechins (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate) possess strong bactericidal action due to ROS such as hydrogen peroxide generated through the oxidation of catechins as the active mechanism (Niwano, Tada, & Tsukada, 2017). Also, there has been research on the enhanced antibacterial action of catechins on photoirradiation (Nakamura et al., 2012, 2013, 2015). During the photoirradiation process, solution of polyphenols led to the generation of hydrogen peroxide that was in turn homolytically cleaved to hydroxyl radical. The resultant hydroxyl radical caused oxidative damage leading to bacterial death.

1.3 Non-thermal process and its impact on food quality and safety

Many new preservation techniques are being developed to satisfy consumer demand of nutritious food. Traditionally, foods are thermally treated at temperatures ranging from 60 to 100 °C for a few seconds or a minute to destroy vegetative microorganisms (Smelt & Brul, 2014). The energy transferred to the food during thermal treatment can affect the sensory and nutritional attributes. For example, blanching is carried out to inactivate harmful enzymes before freezing or drying of some fruits and vegetables; however, it can cause adverse consequences on the color, texture, flavor, and nutritive value of fruits and vegetables (J. Wang et al., 2017). The need for maintaining food safety and at the same time lowering the deleterious impact on food nutrition and quality attributes has resulted in increased interest in alternative preservation techniques for inactivating microorganisms and enzymes in foods (Rawson et al., 2011). In the last few decades, non-thermal inactivation techniques have become a significant emphasis area, driven by an increased consumer demand for nutritious, fresh like food products with a high sensory quality and an acceptable shelf life. Alternative inactivation technologies investigated include ionizing radiation, high hydrostatic pressure (HHP), pulsed electric fields, high pressure homogenization, UV decontamination, pulsed high intensity light, high intensity laser, ultrasound, oscillating magnetic fields, high voltage arc discharge and streamer plasma (Jan, Sood, Sofi, & Norzom, 2017). Currently, non-thermal technologies are being employed commercially for acidic foods such as fruit juice, but more research is needed for the processing and packaging of shelf-stable low acid foods (Aneja, Dhiman, Aggarwal, & Aneja, 2014). HHP is commercially used for entrees, guacamole, salsa, and fruit juices, and its use is expected to increase greatly in the future (Norton & Sun, 2008). UV treatment are mainly applied in the liquid food area, such as juice, milk, honey, and liquid egg products (Li & Farid, 2016).

Irradiation is able to inactivate not only vegetative cells of bacteria but also some types of spores in the food products (Kundu, Gill, Lui, Goswami, & Holley, 2014). However, a common misunderstanding among consumers that food becomes radioactive after irradiation has limited the application of irradiation processing in food industry (József Farkas & Mohácsi-Farkas, 2011). Ultrasonic treatment is not very effective on inactivation of microorganisms when used alone, however, it is capable of accelerating the rate of other thermal or non-thermal treatment of foods and reducing nutritional losses (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). The other non-thermal processes (e.g. pulsed electric field, cold plasma) are still in developmental stages with considerable potential (Jan et al., 2017). A summary of selected non-thermal inactivation techniques can be found in **Table 1-5**.

1.3.1 Light based antimicrobial approaches

Light based technologies are one group of the novel food processing technologies that have emerged in food processing due to their broad antimicrobial action, low cost and non-thermal nature. The application includes prolonging the shelf life and inactivating food-borne pathogens in juices and other beverages, fresh produce, meat, poultry and seafood. The part of the electromagnetic spectrum (**Figure 1-3**) that is known as "light" ranges from UV-C (200-280 nm), UV-B (280-320 nm), UV-A (320-400 nm), visible (400-750 nm), near infrared (NIR, 750-1200 nm), and mid/far IR (1200-10000 nm) (Keyser, Muller, Cilliers, Nel, & Gouws, 2008). All these different wavelengths have been used in one form or another for killing various types of microorganisms, such as continuous UV-C light, pulsed light, pulsed UV, and Light Emitting Diodes (LED). The effectiveness of these techniques varies with the wavelength, intensity and treatment durations.

One of the main advantage of light-based approaches is that most of them are broad-spectrum in nature and can effectively destroy all kinds of microbes including bacteria (Gram-positive, Gram-negative, mycobacteria), fungi (yeasts and filamentous fungi), virus (DNA and RNA), and parasites (Yin et al., 2013). Another important advantage of light-based approaches is that the effectiveness of microbial destruction appears to be largely unaffected by the antibiotic resistance status as it is very unlikely for the microorganisms themselves to develop resistance to the light based techniques due to the non-specific nature of the treatment targets (Hamblin & Hasan, 2011; Yin et al., 2013).

Table 1-5: Selected non-thermal physical techniques for processing of fresh produce

Techniques	Effectiveness on fresh produce	Advantages	Limitations	Reference
High pressure processing	Effective in inactivating most vegetative pathogenic and spoilage microorganisms at pressure above 200 MPa	Microbial and enzymatic inactivation; No degradation in flavor and nutrients; No evidence of toxicity; Uniformity treatment through food; Positive consumer appeal	Expensive equipment; Foods should contain about 40% free water for antimicrobial effect; Affects porous integrity	Bermúdez-Aguirre, D., et al, 2013)
Ultrasound	Effective against common foodborne pathogens, vegetative cells, spores and enzymes	Enhance the penetration of solutions to inaccessible sites; heat transfer rate increased; Reduction of process times and temperature	Need to be combined with other process to be effective; Complex due to difficulty to scale-up; May changes on food structure and texture; Penetration affected by solids and air in product	Cao et al., 2010
Cold plasma	Inactivation of pathogens such as *E. coli O157:H7, Salmonella, S. aureus* and L. *monocytogenes*	High efficiency; Low impact on the internal product matrix; No residues; Can be used on vegetables tissues surface; Can be included as part of the packing process	Physical chemical changes in the product may occur; Inactivation is affected by type of microorganisms, inactivation medium, load of bacteria, gas flow, etc.; Limited information about the inactivation mechanism and the interaction with the food materials	Chawla, Patil, & Singh, 2011
Pulsed light	Inactivates spoilage and pathogenic microorganisms	Few residual compounds; Less cost; Low energy input	Food composition affects the efficacy; Decreased efficacy at high contamination level	Choi, Cheigh, Jeong, Shin, & Chung, 2010
Ultraviolet light	Effective in reducing microbiota growth in fruits and vegetables; Germicidal at UV-C range	Absence of residual toxicity; less expensive and easy to use; Induce the synthesis of health-promoting compounds such as anthocyanins	Low penetration depth; Limited application on solid food and opaque surfaces; Increase produce stress and respiration rate; Difficulty in accurately measure the UV dose	Ramos et al., 2013
Irradiation	High efficiency for inactivating pathogenic bacteria and parasites from the surface of fruits and vegetables; Effective in reducing bacterial and molds of climacteric fruits; High penetration ability	Can be performed after packaging; No need for temperature control; Delays ripening and extends shelf life of produce; Lower energy cost	Low acceptance by consumers; Produce quality may be affected at high doses	Goularte, L., et al, 2004

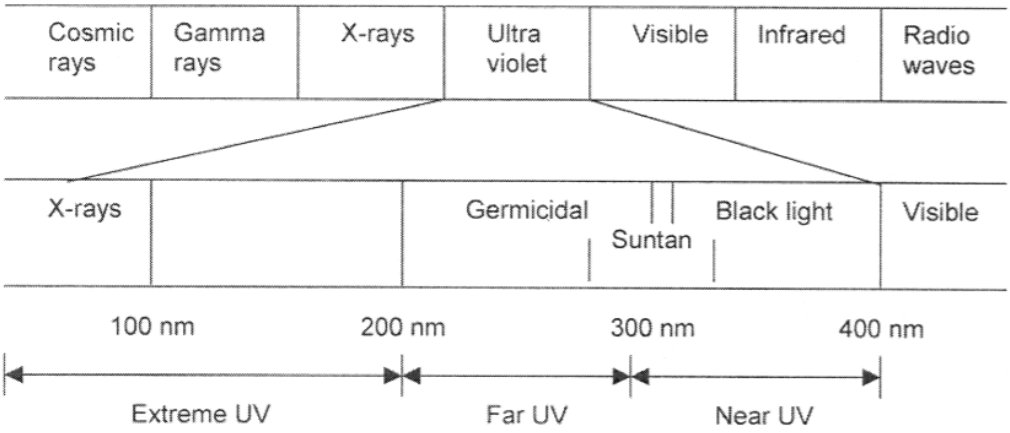

Figure 1-3. Electromagnetic spectrum (adapted from Snowball & Hornsey, 1988)

1.3.1.1 Ultraviolet (UV) light

In the food industry, UV-C treatment has been mainly applied in various processes and products such as air and water disinfection, meat or vegetable processing, on surfaces of fresh products, and liquid products such as fruit juice or beverages (Falguera, Pagán, Garza, Garvín, & Ibarz, 2011). Compared to water and air decontamination, the application of UV light processing of foods is a relatively new area (Koutchma, 2008b). With the growing negative public reaction over chemicals added to foods, UV light has a positive consumer image and holds considerable promise in food processing. While the term "irradiation" is frequently used for UV treatments, UV is considered as light; it is not ionizing radiation. The FDA have concluded that the use of UV-C light for food processing is safe (FDA, 2000) and approved UV-light as an alternative treatment to thermal pasteurization of fresh juice products (Koutchma, 2008a). Research on the germicidal effect of UV irradiation on different microorganisms has been widely studied (Falguera et al., 2011).

The wavelength range for UV processing range is from 100 to 400 nm, and the UV spectrum can be subdivided into three regions: short-wave UV (UV-C) with wavelengths from 200 to 280 nm; medium-wave UV (UV-B) with wavelengths from 280 to 320 nm; long-wave UV (UV-A) with wavelengths from 320 to 400 nm. The most effective wavelengths are located between 200 and 280 nm (the so-called UV-C), especially at 254 nm (Davidson & Harrison, 2002). A summary of the characteristics of UV-A, UV-B, and UV-C for the application of microbial inactivation can be found in **Table 1-6**. UV-C is considered germicidal against most type

Table 1-6: Summary of UV-A, UV-B, and UV-C light for the application of microbial inactivation

Wavelength	Mechanism of inactivation	Advantages	Limitations	Examples of Application	Reference
UV-A	Photosensitization resulting in the formation of free radical and singlet oxygen, ultimately oxidative damage to DNA, proteins and lipids; Membrane damage and change in membrane permeability due to oxidized unsaturated fatty acid	Better penetration into water or other liquid; Rapid bactericidal action in the presence of photosensitizers absorbed into cells; Adequate from solar radiation; Lower energy output	Limited microbicidal properties by itself; the effectiveness can be enhanced by the presence of photosensitizers	Disinfection of drinking water by UV-A and solar light; UV-A/furocoumarin system as sanitation solution; Nanoparticles pre-illuminated by UV-A light against *Escherichia coli* and *Bacillus subtilis*.	Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000
UV-B	Direct damage (lower level than UV-C) to DNA, protein, and other low-molecular weight materials	Properties in between UV-A and UV-C light	Properties in between UV-A and UV-C light	Disinfect biofilm of *P. aeruginosa*; Inactivate norovirus in the presence of TiO_2	Cabinets, Does, & Work, 2013
UV-C	Direct damage of DNA, protein, and lipids	Effective microbiocidal ability in air or on hard surface free from food residues	Lack of penetration in liquid; High intensity can damage food surface	Inactivation of spores from *Aspergillus niger* and *Bacillus subtitlis*; Juice treatment	Castillo, 2017; Park et al., 2016; Pattison & Davies, 2006

of microorganisms, including bacteria, virus, protozoa, molds and yeasts, and algae. For this reason, UV-C light at a wavelength of 254 nm is mostly used for disinfection of surfaces, water, and some food products. The germicidal efficiency decreases as the wavelength increases and at 320 nm is only weakly effective (0.4% of the peak value) (Falguera et al., 2011). The effect of radiation on microorganisms depends on various factors such as species, strain, culture and growth phase. The composition of the food product that is treated also has a great influence (Birmpa, Sfika, & Vantarakis, 2013). Bacteria suspended in air are usually more sensitive to UV-C than bacteria suspended in liquids, due to the different penetration capacity of UV light through different physical media (Skowron, Bauza-Kaszewska, Dobrzański, Paluszak, & Skowron, 2014).

The advantage of UV-C processing is that it does not generate chemical residues, by-products, radiation, and significant amount of heat (Hijnen, Beerendonk, & Medema, 2006). Also, the cost of UV treatment is relatively low (Guerrero-Beltrán & Barbosa-Cánovas, 2004). However, the major limitation of UV-C light treatment is that it does not penetrate the target very deeply, thus limiting its uses to treating liquid foods, such as juices and beverages. For example, the transmission of UV-C light might be influenced due to the optical and physical properties and diverse chemical compositions the liquid foods have, lowering the performance efficiency of the UV processes (Koutchma, 2009). Thus, UV-C light is more frequently used for surface sterilization. In addition, the germicidal effect is obtained only by applying direct UV-C light on the target. It is not effective in the shade or in pores (Guerrero-Beltrán & Barbosa-Cánovas, 2004). Another limitations of UV-C processing is that when used at high intensity, the nutrient content of food products being treated may be affected. The sensitivity of food to UV light vary enormously. The molecules are primarily affected by energy absorption that results in

photochemical reactions. For example, protein oxidation and color profile change were found in UV-C light treated Tilapia samples in a dose-dependent manner (Monteiro et al., 2017). In addition, nutrients, such as vitamin A, carotenes, cyanobalamin (vitamin B12), vitamin D, tocopherols (vitamin E), tryptophan, and unsaturated fatty acid residues in oils, solids fats, and phospholipids, are light sensitive (Spikes, 1981). Certain food pigments are also light-sensitive (Koutchma, 2009).

The primary mechanism of inactivation by UV-C light is the direct altering microbial DNA structure. The main types of the photoproduct in UV-C exposed DNA are pyrimidine dimers, pyrimidine adducts and DNA-protein cross-link (Bintsis et al., 2000). The DNA damage after UV-C exposure prevents microorganisms from replicating and finally leads to cell death (Forney, L. J., & Moraru, 2009). UV light sensitivity of microorganisms is a key factor affecting the efficacy of UV treatment of liquid foods and varies significantly. This variation may be due to: the structure, thickness, and composition of cell wall; the acquisition of UV absorbing proteins; and the differences in the structure of the nucleic acids (Wright, Sumner, Hackney, Pierson, & Zoecklein, 2000). In general, Gram positive bacteria are more resistant to UV light than Gram-negative bacteria (Sommers et al. 1989). The UV sensitivity is also strongly related to the ability of the microorganism to repair UV damage through nucleotide excision repair or photolyase in the presence of visible light. Fungi and yeasts are more resistant during disinfection. In addition to the strain of microorganisms, the concentration should also be taken into account (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

The mode of action of UV-A within cells is different from that of UV-C. Research shows that the most likely effect of UV-A on microorganisms are oxidation reactions triggered through its

photosensitizing reaction (**Figure 1-4**). This reaction involves the generation of ROS such as hydroxyl radical and singlet oxygen, which might be the primary agents of cell damage such as

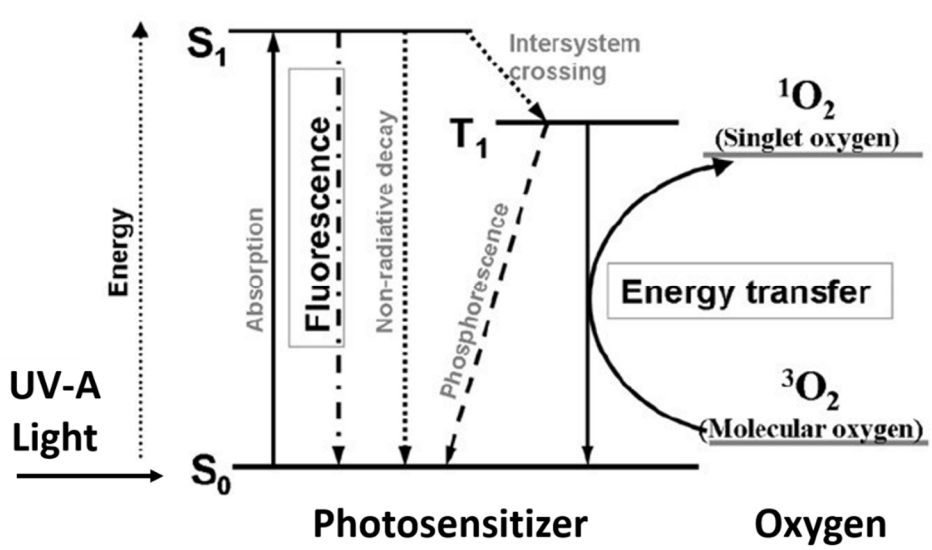

Figure 1-4. The photosensitization process by UV-A light. The relaxation from an excited singlet state by fluorescence emission or by energy transfer to the molecular oxygen after intersystem crossing to the triplet state. (Adapted from Stefflova, K., Chen, J., & Zheng, G., 2007).

the injury of DNA (Kramer & Ames, 1987). Also, membrane damage due to the oxidation of unsaturated fatty acid can induce changes in membrane permeability (Moss, S. H., & Smith, 1981) (Figure 1-4). UV-A is poorly absorbed by living cell compared with UV-C. Although many compounds commonly present in microorganisms including several proteins and lipids have been suggested as possible target molecules, the low lethality of UV-A against microorganisms means that it has little practical value, unless the rate of inactivation can be enhanced. Nevertheless, UV-A has its own advantages: although DNA damaged by UV-C light can be repaired by the enzyme photolyase in bacteria, there is less possibility for microorganisms to repair their damage in the membranes caused by UV-A light, due to the fact that UV-A did not react on DNA but on proteins of various targets (Chevremont, Farnet, Sergent, Coulomb, & Boudenne, 2012). Besides, UV-A light has better penetrating ability, and is much safer to use than UV-C for operators.

One way to enhance the inactivation efficiency of UV-A light is by means of photosensitizer absorbed into the bacterial cell. Once UV-A light is absorbed by the photosensitizer molecules, the molecules jump to triplicate state and transfer energy to generate ROS such as hydroxyl radical and singlet oxygen, which are cytotoxic and able to destroy target microorganisms (Baier et al., 2006). Therefore, instead of directly applying for germicidal purpose as UV-C light, UV-A light is more often used in the photosensitization process to inactivate microorganisms. Photosensitizers can be endogenous and exogenous. Some of the endogenous photosensitizers are flavins, NADH/NADPH, and sterols that are naturally present in cells (Viteri, Edwards, Fuente, & Silva, 2003). Exogenous molecules identified that can serve as effective UV-A photosensitizers to inactivate bacteria includes riboflavin (Makdoumi, Bäckman, Mortensen, & Crafoord, 2010), titanium dioxide (H. A. Foster, Ditta, Varghese, & Steele, 2011), vitamin K (Xu

& Vostal, 2014), etc. Recently, UV-A light has also been explored as a hurdle to be applied in combination with other chemical treatments or physical techniques to enhance antimicrobial activity. For example, in the presence of UV-A light, phenolic compounds such as gallic acid showed enhanced antimicrobial activity against *E. coli* O157:H7 in both cell suspensions and surface of spinach leaves (Cossu et al., 2016). UV-A can also be paired together with UV-C light to show greater penetrating ability and thus enhanced antimicrobial activity (Akgün & Ünlütürk, 2017). Like UV-A, UV-B light can cause oxidative stress in cell. In addition, it also affect microbial cells by causing direct DNA damage, mostly through formation of pyrimidine dimers that block DNA replication and RNA transcription, but is far less effective than UV-C light (A. L. Santos et al., 2013).

1.3.2 Hurdle technology

Thermal processing is a common method of destroying vegetative microorganisms to ensure food safety, but this technique may cause undesirable nutritional and quality effects (Hintz et al., 2015). Non-thermal technologies encompass all preservation treatments that are effective at ambient or sublethal temperatures such as antimicrobial additives, pH adjustment and modified atmospheres. For novel non-thermal technologies, the term refers to techniques such as high hydrostatic pressure, pulsed electric fields, high-intensity ultrasound, ultraviolet light, pulsed light, ionizing radiation and oscillating magnetic field, which are intended for application to microbial inactivation during food processing (Butz & Tauscher, 2002). These novel technologies can inactivate microorganisms to varying degrees. However, with the high treatment intensities required to inactivate significant numbers of microorganisms, certain non-thermal processes can also affect the sensory or functional properties of foods (Wood, O. B., & Bruhn, 2000). For example, high hydrostatic pressure can alter the structure of proteins and

polysaccharides, causing changes in the texture, physical appearance and functionality of foods (Naderi, House, Pouliot, & Doyen, 2017). High-intensity ultrasound also can denature proteins and produce free radicals that can adversely affect the flavor of fruits or foods that contain high level of fat (Aneja et al., 2014). Irradiation does not notably alter proteins and fats but higher doses may cause color changes and nutrient loss (e.g. vitamin C) in some food products (Jo, Lee, & Ahn, 1999). In addition, some emerging non-thermal technologies have been considered as too energy expensive or costly for use in food processing (Raso, Pagán, Condón, & Sala, 1998). Lastly, the development of highly resistant microbial sub-populations can limit the efficacies of emerging non-thermal technologies (Leistner, 1992). Thus, to overcome these deficiencies of one-factor processing, a trend in developing innovative hurdle technologies has emerged.

The hurdle concept is a minimal processing technique that exploits synergistic antimicrobial effects between different preservation treatments at lower individual intensities, while their impact on sensory and nutritive properties of the food is minimized (Leistner, 1992). With hurdle technologies, it is also possible to reduce the energy input and treatment intensities required (Lucera et al., 2012). The use of multiple hurdles usually relies on the combination of different antimicrobials and/or some antimicrobials with a non-thermal processing or a moderate thermal treatment (Davidson & Harrison, 2002). Among these combinations, naturally occurring antimicrobials has proven to be an effective hurdle when combined with non-thermal processing techniques. Selected examples of such combinations can be found in **Table 1-7**.

By placing a number of sublethal stresses on a microbial cell, the organism expends energy to overcome the hostile environment, potentially leading to metabolic exhaustion and death (Leistner, 2000). Hurdles that work on the same targets of cell have an additive inhibitory effect,

Table 1-7: Selected examples of combined use of non-thermal techniques and natural compounds

Combination of hurdles	Microorganism	Food products	Reference
Low dose irradation (2.3 kGy) + pediocin	*Listeria. monocytogenes*	Frankfurters	Chen et al., 2004
Gamma irradation (5 kGy) + nisin (80 IU/g) + heat (90 C, 10 min)	*Spores of Bacillus. cereus*	Sous vide meal	Farkas et al., 2002
Pulsed electric field (35 kV/cm for 1500 ls at 321 Hz and 4 ls pulse length in bipolar) + 1.0% of malic acid	*E. coli O157:H7 & L. monocytogenes*	Apple juice	Raybaudi-Massilia et al., 2006
Pulsed electric field (1,000 µs, 35 kV/cm) + enterocin (+0.61 AU/mL)	*Planctogystia. parvulus*	Apple juice	Martínez-Viedma et al., 2010
High hydrostatic pressure (500 MPa treatment for 5 min at 20 °C) + nisin (500 IU /mL)	*Listeria. innocua & Pseudomonas. fluorescens*	Milk	Black et al., 2005
High hydrostatic pressure (0.1-600 Mpa, 5 min at 25 °C) + mint essential oil (0.1 %).	*L. monocytogenes & L. innocua*	Yogurt	Evrendilek & Balasubramaniam, 2011
Ultrasonication (600 W, 20kHz, 95 µm, 45◦C) + vanillin (1000 ppm)	*L. monocytogenes*	Orange juice	G´omez, P.L., et al., 2011
Pulsed light (180-1100 nm, 12 J/cm2) + malic acid (2% w/v)	*L. innocua & E. coli*	Fresh-cut products	Ramos-Villarroel, A.Y., et al., 2015

while those affecting multiple cellular targets result in synergistic effects. Attacking various cellular targets (e.g. cell membrane, enzyme, DNA, intracellular pH, redox potential, etc.) will have a synergistic effect by forcing the organism employ multiple repair mechanisms simultaneously; two or more hurdles could overcome cellular defenses more easily than one hurdle alone. Attacking multiple cellular targets may also help to reduce the possibility of stress adaptation associated with sublethal treatments (Yousef, 2001). Thus, a good understanding of the mechanisms of each hurdle applied is important for selecting effective antimicrobial combinations for food processing. Further research of the antimicrobial mechanisms of emerging non-thermal technologies as well as their effectiveness when combined with traditional food preservation hurdles is needed so that new food preservation strategies can be developed on a sound scientific basis (Ross et al., 2003).

Light-based hurdle techniques have been explored in previous studies, including the application of UV irradiation, blue light irradiation, pulsed light, and other light based-approaches. The development of these light treatments in combination with traditional physical and chemical preservation methods, or other innovative techniques can be used to enhance the sensorial, nutritional, as well as microbiological quality of food products. For example, UV-C light was combined with subsequent chilling or modified atmosphere packaging as a postharvest treatment for reducing the microbial load on the surface of fresh-cut fruits (Fonseca, J. M., & Rushing, 2006). High intensity pulsed light was applied in combination with pulsed electric fields to inactivate *E. coli* in apple juice (Caminiti et al., 2011). Previous light-based hurdle concepts have also been explored based on naturally occurring phenolic compounds. Nakamura et al. observed the bactericidal effects of various types of photo-irradiated polyphenols such as caffeic acid, GA, and proanthocyanidin by blue light against Gram-positive and Gram-negative bacteria

(Nakamura et al., 2015). Synergistic interactions have been observed between benzoic acid and curcumin with UV-A or visible light (de Oliveira, Tosati, Tikekar, Monteiro, & Nitin, 2018; Ding, Alborzi, Bastarrachea, & Tikekar, 2018). These studies suggest that other alternative technologies are likely to be identified by exploring other combinations of light-based hurdle techniques with naturally-derived compounds.

1.4 Introduction to bacterial adaptive response

1.4.1 Bacteria stress adaptation and impact on food safety

It has been known for decades that microorganisms that experience stressful environment are able to survive subsequent conditions that are considered lethal. Increase in the resistance of an organism to deleterious factors following exposure to mild stress is commonly described as stress adaptation (Wesche, Gurtler, Marks, & Ryser, 2009a). When used in the field of microbiology, the term 'stress' refers to the imposition of detrimental factors of conditions that adversely affect microbial growth or survival (Yousef, A. E., & Juneja, 2002). In all links of the food chain from production to digestion, bacteria are exposed to various stresses, including uncontrollable pre-harvest environmental factors and the deliberate application of preservation factors during post-harvest handling. Generally, stresses to these microorganisms during food production and processing include: 1) Physical treatments such as heat, pressure, electric pulses, ultrasonic waves, light/radiation, and osmotic shock; 2) Addition of chemicals such as acids, salts, and oxidants; and 3) Biological stresses such as competition, microbial metabolites and antagonism (Yousef, A. E., & Juneja, 2002). For example, in the food production environment, sunlight, which contains ultraviolet radiation, may stress or kill bacteria. Heat generated by sunlight may also lead to microbial stress. Acidity of fermented vegetation, salinity of seawater, and dryness of arid climate are examples of other stresses that bacteria may encounter in the

environment. Additionally, bacteria live in environment that lacks essential nutrients for growth or survival stresses, and thus injures or kills bacteria, depending on the severity and duration of starvation (Aertsen & Michiels, 2004). In summary, bacteria in both the environment and food are frequently exposed to physical, chemical and nutritional stresses of varying magnitudes, which can induce bacterial stress adaption towards those stresses.

The stresses bacteria experienced of different levels can be roughly categorized as minor, moderate, severe and eventually lethal stress (Storz, G., 2000). With a minor stress, bacterial cells can completely adapt to the changed conditions, and growth rate is not affected. Low levels of stress may cause a transient adaptation (adaptive response) accompanied by a temporary physiological change that often results in increased stress tolerance (Maurelli, 1989). Lethal stress, however, can cause the death of bacterial cells, but a fraction of them may survive. When lethal stress is experienced by only a fraction of the population, accompanying gene responses and adaptive mutations may actually improve the survival of the overall population. Moderate stress may result in injury ranging from mild to severe (Archer, 1996). The relationship between the different levels of stresses, degrees of injury and the ability of cells to adapt under these conditions is not well defined (Mackey, 2000). **Figure 1-5** gives a brief summary of this relationship. For all practical purposes, sublethal injury means injury in the absence of death with the cell easily undergoing some type of stress adaptation.

Bacterial stress tolerance responses can also result in cross-protection against a wide variety of lethal exposures different from that inducing the adaptive response. For example, thermo-tolerance can be induced by stresses other than heat. Starvation or heat stress can protect *E. coli* against further oxidative challenges (Jenkins, Schultz, & Matin, 1988). More detailed examples of cross-protection can be found in **Table 1-8**. The response to a given stress also differs among

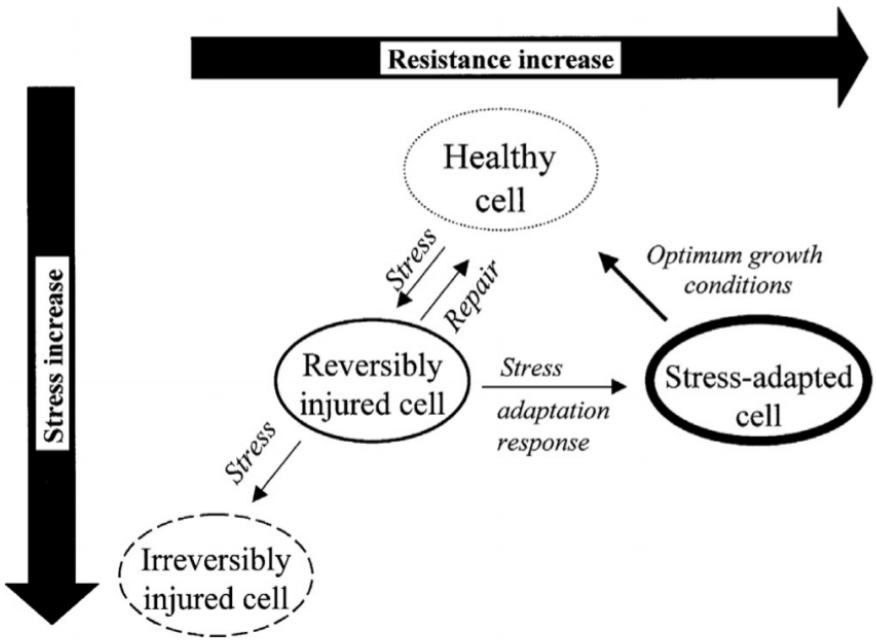

Figure 1-5. Microbial stress, injury, adaptation, and resistance to processing (Lado & Yousef, 2002).bacterial species. Generally, it has been suggested that compared with Gram-positive bacteria, Gram-negative bacteria are more sensitive to cold shock, chilling, and freezing but are more resistant to weak acid preservatives (Beales, 2004).

Table 1-8: Selected examples of bacterial cross-protection

Bacteria strain	Adaptation conditions	Challenge conditions	Response	Reference
Cronobacter. sakazakii	Heat (47.5 °C, 1h)	Pulsed electric field (25 kV cm-1, 35 °C)	Significantly increased protection towards PEF treatment	Arroyo, C., 2011
Listeria. monocytogenes	Heat (48 C for 1 h)	Pulsed UV light (UV dose 0.08 mJ/cm2)	Resistance towards pulsed UV treatment was significantly increased	Bradley, D., 2011
L. monocytogenes	NaCl (1.5 M/ml)	Heat (60 °C)	Significantly increased time to achieve a 4-D reduction	Yousef, A., E., 2003
Salmonella. Typhimurium	Cold shock (5 °C, 5 h)	Acid (pH 4, 90 min)	Significantly enhanced survival during subsequent acid challenge	Shah et al., 2013
L. monocytogenes	Acid (pH 5, 60 min)	Lauric arginate (50 ppm)	Acid adaptation induced cross protection against lauric arginate	Shen, Q., et al., 2015
Staphylococcus. aureus	Acid (pH 4.5, 24 h)	Non-thermal plasma (60 W, 45 s, 2 mm)	Acid stress induced protective effect against the following NTP exposure	Liao, X., et al., 2018
S. aureus	Alkali (pH 9.5, 30 min)	Hydrogen peroxide (50 mM)	Alkali shock induced cross-protection against the following hydrogen peroxide challenge	Cebrián, G., et al., 2010
Salmonella. Enteritidis	Combination (pH 5.5, aw 0.99, 32.5 °C)	Acid (pH 3.78, 25 °C) in apple juice	Prior exposure to a combination of sublethal stresses resulted in acid adaptation	Gabriel, A. A., 2013

The magnitude of a protective stress response also varies with the type of treatment. For example, it was found that nutrient-starved *E. coli* had greater resistance to oxidative and heat challenge than that for heat-adapted cells (Jenkins et al., 1988). Such cross-protection responses are particularly important when minimal processing or hurdle technique is used, where one sublethal stress may lead to the induction of multiple stress responses that reduce the efficacy of subsequent treatments. Therefore, the cross-protection must be considered when implementing new processing technologies or processing hurdles (Wesche, Gurtler, Marks, & Ryser, 2009b).

During traditional food processing such as pasteurization, bacterial cells are more likely to be killed than injured or stressed. However, with the current increasing use of minimally-processing techniques, there are conditions that constitute a mild stress, increasing the survival of bacteria after treatment (Z. Chen, 2017). The survived bacteria become stress-adapted populations that are capable of revisiting similar or different stresses and, in many cases, survive typical injurious or lethal conditions. Although stress factors induced cellular responses vary with the type, magnitude, and method of stress application and there can be multiple outcomes, the adaptive response of microorganisms to stress is of a significance threat in food safety. Therefore, increasing use of alternative novel processing technologies to heat is drawing attention about the potential stress adaptation of foodborne pathogens, and the potential health hazards it constitutes to consumers (Yousef, A. E., & Courtney, 2003). There are also indications that adaptation of bacterial pathogens to stress may increase their virulence and thus ability to cause diseases, although further in-depth studies are needed to investigate this association between resistance and virulence (Beceiro, Tomás, & Bou, 2013).

1.4.2 Mechanism of the development of stress adaptive response

When exposure to an environmental trigger, an adaptive resistance may occur due to an temporary change in gene or protein expression (Rando & Verstrepen, 2007). Microorganisms can sense a stress through many ways, such as that changed membrane fluidity, altered cell protein structure, and damaged RNA (M. R. E. Santos et al., 2016; Willi et al., 2018). For example, a gene regulator OxyR can sense reactive oxygen species via cysteine residues that are oxidized to form a disulphide bridge, which then positively regulates oxidative stress response (Liu et al., 2013). Once bacteria sense the stress, the adaptive response is triggered and the cells respond in various ways. Basically, it involves the genetic regulation of a series of transcription, leading to the synthesis of proteins such as chaperones and proteases that protect against the imposed stress (Fruci & Poole, 2016). For example, in the heat shock response, many chaperones can be developed to control protein folding and protecting the cell from accelerating temperature (Maleki, Khosravi, Nasser, Taghinejad, & Azizian, 2016). The same proteins may be involved in both transient and long-term responses (De La O Leyva-Pérez et al., 2015). The outcomes of microbial stress response include the production of protein to repair cellular damage or eliminate the stress, increase in resistance to other adverse factors, or adaptive mutations. Cells can also transform to a dormant state to reduce metabolic activity and to minimize the effect of harsh conditions (Vliet, 2015). The regulation strategies and outcomes vary among the strains of bacteria and the type of stresses experienced. In addition to a general stress response that helps protect cells from a variety of stresses, cells also have self-protective mechanisms against specific stresses. There can be overlaps between the proteins involved in the general stress response and some other specific stress responses (Battesti, Majdalani, & Gottesman, 2011).

1.4.2.1 General stress resistance

General stress response system can protect against multiple stresses. Activation of the general stress response can be induced by several different stresses and usually results in reduced growth rate or entry into stationary phase (Hengge-Aronis, 1999). The best-characterized general stress response systems are controlled by alternative sigma factors RpoS (σ^S) in Gram-negative bacteria and σ^B (SigB) in Gram-positive bacteria (Kazmierczak, M. J., Wiedmann, M., & Boor, 2005). For *E. coli*, stress adaptive response is coordinated by RpoS, which controls the expression of more than 50 genes involved in the general stress response (Battesti et al., 2011). RpoS can be induced by several different stresses, including nutrient starvation, osmotic shock, high and low temperatures, pH stress, and oxidative stress. Very little of RpoS can be detected in non-stressed *E. coli* cells (Rowbury, 2003). Bacteria lacking in the gene for RpoS are more sensitive to different food processing conditions, including heat shock, starvation, acid, and ethanol exposure (Abee & Wouters, 1999).

1.4.2.2 Heat

Heat treatment is one of the most commonly used food processing methods that can eliminate foodborne pathogens from foods. However, some bacteria may develop heat adaptation under sublethal heat stress and become more resistant towards subsequent lethal temperature (Lindquist, 1992). Many heat-induced stress proteins are protein chaperones that assist in folding assembly of heat-damaged proteins or are ATP-dependent proteases that degrade damaged proteins (Verghese, Abrams, Wang, & Morano, 2012). In addition, some bacteria alter their cell membrane in response to heat by increasing the ratio of *trans* to *cis* fatty acids in the membrane, in order to decrease fluidity caused by increasing temperatures (Los & Murata, 2004). In *E. coli*, the alternative sigma factor, σ^{32}, controls the expression of the majority of heat-induced genes.

Approximately 50 genes are induced by σ^{32} when denatured proteins are detected in the cytoplasm. If the bacteria is not under heat stress, σ^{32} is commonly present at low levels (Morita et al., 1999).

1.4.2.3 Acid

Acid is one of the most frequently encountered harsh condition foodborne bacteria encounter. Pathogens such as *E. coli* and *Salmonella* must endure extreme low pH (2.5) in the stomach as well as fatty acids present in the intestine (pH 4-6). Outside the host, bacteria may encounter acid stress from industrial waste or decaying organic matter (Bearson, S., Bearson, B., & Foster, 1997). The mechanisms of microbial inactivation by inorganic and organics acids are different, although both result in intracellular acidification that cause damage to biochemical processes (Chung, Bang, & Drake, 2006). To survive, bacteria sense environmental acidic pH and then initiate a signaling cascade allowing adaptation to these conditions (Fang, Frawley, Tapscott, & Vázquez-Torres, 2016). The acid tolerance response (ATR), is a phenomenon when bacteria are exposed to moderately low pH, inducing the synthesis of proteins that promote survival at extremely low pH. ATR differs in exponential and stationary phase cells. For example, *E. coli* cells in the stationary phase were more acid tolerant than cells in the log phase (Buchanan RL, 1997). This response has been identified in many bacteria and differs among different species (J. W. Foster, 1993).

1.4.2.4 Oxidative stress

Bacteria may be exposed to increased levels of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals and superoxide during food processing or in food products. Such oxidants can cause damage to cellular proteins, lipids and nucleic acids (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Therefore, many of the known proteins induced by

oxidative stress have antioxidant roles, while others are involved in the repair of oxidative damage, particularly damage to nucleic acids (Storz, Tartaglia, Farr, & Ames, 1990). In *E. coli*, most oxidative stress-induced genes are part of the *oxyR* and *soxRS* regulons induced by hydrogen peroxide and superoxide, respectively (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). OxyR sense oxidative damage via cysteine residues that are oxidized to form a disulphide bridge, altering the protein structure into the active form (Ke & Berkmen, 2014). There is significant overlap between the oxidative stress-induced proteins and those induced by RpoS, suggesting that oxidative damage is significant in stationary phase or generally stressed cells (Farr & Kogoma, 1991).

1.5 Statement of problem

Based on the extensive literature review performed, it is evident that-

1. Foodborne disease is a growing public health problem worldwide, and several of them are associated with the consumption of fresh fruits and vegetables. While various chemical and physical technologies are used in the food industry to address these problems, traditional food process and preservation methods have been questioned due to the damage to food quality, limited antimicrobial efficiency, or side-effect on human health.
2. The long-term use of chlorine, the most commonly used sanitizer so far, for fresh produce disinfection has been associated with the formation of carcinogenic compounds. Also, these chemical agents may have limited efficacy especially in the presence of organic content. Moreover, some microorganisms have been shown to adapt and gain resistance to the existing food process techniques. Therefore, there is a current need to explore

environmental-friendly, and highly efficient antimicrobial techniques to produce fresh and microbiologically safe foods to consumers.

3. The interest towards food-grade, naturally occurring compounds as antimicrobials is increasing around the world. One problem with many naturally occurring antimicrobial compounds is that they are not effective enough to be used alone to prevent food safety and spoilage problems. The development of emerging, non-thermal technologies in food processing addresses specific consumer needs toward safe, health, and minimally processed foods. Examples include high hydrostatic pressure (HHP), pulsed electric fields, ultrasound (US), cold plasma, and irradiation. Previous literature have demonstrated that physical preservation processes enhance the effectiveness of natural antimicrobials in inactivating target microorganism. The most desirable outcome of the combination treatment is its synergistic effect. Natural or food-grade antimicrobials have the potential to be applied in combination with new non-thermal techniques to enhance the effectiveness of food processing and maintain the high quality of the products.

To address these needs and gaps in the literature, the overall goal of this project was to develop novel antimicrobial treatments based on the synergistic interaction between gallic acid (GA, 3,4,5-trihydroxyl-benzoic acid), a Generally Recognized As Safe (GRAS) polyhydroxyphenolic compound, and UV light and GA for the application on fresh produce disinfection.

1.6 Specific objectives

1. Investigate the antimicrobial mechanism of a previously developed UVA+GA treatment by analyzing GA uptake, intracellular ROS generation, enzyme inhibition, and membrane injury of *E. coli* O157:H7. In addition, the effect of solution pH, the presence of ethylenediaminetetraacetic acid (EDTA) on the antimicrobial activity of the GA+UV-A

system will be investigated. Since pyrogallol (Py) and propyl gallate (PG), derivatives of GA, are structurally similar to GA, the antimicrobial activity of these two compounds will also be evaluated.

2. Develop a novel light-enhanced antimicrobial treatment by exposing GA to UV-C light and evaluating its efficacy using *E. coli* O157:H7 as a model bacteria. The bactericidal characteristics of UV-C irradiated GA were evaluated, and the underlying mode of action of the observed enhanced antimicrobial activity investigated.
3. Validate the inactivation efficacy of UVA+GA and UVC-GA treatments on fresh produce against *E. coli* O157:H7, using baby spinach and cherry tomatoes as model produce. The texture and color of the treated produce were also be evaluated.
4. Investigate the effect of 1) prior exposure to sublethal stress (heat, acidity, osmotic stress, and oxidative stress) on the development of resistance towards UVA+GA and UVC-GA treatments, and 2) exposure to UVA+GA or UVC-GA treatments on the development of cross-resistance to other stresses, using *E. coli* O157:H7 as a model microorganism.

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Chapter 2: On mechanism behind UV-A light enhanced antibacterial activity of gallic acid and propyl gallate against *Escherichia coli* O157:H7

Hypotheses

1) The combination of GA and UV-A light (UVA+GA) has synergistic antimicrobial activity against *E. coli* O157:H7.
2) This antimicrobial activity is affected by various factors such as concentration of GA, solution pH, and the presence of metal ions.
3) This antimicrobial activity is a result of complementary stresses affecting various aspects of the cell, ultimately leading to the death of bacteria.

Publication status

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Abstract

Possible mechanisms behind the enhanced antimicrobial activity of gallic acid (GA) and its ester propyl gallate (PG) in the presence of UV-A light against *Escherichia coli* O157:H7 were investigated. GA by itself is a mild antimicrobial and has pro-oxidant ability. We found that the presence of UV-A light increases the uptake of GA by the bacteria. Once GA is internalized, the interaction between GA and UV-A induces intracellular ROS formation, leading to oxidative damage. Concurrently, UVA+GA also inhibits the activity of superoxide dismutase (SOD), magnifying the imbalance of redox status of *E. coli* O157:H7. In addition to ROS induced damage, UV-A light and GA also cause injury to the cell membrane of *E. coli* O157:H7. UV-A exposed PG caused oxidative damage to the cell and significantly higher damage to the cell membrane than UVA+GA treatment, explaining its higher effectiveness than UVA+GA treatment. The findings presented here may be useful in developing new antimicrobial sanitation technologies for food and pharmaceutical industries.

2.1 Introduction

Gallic acid (GA, 3,4,5-trihydroxyl-benzoic acid) is a polyhydroxyphenolic compound widely distributed in various plants, fruit and vegetables (Niemetz & Gross, 2005), and is a Generally Recognized As Safe compound (GRAS) to humans (US FDA, 2015). A variety of biological activities of GA have been demonstrated, including antioxidant (Badhani, Sharma, & Kakkar, 2015), anti-inflammatory (Kim, 2006), and anti-cancer (Bo Ra You, Moon, Han, & Park, 2010). Furthermore, GA has been found to have mild antimicrobial effect by itself against a wide variety of planktonic bacteria, biofilm, and fungi (Borges, Ferreira, Saavedra, & Simões, 2013; Borges, Saavedra, & Simões, 2012; Kang, Oh, Kang, Hong, & Choi, 2008; Nohynek et al., 2006). Although it has been well known that GA provides efficient protection against oxidative damage, it also has been reported to have pro-oxidant potential due to its autoxidation in certain conditions, resulting in the generation of reactive oxidative species (ROS) such as hydroxyl radicals, hydrogen peroxide, and superoxide anion (Badhani et al., 2015). This ROS generation resulted from the pro-oxidant potential of GA has been regarded as one of the contributors to the antimicrobial activity of GA (Barcelo et al., 2014), (Nakamura et al., 2012), and has also been associated with the ability of GA to induce apoptosis of different cell lines (C. Chen, Chen, Yang, Liu, & Hsu, 2013; Chuang et al., 2010; Larry H. Russell Jr, Elizabeth Mazzio, Ramesh B. Badisa, Zhi-Ping Zhu, Maryam Agharahimi, Ebenezer T. Oriaku, 2007; Sakaguchi, Inoue, & Ogihara, 1998; Bo R. You & Park, 2010). Besides the effect of ROS, previous studies also attributed the antimicrobial activity of GA to the cell membrane disintegration and consequent leakage of intracellular constituents of bacteria (Borges et al., 2013; Nohynek et al., 2006). Propyl gallate (Gallate acid propyl ester, PG), a derivative of GA is widely used as a synthetic antioxidant in processed foods, cosmetics, and food packaging materials to prevent rancidity and spoilage

(Nakagawa, Nakajima, Tayama, & Moldeus, 1995). Previously, alkyl gallate such as PG has been found to work as antibacterial and antifungal agents, but the mechanism was assumed not be ROS related (del Valle et al., 2016; Kubo, I., Fujita, K. I., Kubo, A., Nihei, K. I., & Ogura, T., 2004).

Studies have shown that some mild antimicrobials have synergistic antibacterial effects when they are combined with physical intervention such as heat or acid treatment, even at doses that are generally not inherently effective (Ricke, Kundinger, Miller, & Keeton, 2005). A recent study from our group established a novel synergistic antimicrobial method, in which the non-thermal UV-A light treatment and GA (UVA+GA) generated enhanced antibacterial activity against *E. coli* O157:H7 (Cossu et al., 2016). This UVA+GA treatment was effective in achieving > 5 log CFU/mL in *E. coli* O157:H7 in water suspension, and > 3 log CFU/mL when organic matter (2000 mg O_2/L COD) were present. Inactivation against biofilm of *E. coli* O157:H7 was also effective that about 80% decrease in the metabolic activity of the biofilm was observed. However, the mechanism of this synergistic effect has not been fully explored. Previously, this antibacterial effect was attributed to the photo-irradiation of GA by UV-A light and the subsequent generation of reactive oxidative species (ROS), by recognizing GA as a photosensitizer. High concentrations of ROS, including oxygen radicals and reactive non-radicals can cause cellular damage (Deavall, Martin, Horner, & Roberts, 2012). Nakamura et al. studied the antimicrobial action of photo-irradiated GA (4 mg. L^{-1}) by LED (400 nm). Although it was demonstrated that hydroxyl radicals and other ROS formed by photo-oxidation of GA, the concentration was too low to be effective by itself, and there was no direct evidence that these ROS species were responsible for the inactivation (Nakamura et al., 2012, 2015). Thus, there

was a need for a study focused on understanding the mechanism behind the synergistic interaction between UV-A light and GA.

The objective of this study is to investigate the antimicrobial mechanism of action of the UVA+GA simultaneous treatment, by analyzing GA uptake, intracellular ROS generation, enzyme inhibition, and membrane injury of *E. coli* O157:H7. In addition, the effect of solution pH, the presence of ethylenediaminetetraacetic acid (EDTA) on the antimicrobial activity of the UVA+GA system was investigated. Since pyrogallol (Py) and propyl gallate (PG), derivatives of GA, are structurally similar to GA, the antimicrobial activity of these two compounds was also evaluated.

2.2 Methods

2.2.1 Bacteria cultivation

A rifampicin resistant, shiga toxin negative *E. coli* O157:H7 (ATCC #700728, Manassas, VA) was kindly provided by Prof. N. Nitin at University of California-Davis. Stock cultures were maintained at -80 °C in tryptic soy broth (TSB) supplemented with 20% glycerol. The culture was re-activated by transferring to a plate of tryptic soy agar (TSA) and stored at 4 °C. Prior to the experiments, the bacterium was cultured in Tryptic Soy Broth (TSB) at 37°C for 20 h to obtain the bacterial population in early stationary phase before each experiment.

2.2.2 UVA+GA treatment

The overnight bacterial culture was diluted in sterilized GA solution of various concentrations (5, 10, 15 mM) prepared in either DI water or phosphate buffer (100 mM, pH = 7.4), with or without the presence of EDTA (1 mM) to achive a reach a final concentration of approximately 1×10^7 CFU/mL. To evaluate the antimicrobial activity of some GA derivatives, the bacteria were also

exposed to pyrogallol (Py, 15 mM) or propyl gallate (PG, 10 mM). After inoculation, 2 mL of each bacterial suspension was transferred to a well of a 6-well flat bottom polystyrene plate, and exposed to UV-A light for 30 min. The UV-A light source (Spectroline ^{TM}, Westbury NYUSA) was a bench-top, batch type chamber with a peak wavelength of 365 nm and average intensity of 3425 μW/cm^2 applied at the surface from a distance of 17 cm. Bacterial suspensions incubated with select compounds and stored in the dark for 30 min were used as controls. After the treatment, the bacteria suspensions were serially diluted in 0.2 % (w/v) buffered peptone water, and an aliquot of 100 μL suspensions from each dilution was transferred and plated onto Tryptic Soy Agar (TSA, Difco^{TM}, Detroit MI USA) plates. The plates were incubated at 37 °C for 24 h before enumeration.

2.2.3 Analysis of GA uptake by E. coli *O157:H7*

The uptake of GA was evaluated using diphenylboric acid 2-aminoethyl ester (DPBA), a flavonoid specific dye that becomes fluorescent upon conjugation with flavonoid compounds and can permeate the bacterial membrane. A volume of 1 mL of overnight culture of *E. coli* O157:H7 was transferred to a 1.5 mL Eppendorf tube and centrifuged twice at 10,000 rcf for 2 min to obtain a pellet (~ 1×10^9 CFU/mL). A volume of 1 mL of solution of GA (15 mM) + EDTA (1 mM), GA (15 mM), EDTA (1 mM), GA in phosphate buffer solution (100 mM, pH = 7.4), or PG (10 mM) was added to the pellet and mixed. To evaluate the effect of pH on GA permeability, GA (15 mM) in phosphate buffer at the pH of 7.4 was also used to treat the pellet. DI water was added to the pellet as control. Then, 2 mL of the suspension was transferred to a 6-well plate and exposed to UV-A light for 30 min as described previously. Control samples were treated in the exact same manner except for the UV treatment. After the incubation, each suspension was transferred back to an Eppendorf tube and centrifuged at 10,000 rcf for 2 min. The supernatant

was removed and the pellet was washed twice with DI water. Then, 450 µL of DPBA solution (0.2% w/v in DMSO) was added to the pellet and mixed. The final suspension of 100 µL was transferred to a 96-well plate and fluorescence intensity was measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at excitation/emission wavelength of 405/465 nm. The fluorescence intensity ratio was corrected using the following equation:

$$Corrected\ fluorescence = fluorescence\ intensity_{\ treated\ sample} - fluorescence\ intensity_{\ control} \quad (1)$$

2.2.4 Analysis of intracellular oxidative stress

The intracellular oxidative stress of *E. coli* O157:H7 was analyzed using two distinct approaches. CellROX® Green Reagent is a novel fluorogenic probe for oxidative stress measurement in live cell. The cell-permeant dye is weakly fluorescent while in a reduced state and exhibits bright green photostable fluorescence upon oxidation by reactive oxidative species (ROS) and subsequent binding to DNA. A volume of 1 mL of overnight culture of *E. coli* O157:H7 at stationary phase was transferred to an Eppendorf tube, washed with DI water, centrifuged for 2 min at 10,000 ×g, and exposed to UV-A or kept under the dark with GA (15 mM), PG (10 mM), GA in phosphate buffer (pH = 7.4), HCl (pH = 3.1), or DI water. Hydrogen peroxide (HP, 1.5%), was used as a positive control. After the treatment, CellROX® Green probe was added to the bacterial suspension at a concentration of 5 µM and incubated at 37 °C for 30 min without light exposure. Then, the bacterial suspension was washed three times with sterile phosphate buffered saline (PBS) solution, and re-suspended in 500 µL of PBS solution. This final suspension was transferred either to a 96-well plate for intensity measurements at 485/520 (ex/em), or to a

fluorescence microscope for imaging. The fluorescence intensity was corrected using equation (1).

For fluorescence imaging, 5 μL of the stained bacteria suspension was placed between a slide and a cover slip for microscopy evaluation. Zeiss Axio Observer Z1 fluorescence microscope (Oberkochen, Germany) at University of Maryland (Department of Microbiology) was used for observation of the stained bacteria at a magnification of 100×. The light source was a TL Halogen lamp. Filter wavelength of excitation/emission were 450-495 nm / 500-550 nm respectively. The fluorescence intensity of images were measured using image processing software ImageJ (Jensen, 2013) after the images were set to identical threshold.

The intracellular oxidative stress was also analyzed by measuring free thiols in bacteria using Thiol Detection Assay Kit (Cayman Chemical Company, Ann Arbor, MI). A volume of 1 mL of overnight culture was transferred to a sterile Eppendorf tube, washed with DI water, centrifuged for 2 min at 10,000 ×g, and treated with or without the exposure of UV-A light in the presence of GA (15 mM), PG (10 mM), HCl (pH = 3.1). Bacterial pellet suspended in DI water only without UV-A light treatment was set as the control. Hydrogen peroxide (1.5%), was used as a positive control. After the treatment and washing with DI water, 1 mL of cold lysis buffer (Tris-HCl 10 mM with EDTA 1 mM) was added to the pellet and mixed thoroughly. Then, 500 μL of suspension was transferred to a new Eppendorf tube containing approximately 400 μL of silica beads. The mixture was vortexed for 10 min before centrifuging at 15,000 rcf at 4 °C. The supernatant was diluted 5-fold in thiol assay buffer included by the assay kit. Finally, 50 μL of diluted supernatant were transferred to a 96-well plate, to which 50 μL of thiol fluorometric detector were added. The plate was incubated under the dark for 5 min, before measuring

fluorescence at the excitation and emission wavelength of 385 and 515 nm respectively. The fluorescence intensity ratio was normalized using the following equation:

$$\%\, Relative\ fluorescence = \frac{fluorescence\ intensity_{\ treated\ sample}}{fluorescence\ intensity_{\ control}} \times 100 \qquad (2)$$

2.2.5 Analysis of superoxide dismutase activity

Activity of superoxide dismutase (SOD) within *E. coli* O157:H7 was analyzed using Superoxide Dismutase Assay Kit according to the manufacturer's protocol (Cayman Chemical, MI). *E. coli* O157:H7 suspensions were diluted in 15 mM GA, 10 mM PG, HCl with the same pH of 15 mM GA, or DI water as control. After incubation in the presence or absence of UV-A light, the samples were washed with DI water. Then, samples were homogenized in 20 mM HEPES buffer (pH = 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose), followed by centrifugation at 1,500 ×g for 5 min at 4 °C, and recovery of the supernatant. To quantify SOD activity, 200 µL of diluted SOD radical detector (included in the assay kit), 10 µL of sample, and 20 µL of diluted xanthine oxidase (included in the assay kit) were successively added to a 96-well plate, covered with the plate's lid, and incubated on a shaker for 30 min to mix at room temperature. The absorption was analyzed using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at a wavelength of 440 nm. The SOD activity was determined by referring to a SOD activity standard curve established using the same assay kit.

2.2.6 Analysis of membrane damage

Membrane damage of *E. coli* O157:H7 during the treatment was analyzed by using the fluorescence probe propidium iodide (PI) and through scanning electron microscope (SEM). PI is a red-fluorescent nuclear and chromosome counterstain that penetrates only bacteria with damaged membranes and is frequently used to detect cell membrane damage (Virto, Mañas,

Álvarez, Condon, & Raso, 2005)·(Davey & Hexley, 2011). Test solutions consisting of bacteria (~ 1×10^9 CFU/mL) suspended in GA solution (15 mM), PG solution (10 mM), EDTA solution (1 mM), or HCl solution (pH = 3.1) were treated under UV-A light exposure or incubated under the dark for 30 min. Bacteria in DI water alone in the dark was used as live control. After treatment, samples were washed with DI water and centrifuged for 2 min at 10,000 ×g. Then, a volume of 50 µL of PI was added to each sample to reach a concentration of 5 µM, following dark incubation at room temperature for 15 min. Subsequently, samples were washed and suspended in 500 µL 1× PBS. A volume of 100 µL of this sample were transferred to a 96-well plate, and the fluorescence intensity was measured using a plate reader with excitation and emission wavelength of 490/635 nm. The fluorescence intensity was corrected using equation (1).

Membrane damage was also visualized by SEM imaging based on the method described by Kihm et al.(Kihm, Leyer, An, & Johnson, 1994). *E. coli* O157:H7 (approximately 1×10^7 CFU/mL) suspended in 15 mM GA solution, 10 mM PG solution or DI water were exposed to UV-A light or incubated in the dark for 30 min. Then, cells were recovered by filtering through a 0.2 µm sterile filter, fixed by incubating in 0.25% glutaraldehyde for 1 h, rinsed three times in DI water, dehydrated six times in ethanol of increasing concentration, and stored in a desiccator overnight for dehydration prior to imaging. To observe cell morphology under SEM, bacteria were coated with gold (20 nm) with a sputter coater. After coating with gold, the morphology of bacteria was studied using a SEM at an accelerating voltage of 10 kV.

2.2.7 Statistical analysis

Experiments were performed in triplicate. Statistical analysis of the data was performed using the two-tailed unpaired *t*-test ($\alpha = 0.05$) by Microsoft Excel 2016 (Microsoft Inc., Redmond WA, USA). When appropriate, statistical significance was determined through analysis of variance

performed by JMP 13.1.0 (SAS Institute Inc., Cary NC, USA) followed by pairwise comparisons through Tukey's HSD test ($\alpha = 0.05$).

2.3 Results

2.3.1 Antimicrobial activity of UVA+GA against E. coli O157:H7: effect of GA concentration, solution pH, structure of derivatives, and the presence of EDTA

Figure 2-1a illustrates the antimicrobial activity of GA at various concentrations (5, 10 and 15 mM) dissolved in DI water or phosphate buffer (100 mM) at pH 7.4 in the absence or presence of UV-A light. GA alone in the absence of UV-A light and UV-A alone in the absence of GA did not show significant antibacterial effect (< 0.5 log CFU/mL). In the presence of UV-A light, 5 mM GA, caused a decrease of less than 1 log CFU/mL (0.41 ± 0.12), and was not significantly different ($P > 0.05$) from that of control. At 10 or 15 mM, the reduction in *E. coli* O157:H7 increased to 2.06 ± 0.19 and 4.41 ± 0.21 log CFU/mL, respectively. The dependence of microbial inactivation on the concentration of GA was consistent with our previous study using synergistic interaction of GA and UV-A light against *E. coli* O157:H7 in simulated fresh produce (Cossu et al., 2016). Interestingly, UVA+GA did not show significant ($P > 0.05$) antibacterial activity when the solution was prepared in phosphate buffer of pH 7.4.

To investigate if derivatives of GA had comparable antibacterial activity when treated under UV-A light, we performed similar experiments with pyrogallol (Py) and propyl gallate (PG) (**Figure 2-1b**). Py+UV-A (15 mM) treatment did not cause any reduction of *E. coli* O157:H7 at either its natural pH (pH = 5.5) or at a pH similar to 15 mM GA (pH = 3.1). In contrast, PG+UV-A (10

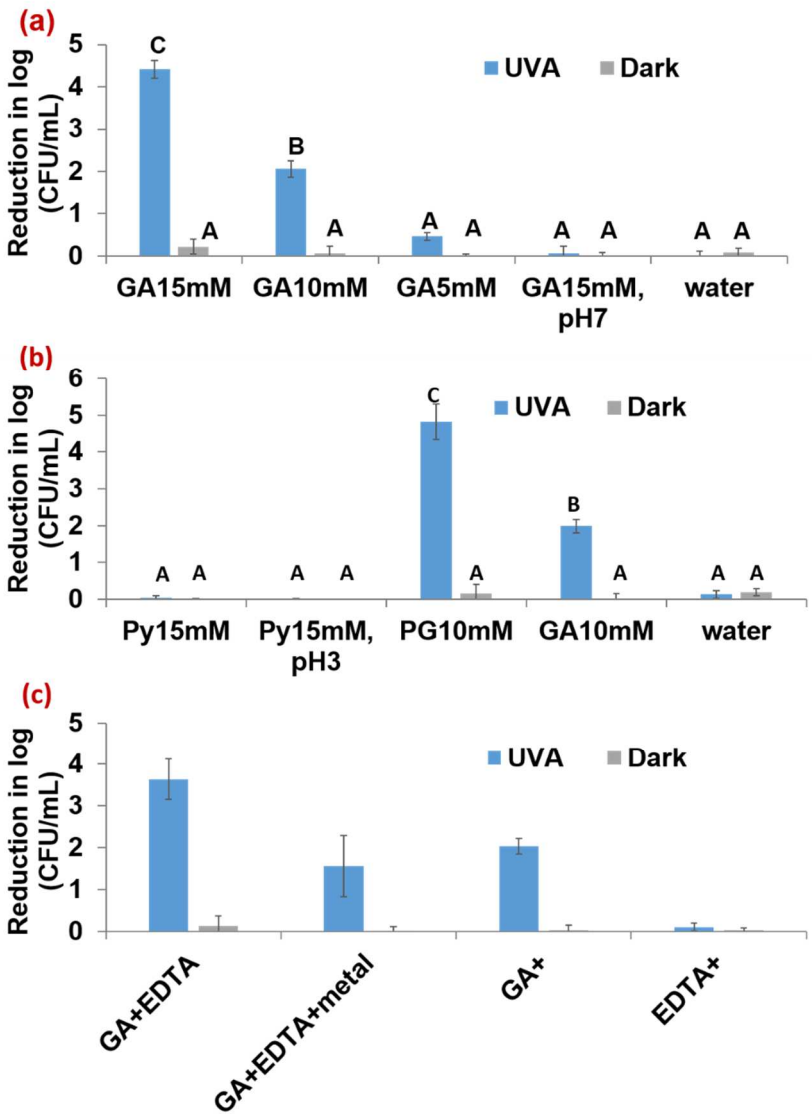

Figure 2-1. Evaluate the effect of GA concentration, solution pH, derivatives of GA and EDTA on the antimicrobial activity in the absence or presence of UV-A light. Logarithmic reduction of *E. coli* O157:H7 in the absence or presence of UV-A light and presence of **(a)** GA (0, 5, 10, 15 mM) and GA (15 mM) in phosphate buffer pH 7.4, **(b)** Pyrogallol (Py, 15 mM) or Propyl Gallate (PG, 10 mM), and **(c)** 15 mM GA+EDTA (E, 1 mM) with or without addition of 2 mM Mg^{2+} and 2 mM Ca^{2+} (MC). Mean ± SD. Means sharing the same letter are non-significant at $P < 0.05$ according to Tukey's HSD test.

mM) caused more than 6 log CFU/mL in reduction, indicating a stronger antibacterial activity than UVA+GA.

Figure 2-1c shows the effect of addition of 1 mM EDTA to GA (10 mM) solution. While 10 mM GA with UV-A light caused a 2.31 ± 0.57 log CFU/mL reduction, addition of 1 mM EDTA to 10 mM GA significantly ($P < 0.05$) increased the microbial inactivation to 3.64 ± 0.48 log CFU/mL ($P < 0.05$) in the presence of UV-A light. When a mixture of 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was added to the bacteria solution containing 1 mM EDTA and 10 mM GA and exposed under UV-A light for 30 min, a microbial reduction of 1.56 ± 0.74 log CFU/mL was observed. This was significantly ($P < 0.05$) lower than that in the absence of metal ions, and not significantly ($P > 0.05$) different from the logarithmic reduction obtained from the 10 mM UVA+GA treatment.

2.3.2 Analysis of GA uptake by E. coli O157:H7

Diphenylboric acid 2-aminoethyl ester (DPBA) is a flavonoid specific dye that becomes fluorescent upon conjugation with flavonoid compounds (J. Chen et al., 2015). In the present study, it was used to detect the uptake of GA in *E. coli* O157:H7. Higher fluorescence intensity indicates higher association of GA with bacteria. **Figure 2-2a** shows that *E. coli* O157:H7 treated with 15 mM UVA+GA light had significantly ($P < 0.05$) higher level of fluorescent intensity than that incubated in the dark, suggesting UV-A exposure increased the uptake of GA. Also, *E. coli* O157:H7 treated by GA+EDTA showed higher fluorescence intensity than by GA itself, suggesting GA uptake increased in the presence of EDTA. Fluorescence intensity within *E. coli* O157:H7 treated by GA in neutral pH (pH = 7.4) was not significantly different ($P > 0.05$) from that of control, indicating GA was not taken up at pH 7.4. The GA uptake results were consistent with the inactivation results. It should be noted that although fluorescence intensity in *E. coli*

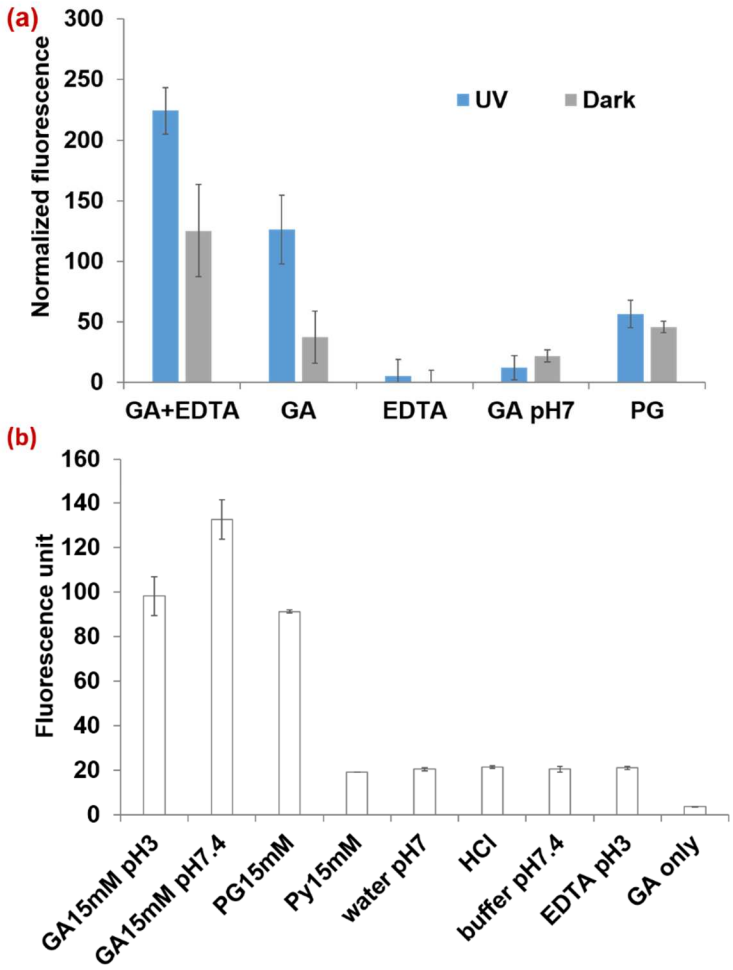

Figure 2-2. Measurement of uptake of gallic acid (GA) and its derivatives in *E. coli* O157:H7 as measured by binding with DPBA. (a) *E. coli* O157:H7 treated by GA (15 mM) +EDTA (1 mM) solution, GA (15 mM), EDTA (1 mM), GA in phosphate buffer solution (100 mM, pH 7.4), or PG (10 mM) in the presence and absence of UV-A light. Absolute fluorescence values were corrected by subtracting the fluorescence values for samples incubated in water and in dark. **(b)** The assessment of DPBA sensitivity to binding various compounds in the absence of bacteria. Mean ± SD.

O157:H7 treated by GA+EDTA incubated in the dark was not significantly different from that treated by UVA+GA ($P > 0.05$), GA+EDTA without UV-A reduced the microbial population by 0.14 ± 0.24 log CFU/mL, while UVA+GA treatment had a 2.3 ± 0.57 log CFU/mL reduction. Thus, the extent of uptake may not be the only factor that affects the antibacterial effect of the treatment. It should also be noted that bacteria treated by PG+UV-A (10 mM) did not show a higher fluorescence intensity than that from UVA+GA, although the antimicrobial activity of PG+UV-A was significantly ($P < 0.05$) higher than that of UVA+GA exposure, indicating that inactivation of the bacteria by PG+UV-A followed a different mechanism than UVA+GA. To verify the specificity of the complexation between DPBA and GA, experiments were also performed by measuring only the intensity of DPBA dissolved in water, HCl (pH = 3.1), phosphate buffer, EDTA (pH = 3.1), and GA in acidic or neutral pH without bacteria. The results (**Figure 2-2b)** showed the relative affinity of DPBA to selected compounds or solvents. When GA of the same concentration was dissolved in a neutral pH buffer, the DBPA intensity was higher than that in acidic solution, indicating that GA was still able to complex with DBPA at neutral pH. Thus, the low fluorescence intensity in bacterial samples exposed to GA in phosphate buffer was due to the low extent of uptake of GA by bacteria at neutral pH. Therefore, the uptake of GA by bacteria was an important factor for GA to exert antibacterial activity with UV-A light. Similarly, PG was also able to bind with DPBA to produce fluorescent signal. However, based on the results shown in **Figure 2-2a**, where fluorescence intensity from PG+UV-A treatment was significantly lower ($P < 0.05$) than that of UVA+GA or GA+EDTA+UV-A, it is likely that the mechanism of inactivation for PG is distinct from GA. Py by itself did not show significant affinity to DPBA, therefore the uptake of Py by bacteria was not detected.

2.3.3 Analysis of intracellular oxidative stress

Generation of reactive oxygen species (ROS) in the bacteria is hypothesized to be one of the reasons for antimicrobial activity of the UVA+GA treatment against *E. coli* O157:H7. To evaluate this hypothesis, we used a fluorescent probe, CellROX® Green Reagent for measuring oxidative stress in cells (Choi, Yang, & Weisshaar, 2015; Cornejo-Corona, Thapa, Browne, Devarenne, & Lozoya-Gloria, 2016; Lam et al., 2016; Xiao & Miwa, 2016). **Figure 2-3a** shows the results from CellROX® fluorescence intensity measurement. The higher fluorescence intensity indicates higher concentrations of intracellular ROS. Bacteria treated by hydrogen peroxide (1.5%) in dark for 30 min were used as the positive control. *E. coli* O157:H7 treated by 15 mM UVA+GA light for 30 min had significantly ($P < 0.05$) higher intensity than UV-A alone or GA incubated in the dark, indicating higher concentration of ROS. Fluorescence intensity was also measured when the bacteria were exposed to a sub-lethal treatment of UVA+GA for 5 min (< 1 log CFU/mL reduction). A significant increase ($P < 0.05$) in fluorescence intensity was observed, for both GA in dark and UVA+GA, indicating that the generation of ROS preceded the inactivation of the bacteria.

To visualize the ROS generated by UVA+GA interaction, CellROX® fluorescent reagent treated bacteria were observed under a fluorescence microscope (**Figure 2-3b)** and the fluorescence intensity within bacteria was quantified (10 bacteria per treatment) in **Figure 2-3c**. *E. coli* O157:H7 treated by UVA+GA light had the highest fluorescent intensity, followed by bacteria exposed to GA in dark. HCl with or without UV-A light treated samples had similar and weaker fluorescent intensity than the treatment. Bacteria in DI water exhibited the weakest fluorescence. Thus, the fluorescence spectroscopic results and imaging results were qualitatively consistent. Since the level of unoxidized free thiols is a good indicator for intracellular oxidative

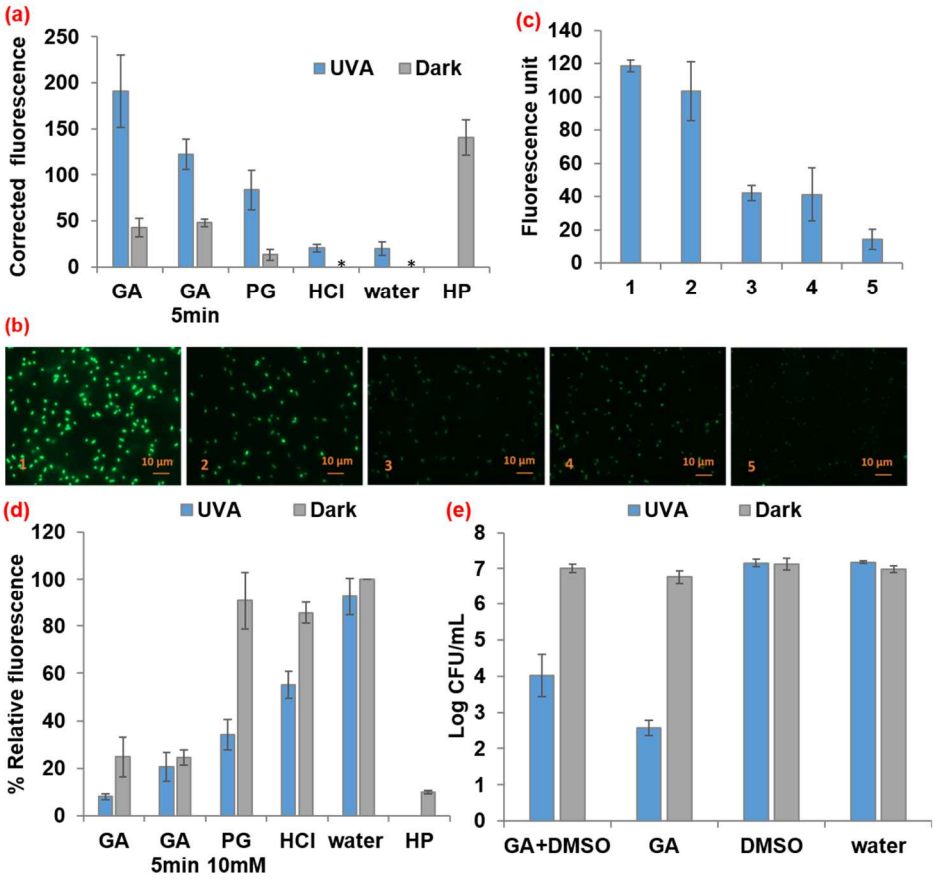

Figure 2-3. Measurement of oxidative stress experienced by bacteria by the select treatments. (a) Detection of reactive oxidative species (ROS) within *E. coli* O157:H7 using CellROX® Reagent Green upon treatment by GA (15 mM), PG (10 mM), HCl (pH 3.1), water, or hydrogen peroxide (HP, 1.5%), in the absence or presence of UV-A light for 30 or 5 min. Absolute fluorescence values were corrected by subtracting the fluorescence values for samples incubated in water and in dark. * indicates that corrected fluorescence value was zero. **(b)** Fluorescent microscopy images (×100) of bacteria incubated with CellROX® Reagent Green and (1) 15 mM GA+UV-A, (2) 15 mM GA in dark, (3) HCl (pH = 3.1) +UV-A, (4) HCl (pH = 3.1) in dark, (5) DI water in dark **(c)** Average maximum fluorescence intensity within the bacteria imaged using fluorescence microscopy. **(d)** Measurement of free thiols content from *E. coli* O157:H7 suspensions treated by GA (15 mM), PG (10 mM), HCl (pH 3.1), or water, with the presence or absence of UV-A light

for 30 or 5min. Hydrogen peroxide (HP, 1.5%), was used as a positive control. **(e)** Logarithmic reduction of *E. coli* O157:H7 by 15 mM GA+UV-A light, with or without 5% DMSO. Mean ± SD.

stress (Kundi et al., 2015; Zhangrong Lou et al., 2013; Terrill et al., 2013), we measured the free thiols content in the cell **(Figure 2-3d)** to further demonstrate the oxidative stress was associated with bacterial inactivation. The relative percentage of free thiol in bacteria treated by UVA+GA was 8.09 ± 1.24% (compared to control), which was significantly lower ($P < 0.05$) than that of the control (normalized to 100%). UV-A light, in the absence of GA only slightly and not significantly lowered ($P > 0.05$) the free thiol level (92.66 ± 7.78%). Bacteria incubated in HCl (pH = 3.1) in the dark had less free thiol content compared to control (85.66 ± 4.50%). However, when exposed to UV-A light for 30 min, HCl significantly lowered ($P < 0.05$) the thiol content to 55.13 ± 5.81%, but this content was still significantly higher ($P < 0.05$) than that of UVA+GA treated sample. Thus, UVA+GA caused more oxidative damage than that of the combination of low pH and UV-A. Results of the thiol content assay were consistent with results of CellROX® assay. A sub-lethal treatment of UVA+GA for 5 min lowered the free thiol content of *E. coli* O157:H7 to 20.64 ± 6.08% of control, indicating that thiol oxidation preceded inactivation. Thus, this result was also consistent with CellROX® assay.

To further investigate if the ROS generated by UVA+GA interaction is the main contributor to antimicrobial activity of this treatment, a known hydroxyl radical quencher, DMSO (Price, Reiners, Santiago, & Kessel, 2009) was added to GA solution to 5% v/v (**Figure 2-3e**). In the presence of DMSO, UVA+GA treatment caused a 3.12 ± 0.58 log CFU/mL reduction of *E. coli* O157:H7, approximately 1.5 log CFU/mL lower than that caused by UVA+GA light without DMSO (4.41 ± 0.21 log CFU/mL). Although the presence of DMSO significantly ($P < 0.05$) attenuated the antibacterial efficacy, it did not fully eliminate it.

2.3.4 Analysis of SOD activity

The activity of superoxide dismutase (SOD) within *E. coli* O157:H7 after various treatments is shown in **Figure 2-4**. After incubation in GA (15 mM) for 30 min in the dark, the SOD activity within *E. coli* O157:H7 increased 7.6-fold ($P < 0.05$). However, when UV-A light was simultaneously present with GA, the SOD activity was low and the activity was not significantly different ($P > 0.05$) as control (bacteria treated in water in the dark for 30 min). Bacteria treated in HCl did not show significant increase in SOD activity either ($P > 0.05$). In a sub-lethal treatment of 5 min exposure, samples treated by GA alone had higher SOD activity than that subjected to UV-A light, which was consistent with the results of the 30-min treatment. Bacteria treated by PG (10 mM) in the dark for 30 min also showed increased SOD activity (4.95 fold than control) while treatment in the presence of UV-A showed diminished SOD activity, similar to UVA+GA.

2.3.5 Measurement of membrane damage

E. coli O157:H7 membrane damage was assessed using fluorescent staining probe, propidium iodide (**Figure 2-5a**). The presence of UV-A light significantly increased ($P < 0.05$) the fluorescence intensity for all the treatments, compared to the corresponding treatment without UV-A light exposure. The fluorescence intensity of bacteria treated by GA (15 mM)+UV-A light (35.74 ± 9.70) was significantly higher ($P < 0.05$) than that by GA incubated in the dark (9.50 ± 0.72) or control (corrected to 0), but not significantly different ($P > 0.05$) to UV-A alone (16.97 ± 8.64). The fluorescence intensity of GA increased in the presence of EDTA, and further increased significantly ($P < 0.05$) in the presence of UV-A light. PG (10 mM)+UVA treated sample had significantly higher ($P < 0.05$) fluorescence intensity than that of UVA+GA. However, samples treated with EDTA or HCl also showed higher fluorescence intensity of PI in

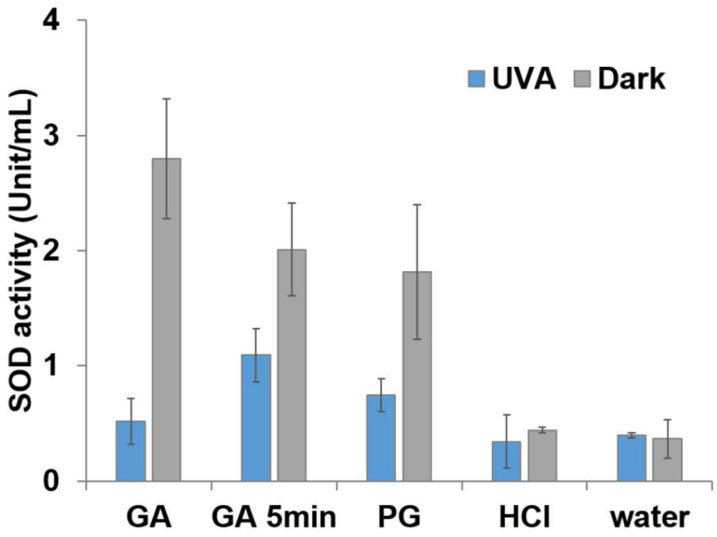

Figure 2-4. Superoxide dismutase activity (Unit/mL) of *E. coli* O157:H7 suspensions treated by UV-A light and selected compounds. *E. coli* O157:H7 was treated by GA (15 mM), PG (10 mM), HCl (pH=3.1), or water for 30 or 5 min. Mean ± SD.

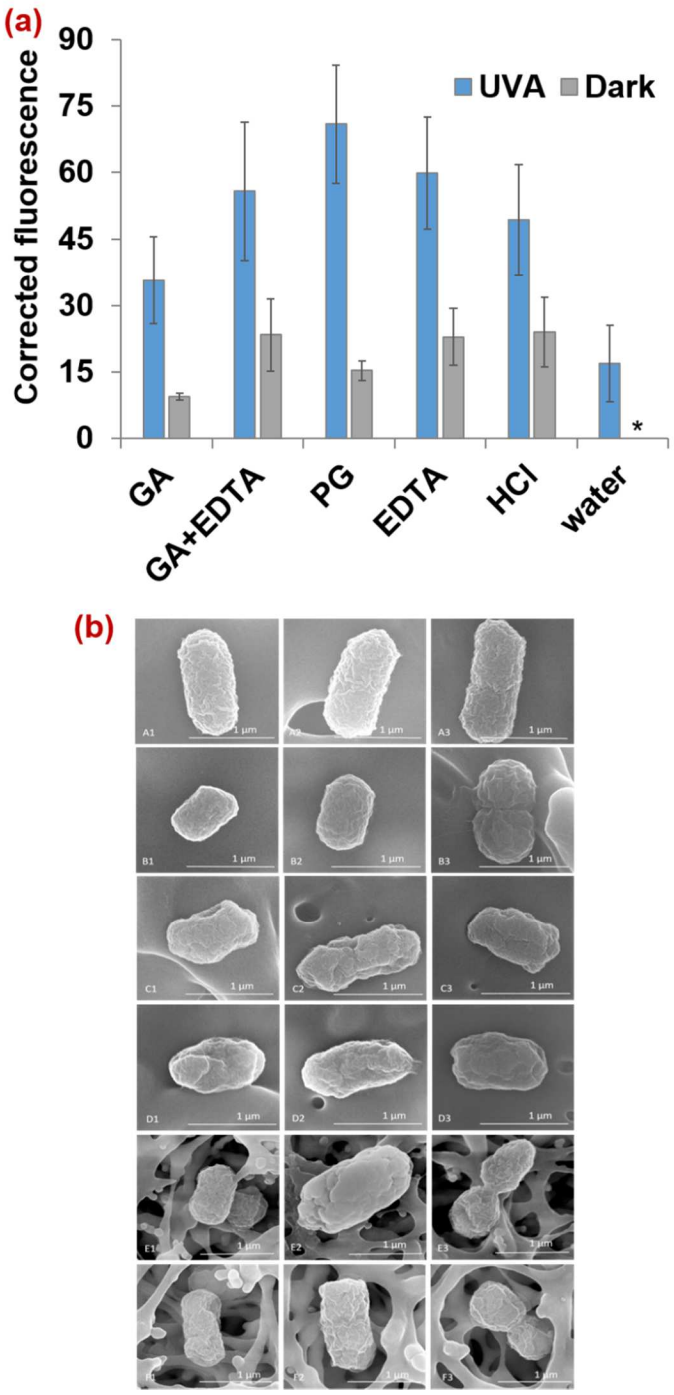

Figure 2-5. Analysis of cell membrane injury in *E. coli* O157:H7 treated by UV-A light with select compounds. (a) Permeability of *E. coli* O157:H7 to propidium iodide (PI) after treatment with GA (15 mM), GA (15 mM)+EDTA (1 mM) , PG (10 mM), HCl (pH 3.1) or water for 30 min with or without UV-A exposure. Mean ± SD. Absolute fluorescence values were corrected

by subtracting the fluorescence values for samples incubated in water and in dark. * indicates that corrected fluorescence value was zero. **(b)** SEM images showing morphology of *E. coli* O157:H7 treated for 30 min by (A1-A3) water, (B1-B3) water +UV-A light, (C1-C3) 15 mM GA, (D1-D3) 15 mM GA+UV-A light, (E1-E3) 10 mM PG, (F1-F3) 10 mM PG+UV-A light.

the presence UV-A, these treatments did not show significant ($P > 0.05$) antimicrobial effect. Therefore, membrane damage may only contribute partially to the bacteria inactivation.

Scanning electron microscopy (SEM) was used for surface morphology analysis of *E. coli* O157:H7 treated by UVA+GA or PG+UV-A light (**Figure 2-5b**). *E. coli* O157:H7 treated by 15 mM GA with or without UV-A light had shrinkage and irregular shape of the surface compared to control. However, the morphological difference between the bacteria treated with UVA+GA or GA alone were visually comparable. *E. coli* O157:H7 treated by PG with or without UV-A light showed similar changes in morphology as those treated by UVA+GA, indicating membrane damage occurred during this treatment as well.

2.4 Discussion

The antimicrobial effect of UVA+GA was dependent on the concentration of GA and the solution pH, and the effect was synergistic as neither UV-A light nor GA alone caused substantial inactivation. A previous study attributed the antimicrobial action of photoirradiated GA (4 mg/ L) by LED (400 nm) to the generation of hydroxyl radicals and other ROS formed by photo-oxidation GA (Nakamura et al., 2012, 2015). They observed that approximately 7 μM hydrogen peroxide were generated and some lipid oxidation was also observed. However, the observed concentration of those ROS would be too low to be effective. Additionally, that study did not consider the location of ROS generation and fate of GA. Thus, more investigation was needed to understand the mechanism of inactivation.

An interesting observation was that although GA produced significantly more hydrogen peroxide upon exposure to UV-A light at pH 7.4 than at pH 3.1, the antimicrobial effect was negligible at pH 7.4 (**Figure 2-1a**). A likely explanation is that at a neutral pH, the carboxyl group (pK_a=4.0)

(Eslami, Pasanphan, Wagner, & Buettner, 2010) was dissociated, conferring a net negative charge on the molecule and therefore decreasing its permeability within the bacterial membrane. Phenolic acids cross the cell membrane by passive diffusion in their undissociated form and the antibacterial activity of phenolic acid is dependent on the concentration of the undissociated acid (Campos et al., 2009)·(Luisina Gómez, Welti-Chanes, & Alzamora, 2011). Thus, the uptake of the molecule by the bacteria is a pre-requisite for GA to exert its antimicrobial effect with UV-A light. Consistent with this postulation, GA in pH 7.4 solution did not show a significant uptake (**Figure 2-2a**) and did not generate oxidative stress within the bacteria (**Figure 2-3**). Py (15 mM) did not show any antibacterial activity at either natural (pH = 5.5) or acidic pH (pH = 3.1), probably due to its higher hydrophilicity that might reduce its affinity to cell membrane (Log P value of Py is 0.5 (PubChem Compound Database, n.d.-a) while Log P value for GA is 0.7 (PubChem Compound Database, n.d.-b)), and thus reduce its uptake by bacteria. The importance of GA uptake within cell in bacterial inactivation was further highlighted by the effect of addition of EDTA in UVA+GA system. EDTA permeabilizes outer membrane by binding divalent cations (in particular Mg^{2+} and Ca^{2+}) that are essential for stabilizing the strong negative charges of the lipopolysaccharides (LPS) molecules (Clifton et al., 2015; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Oviedo & Rodríguez, 2003). Since cellular impermeability through the outer membrane is particularly important in the resistance of Gram-negative bacteria (Hancock, 1997)·(Clifton et al., 2015), EDTA enhanced the antibacterial efficacy of UVA+GA treatment probably by disintegrating the outer membrane of bacteria and increasing the permeability of GA. This hypothesis was supported by the DPBA assay where GA uptake was shown to be significantly higher ($P < 0.05$) in the presence of EDTA, and the higher uptake of GA by EDTA under UV-A light exposure enhanced the antimicrobial effect (**Figure 2-**

1c & Figure 2-2a). Attenuation of EDTA effect by addition of excess Mg^{2+} and Ca^{2+} further validates this hypothesis.

CELLROX® is a cell permeable dye that can be oxidized by superoxide and hydroxyl radicals, inducing strong fluorescence in the presence of dsDNA (Choi et al., 2015). CELLROX® assay and free thiols oxidation assay were both performed to corroborate the generation of ROS in *E. coli* O157:H7 as a result of UVA+GA treatment (**Figure 2-3a & Figure 2-3b**). Results of these assays indicated that GA or PG induced higher ROS generation when UV-A was present than by themselves, even when the concentration of GA was reduced to a sub-lethal condition to the bacteria. The concentration of ROS detected in bacteria was consistent with the antimicrobial activity of the treatment. Since acidified water upon exposure to UV-A light did not significantly ($P > 0.05$) increase the ROS concentration, ROS produced within the bacteria were due to a specific interaction between GA and UV-A. The attenuation of antibacterial effect of UVA+GA by DMSO, a hydroxyl radical scavenger (**Figure 2-3e**) further supports the assumption that ROS were involved in the antimicrobial activity. Studies have shown that the generation of ROS may be the mechanism of action for some antibiotics (Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007). Previous studies have also suggested that GA plays a role as a pro-oxidant inducing intracellular ROS generation and subsequent mammal cell apoptosis of a variety of cell lines and bacteria self-destruction (Chuang et al., 2010)·(Zhao, Hong, & Drlica, 2015)·(Brynildsen, Winkler, Spina, MacDonald, & Collins, 2013). Therefore, we inferred that the ROS observed in the present study may be produced directly by GA upon oxidation by UV-A light in bacteria, or by indirectly mediating ROS formation through activating a variety of intracellular metabolic pathways. Also, GA as a weak organic acid dissociates when diffusing across membrane in the neutral pH environment of cytosol to protons and acid anion. The high

anion accumulation may generate high turgor pressure and influence ROS generation, increasing oxidative stress (King, Lucchini, Hinton, & Gobius, 2010). When exposed to high levels of oxidative stress, bacteria can become more sensitive to other stresses such as low pH (Wesche, Gurtler, Marks, & Ryser, 2009), thus causing more damage to the cell.

In addition to intracellular ROS generation, the impairment of the redox detoxification and repair systems is another possible mechanism for bacteria to become more susceptible to oxidative attack (Brynildsen et al., 2013). Micromolar hydrogen peroxide is rarely lethal unless key oxidative defenses are also disabled (Imlay, 2015). It appeared that the simultaneous presence of GA and UV-A light inhibited the activity of SOD (**Figure 2-4**), an essential enzyme for defending oxidative toxicity. GA is known to undergo autoxidation to produce ROS such as hydrogen peroxide and superoxide radicals (Bo R. You & Park, 2010)·(Russell Jr et al., 2012). Therefore, it was reasonable to observe that SOD activity was significantly increased ($P < 0.05$) when GA was treated against *E. coli* O157:H7, indicating superoxide radicals were generated that activated SOD. This finding was consistent with the fact that SOD is strongly induced when *E. coli* is treated with antibiotics that generate intracellular superoxide (Imlay, 2009). However, when the bacteria were exposed to both GA and UV-A light, they showed lower activity of SOD than in the absence of UV-A light. The effect was evident in both sub-lethal and lethal treatment, but was far more pronounced at lethal treatment condition (30 min). It is likely that the amount of ROS produced by UVA+GA treatment were too high to be quenched by SOD and related enzymes and these ROS also directly inactivated SOD, possibly through oxidation. In addition to this, a recent study demonstrated that activity of metabolic enzymes was also reduced by UV-A light and some organic acids including GA, indicating the reduction of metabolic activity and

ATP level within bacterial cells could be other possible reasons for the antimicrobial effect of GA and UV-A treatment (de Oliveira, Cossu, Tikekar, & Nitin, 2017).

Since GA may permeate outer membrane and trigger oxidative stress in bacteria, it is likely that cell membrane was damaged during the treatment. Previous studies have observed that GA and some other phenolic compounds disintegrate bacterial outer membrane (Borges et al., 2013; Lacombe, Wu, Tyler, & Edwards, 2010; Zaixiang Lou, Wang, Zhu, Ma, & Wang, 2011; Nohynek et al., 2006). Results in **Figure 2-5a** show that UV-A light alone caused membrane injury. The damage increased when UV-A was used in combination with GA, PG, EDTA, or HCl (pH = 3.1). However, it is interesting to observe that although the magnitude of membrane damage of *E. coli* O157:H7 was similar when treated by EDTA, PG, HCl, and EDTA+GA, only PG and GA+EDTA treatments caused substantial inactivation (> 3 log CFU/mL in reduction) of *E. coli* O157:H7, suggesting the membrane damage by itself was not strongly correlated with the antimicrobial effect of the above treatments.

Although PG has a similar structure to that of GA except for the esterification of the carboxylic acid group, it exhibited characteristics distinct from that of GA in the absence and presence of UV-A light. PG showed higher antibacterial activity and lower uptake level than GA when UV-A was present. These differences can be attributed to its higher hydrophobicity (Log P for PG is 1.8 (PubChem Compound Database, n.d.-c)) due to the longer hydrocarbon tail on the carboxylic group that can increase its affinity to cell membrane. Thus, PG may preferentially localize within cell membrane than distribute within the cytoplasm. In the absence of UV-A light, PG had a marginal effect on ROS production (**Figure 2-3a**), and had no effect on thiol oxidation (**Figure 2-3d**), but increased SOD activity, similar to GA (**Figure 2-4**). This finding is partially consistent with a previous study that showed the antimicrobial activity of alkyl gallates (in the

absence of UV-A light) was not due to the ROS related pro-oxidant action, but likely comes in part from the inhibition of the enzyme and membrane respiration chain by moving into the membrane lipid bilayer portions (Kubo, I., et al, 2004). PG in combination with UV-A showed significantly elevated level of ROS compared to PG in dark ($P < 0.05$), had a similar impact on the SOD activity as UVA+GA did, and a significantly higher ($P < 0.05$) membrane injury than UVA+GA. Our results show that, in addition to ROS production and SOD inhibition, membrane injury was significantly enhanced ($P < 0.05$) by the simultaneous exposure to UV-A light and PG. Although antibacterial activity of PG is explored previously, albeit scarcely, its synergistic interaction with UV-A has not been reported before.

The biological damage caused by UV-A light is usually attributed to enhanced production of ROS that results in oxidative damage to lipids, proteins and DNA (Santos et al., 2013),(Zeeshan & Prasad, 2009). Also, UV-A irradiation has been shown to cause membrane dysfunction and increase membrane permeability of bacteria (Bosshard, Bucheli, Meur, & Egli, 2010). In our experimental condition, UV-A light itself was not an effective bactericidal against *E. coli* O157:H7 (< 1 log CFU/mL reduction). One role UV-A light played in the combination treatment of UVA+GA was increasing the permeability of GA into the cells. Nevertheless, both EDTA and UV-A light increased the uptake of GA to the cell (**Figure** 2-**2a**). However, EDTA and GA in the dark incubation did not have lethal effect. Only in the presence of UV-A light (UVA+GA or GA+EDTA+UV-A treatment) could the internalized GA exert antibacterial activity. Therefore, the contribution of UV-A was more than increasing the GA uptake. It may have also increased the oxidative stress generated from GA, possibly through its photo-oxidation or altering the bacterial metabolism as discussed earlier.

2.5 Conclusion

Correlating the results from complimentary experiments, we propose that the antimicrobial mechanism of this combined treatment against *E. coli* O157:H7 is as follows: GA by itself is a mild antimicrobial and has a pro-oxidant ability. The presence of UV-A light increases the uptake of GA. Once GA is internalized, the interaction between GA and UV-A directly or indirectly induces intracellular ROS formation, leading to oxidative damage. Concurrently, the activity of ROS defending enzyme, such as SOD, is also inhibited, magnifying the oxidative damage to *E. coli* O157:H7. Other than oxidative stress, the acidification effect of GA and membrane damage of UV-A is also associated with the inactivation of *E. coli* O157:H7. It is also plausible that these combinations of stresses may have an impact on the bacterial DNA and metabolism. These complimentary stresses affect various aspects of cell metabolism and structure, ultimately lead to the death of bacteria. PG showed a stronger antimicrobial activity in the presence of UV-A light than UVA+GA. In addition to the generation of oxidative stress, a higher level of bacterial membrane damage was responsible for the antimicrobial effect of PG+UV-A treatment.

Reference

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Chapter 3: UV-C exposed gallic acid exhibits enhanced antimicrobial activity via generation of reactive oxidative species and quinone

Hypotheses

1) GA exposure to UV-C light (UVC-GA) shows enhanced antimicrobial activity
2) New compounds (e.g. quinone) and ROS are generated from GA solution after UV-C light exposure, which contribute to the enhanced antimicrobial activity of UVC-GA

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Abstract

UV-C (254 nm) exposed gallic acid (UVC-GA) was investigated for its enhanced antimicrobial activity. 15 mM GA solution was exposed to UV-C for 30 min and subsequently incubated with *E. coli* O157:H7 for 30 min to achieve a 3.2±0.2 log CFU/mL reduction. The antimicrobial activity persisted for up to 4 weeks following the treatment, and is affected by the irradiation duration, wavelength, and pH of solution. The addition of benzenesulfinic acid (BSA) to UVC-GA lowered its antimicrobial activity, indicating that quinones contributed to its overall antimicrobial effect. In addition, the attenuated antimicrobial activity of UV-C exposed GA in the presence of ROS quenchers, the generation of hydrogen peroxide, and increased levels of intracellular oxidative stress detected in *E. coli* O157:H7 illustrated that ROS also played a role in the antimicrobial effect of UVC-GA. UVC-GA could be applied as a novel antimicrobial in food systems.

3.1. Introduction

The exploration of novel antimicrobial compounds for food use has gained much attention due to the rise of microorganisms that are not only antibiotic resistant but also more tolerant to several food processing and preservation methods (Gyawali & Ibrahim, 2014). In addition, there is an increasing demand of natural antimicrobial compounds that provide an effective alternative method to reduce pathogenic and spoilage microorganisms in food and to increase the safety and quality of foods products (Gutiérrez-Larraínzar et al., 2012; Zhang & Yang, 2017). Phenolic compounds are one of the most diverse groups of plant-derived compounds found in a wide variety of fruits, vegetables, seeds, tea, and wine, and have shown to possess antimicrobial properties (Cetin-Karaca & Newman, 2015). However, when used alone, the bactericidal activity exhibited by those compounds is rarely sufficiently effective to achieve a rapid reduction of human pathogens in a relatively short time (Ricke, Kundinger, Miller, & Keeton, 2005). To potentiate the antimicrobial efficiency of naturally derived antimicrobials such as phenolic compounds, one strategy is to explore the synergy using combinations of mild antimicrobials with other interventions (Sanhueza et al., 2017).

Gallic acid (GA; 3,4,5-trihydroxyl-benzoic acid), a polyhydroxyphenolic compound naturally derived from various plants, fruit and vegetables (Varela-López, Bullón, Giampieri, & Quiles, 2015), and is Generally Recognized As Safe (GRAS) for human consumption (US FDA, 2015). GA by itself has inhibitory effect towards a wide variety of planktonic bacteria, biofilm, and fungi, which has been associated with its ability to generate reactive oxidative species (ROS) and membrane disintegration to the bacteria (Borges, Ferreira, Saavedra, & Simões, 2013; Nohynek et al., 2006). However, the bactericidal effect of GA was weak against some food pathogens such as *E. coli* O157:H7, in terms of the high concentration of GA used, the long duration of treatment,

and inability to inactivate more robust stationary-phase bacteria. A previous study found only 1 log CFU/mL reduction in *E. coli* CETE 434 that was in exponential phase following exposure to 15 mM GA for 60 min (Borges et al., 2013). Another study found that more than 2500 mg/L of GA was needed as the minimum inhibitory concentration against exponential phase *Staphylococcus aureus* and *E. coli* O157:H7 in 60 min (C. Xu et al., 2014). Thus, the potential use of GA alone by the food industry as an efficient natural antimicrobial is limited at this point.

Light-based antimicrobial technology is one of the novel non-thermal antimicrobial approaches, including the application of ultraviolet irradiation, blue light irradiation, and other light based-approaches (Yin, R., Dai, T., Avci, P., Jorge, A.E.S., de Melo, W.C., Vecchio, D., Huang, Y.Y., Gupta, A. and Hamblin, 2013). Work has been previously carried out to establish the light-mediated antimicrobial techniques based on naturally occurring phenolic compounds. Nakamura et al. observed the bactericidal effect of various types of photoirradiated polyphenols such as caffeic acid, GA, and proanthocyanidin by blue light against Gram-positive and Gram-negative bacteria (Keisuke Nakamura et al., 2015). Cossu et al. developed a synergistic interaction between GA and UV-A light to inactivate *E. coli* O157:H7 in spinach wash water and in biofilm (Cossu et al., 2016). Similar studies have been reported that investigated bacterial inactivation in wash water by combining benzoic acid or curcumin with UV-A or visible light (E. F. de Oliveira, Tosati, Tikekar, Monteiro, & Nitin, 2018; Ding, Alborzi, Bastarrachea, & Tikekar, 2018). However, the studies reported so far require the simultaneous involvement of both the light source and the compounds to be irradiated by the light to exert antimicrobial activity, which may limit their application due to the requirement of the on-site light source.

The objective of this study was to explore a novel light-enhanced antimicrobial by using post-UV light irradiated GA. *E. coli* O157:H7 was selected as the model bacteria since it is one of the

most tolerant pathogen to acidic antimicrobials (G. Wang & Doyle, 1998), and continues to be one of the major foodborne pathogens that caused worldwide threat to public health and significant economic losses for the food industry. The bactericidal characteristics of UVC-GA were evaluated. Also, the underlying mode of mechanism of this observed enhanced antimicrobial was also evaluated.

3.2 Methods

3.2.1 UV-C Light source and preparation of UV-C treated GA solution

The UV-C light source (Spectroline ^{TM}, Westbury, NY, USA) is a bench-top, batch type chamber with a peak wavelength of 245 nm and an average intensity of 4540 $\mu W/cm^2$ applied at the surface from a distance of 17 cm. GA was diluted in distilled water to a final concentration of 15 mM, and transferred to a crystal petri dish (KIMAX®, NJ, USA), incubated under UV chamber for a selected duration of time. After that, GA solutions were filtered through a 0.25 μm sterile filter and were ready for use. For experiment revealing the stability of the antimicrobial activity of UVC-GA, the post-irradiated GA solution was stored in the dark at room temperature for selected duration (overnight or 1-4 weeks).

3.2.2 Model bacteria selection, cultivation, and bactericidal efficacy determination

Shiga toxin negative *E. coli* O157:H7 (ATCC #700728, Manassas, VA) was selected as a model Gram-negative pathogen. The bacteria were cultured in Tryptic Soy Broth (TSB) at 37 °C for 20 h to obtain the bacterial population in stationary phase before each experiment. To determine the antimicrobial efficacy of selected treatment against bacteria, the bacteria were diluted, and the suspensions were incubated in solutions with selected compounds to reach an initial concentration of 7 log CFU/mL. After incubation, the bacterial suspension was serially diluted in

buffer peptone water (BPW) and plated on Tryptic Soy Agar (TSA). The number of colony-forming unites (CFU) was determined by plate counts after incubation at 37 °C for 24 h.

3.2.3 Ultraviolet–visible (UV–vis) absorption spectra of GA with or without UV-C light exposure

Ultraviolet–visible (UV–vis) absorption spectra were recorded using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) from 200-800 nm, at the interval of 1 nm.

3.2.4 Detection of semi-quinone radical and hydroxyl radical from UVC-GA

3.2.4.1 Electron Paramagnetic Resonance (EPR) analysis of GA semi-quinone radical from UVC-GA solution

GA solution (15 mM) of 20 mL was loaded into a disposable petri dish (diameter, 100 mm) and then subjected to UV-C radiation (254 nm, 10W, Atlantic Ultraviolet Inc, Hauppauge, N.Y, USA) for 30 min at room temperature. After 30 min, sample was loaded into a 50 µL calibrated pipette (VWR internation, USA) and the EPR spectrum was immediately recorded on a Miniscope MS 400 X-band spectrometer (Magnettech, Berlin, Germany) at room temperature. The measurement conditions for EPR were as follow: field sweep, 335.51-337.24 mT; field modulation width, 0.02 mT; sweep time, 60 s; microwave frequency, 9.743 GHz; receiver gain, 200-900. Positive control was prepared by adjusting the pH of GA solution to pH 11.8 using sodium hydroxide (10N), without UV-C treatment.

3.2.4.2 EPR spin trapping analysis of hydroxyl radical from UVC-GA solution

α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone (POBN) was dissolved directly into GA solution, followed by Fe(II). The final concentration of GA, POBN and Fe(II) were 15 mM, 20 mM and 50 µM respectively. The sample was kept in an enclosed quartz tube (190-800 nm) and was exposed to UV-C radiation (254 nm, 10W, Atlantic Ultraviolet Inc, Hauppauge, N.Y, USA) for

30 min at room temperature. After the UV radiation, the sample was loaded into a 50 µL calibrated pipette (VWR international, USA), and the EPR spectrum was immediately recorded on a Miniscope MS 400 X-band spectrometer (Magnettech, Berlin, Germany) at room temperature. The measurement conditions for EPR were as follow: field sweep, 330.96-341.31 mT; field modulation width, 0.1 mT; sweep time, 60 s; microwave frequency, 9.743 GHz; receiver gain, 900. Positive control was prepared by using hydrogen peroxide solution diluted in DI water containing POBN (20 mM final concentration). Fe(II) (50 µM final concentration) was then added to initiate Fenton reaction. The final concentration of hydrogen peroxide in the positive control was 183 µM. The control was kept in a capped glass culture tube at room temperature for 30 min, without exposure to UV-C, prior to EPR measurement (parameters as above mentioned).

3.2.5 High pressure liquid chromatography (HPLC)

UVC-GA solution was analyzed by HPLC. Samples of both 15 mM GA and GA with previous UV-C light exposure for 60 min were collected and diluted 10 fold in DI water. HPLC analysis was conducted on a Shimadzu® LC-2010A (Kyoto, Japan), with an LC-2010 pump, a LC-2010 auto sampler, a LC-2010 UV-Vis detector, and LabSolution V 5.81 software. The column used was C18 (250 mm × 4.6 mm, 10 µm) from Phenomenex® (Torrance, CA, USA). The chromatographic separation was carried out in isocratic elution using a mobile phase composed of 0.1 N phosphoric acid (84%) and acetonitrile (16%), at a flow rate of 0.6 mL/min. The wavelength of detection was 222 nm. The column temperature was 40 °C and the injection volume was 10 µL.

3.2.6 Effect of ROS scavengers on the antimicrobial activity of UVC-GA

Catalase (300 U/mL), DMSO (0.7 M) and mannitol (0.1 M) were added to 15 mM UV-C light irradiated GA solution as scavengers of hydrogen peroxide, and hydroxyl radical (Bektaşoğlu, Esin Çelik, Özyürek, Güçlü, & Apak, 2006; Li, 2013). Bacterial suspension was then diluted to the solution and incubated for 30 min. After incubation, the antimicrobial efficacy was analyzed based on previous description. The antimicrobial efficacy was compared among UVC-GA without added scavengers, GA and DI water.

3.2.7 Determination of hydrogen peroxide from UVC-GA solution

The generation of hydrogen peroxide was analyzed by ferrous ion oxidation xylenol orange (FOX) method with some modifications. The mechanism of this method was based on the conversion of ferrous ions into ferric ions by hydrogen peroxide that form a complex with xylenol orange (XO), the concentration of which is determined using spectrophotometry (Wolff, 1994). UVC-GA (15 mM) sample, freshly made or storing at dark for 1-4 weeks, was prepared as described above, with or without the addition of catalase (300 U/mL). FOX assay reagent of 100 µL containing 1 mM xylenol orange, 2.5 mM ferrous sulfate, 1 M sorbitol, and 250 mM sulfuric acid was added to 300 µL samples, followed by incubation at room temperature for 30 min. After that, the absorbance of solutions was measured at 560 nm using a Spectroscopy M5e plate reader (Molecular Devices LLC, Sunnyvale CA). Hydrogen peroxide concentrations were determined based on an external standard curve. Amplex™ Red (10-acetyl-3,7-dihydroxyphenoxazine) hydrogen peroxide assay kit (Thermo Fisher Scientific, MA) was used according to the manufacture's protocol to validate the measurement of hydrogen peroxide by FOX assay from UVC-GA solution. Amplex™ Red stock solution was prepared in DMSO, protected from light and used in the same day. A working solution containing 100 µM Amplex™

Red reagent and 0.2 U/mL horseradish peroxidase was added to samples in a 96-well pate with 1:1 ratio. After incubating at room temperature in dark for 30 min, the absorbance was measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at a wavelength of 560 nm. The concentration of hydrogen peroxide was determined by referring to a standard curve established using the same assay kit.

3.2.8 Analysis of intracellular oxidative stress in E. coli O157:H7

CellROX® Green Reagent, a fluorescent probe for measurement of oxidative stress was used to detect intracellular oxidative stress within *E. coli* O157:H7 following various treatments. Upon permeating cell membrane and a subsequent oxidation by intracellular oxidative species, the probe shows green photostable fluorescence (Choi, Yang, & Weisshaar, 2015). Stationary phase *E. coli* O157:H7 (initial concentration of 9 log CFU/mL) prepared as described previously was centrifuged for 2 min at 10,000 ×g, washed with DI water, and exposed to 15 mM GA solution with or without previous UV-C light irradiation for 30 min. Bacteria treated by HCl (pH=3.1) and water were used as a control. Then, CellROX® Green probe was added to each of the samples to reach a final concentration of 5 μM, followed by incubation in dark at 37 °C for 30 min. After that, samples were washed three times, resuspended in phosphate buffered saline solution and transferred to a 96-well plate for intensity measurements at 485/520 (ex/em) using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA). The raw fluorescence intensity values were corrected using an equation described below:

$$Corrected\ fluorescence = fluorescence\ intensity_{\ treated\ sample} - fluorescence\ intensity_{\ control} \quad (1)$$

3.2.9 Analysis of GA uptake by E. coli O157:H7

Diphenylboric acid 2-aminoethyl ester (DPBA) is a cell-permeable dye that shows fluorescence upon binding with specific flavonoid compounds (Schwalm et al., 2003). It was used to measure the uptake of GA within the bacteria in our previous paper (Q. Wang, De Oliveira, Alborzi, Bastarrachea, & Tikekar, 2017). Stationary phase *E. coli* O157:H7 (initial concentration of 9 log CFU/mL) prepared as described previously was centrifuged for 2 min at 10,000 ×g, washed with DI water, and exposed to 15 mM GA solution with or without previous UV-C light irradiation for 30 min. Bacteria treated by DI water was used as control. After the treatment, samples were centrifuged at 10,000 rcf for 2 min, and washed twice by DI water to remove extracellular GA. Then, 450 µL of 0.2% DPBA assay solution prepared in DMSO was added to the bacterial pellet. The well mixed sample was transferred to a 96-well plate and fluorescence intensity measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at excitation/emission wavelength of 405/465 nm. The fluorescence intensity was corrected using equation (1).

3.2.10 Analysis of membrane damage of E. coli O157:H7

Propidium iodide (PI) was used to analyze membrane damage within *E. coli* O157:H7 by UVC-GA according to our previous study (Q. Wang et al., 2017). Stationary phase *E. coli* O157:H7 (initial concentration of 9 log CFU/mL) prepared as described previously was centrifuged for 2 min at 10,000 ×g, washed with DI water, and exposed to 15 mM GA solution with or without previous UV-C light irradiation for 30 min. Bacteria treated by DI water was used as control. Following the treatment, samples were centrifuged for 2 min at 10,000 ×g, washed with DI water, and to which PI was added to reach a final concentration of 5 µM. Samples were incubated for 15 min in dark, followed by washing with DI water and resuspended in 500 µL phosphate

buffered saline solution Finally, each of the samples was transferred to a 96-well plate and fluorescence intensity was measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at excitation/emission wavelength of 490/635 nm using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA). The fluorescence intensity was corrected using equation (1).

3.2.11 Statistical analysis

Statistical analysis of the triplicate data was performed using the two-tailed unpaired *t*-test (α = 0.05) by Microsoft Excel 2016 (Microsoft Inc., Redmond WA, USA).

3.3 Results

3.3.1 Characterization of the antimicrobial activity of UVC-GA

The bactericidal activity of UVC-GA is summarized in **Figure 3-1**. **Figure 3-1a** shows that following UV-C exposure, the antimicrobial activity of GA solution significantly ($P < 0.05$) increased and 3.2±0.2 log CFU/mL reduction was achieved against *E. coli* O157:H7 when the bacteria was incubated with UV-C treated GA for 30 min compared with only a 0.13±0.1 log CFU/mL reduction by GA solution without prior UV-C exposure. Bacteria incubated in UV-C irradiated DI water for 30 min did not show a significant ($P < 0.05$) change in population. Antimicrobial activity of the same UVC-GA solution was evaluated for 4 consecutive weeks to investigate the longevity of its antimicrobial activity. It was found that even after 4-weeks of ambient storage, UV-C treated GA (15 mM) was able to achieve a 3.2±0.1 log CFU/mL

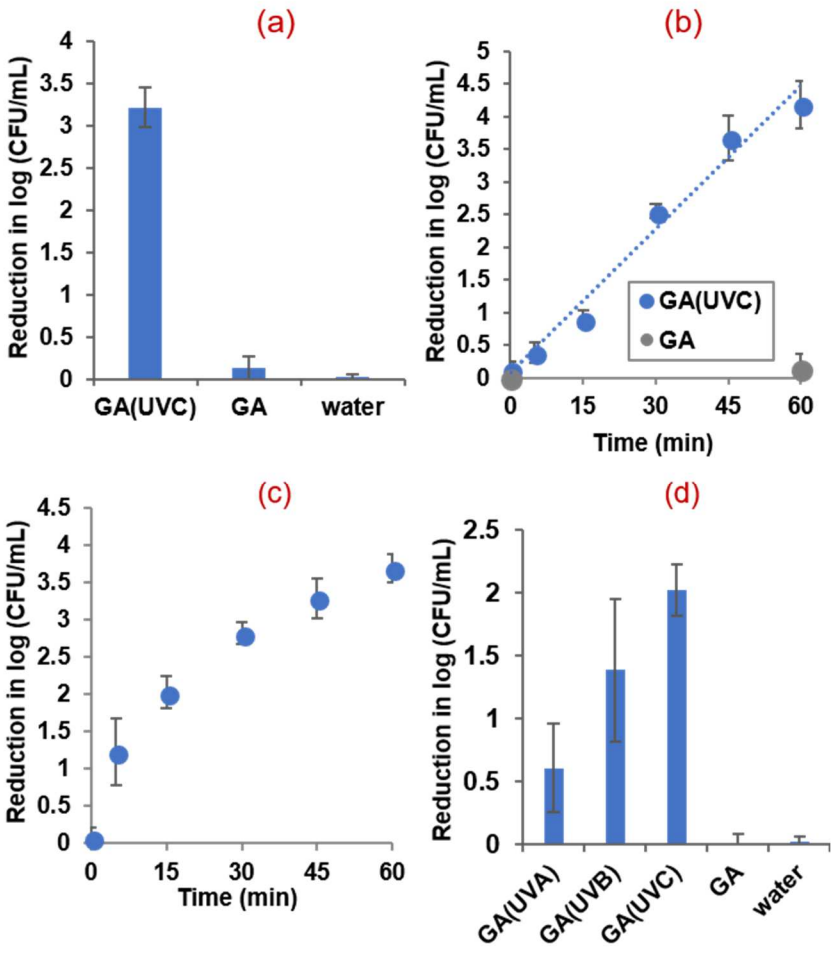

Figure 3-1. Antimicrobial characteristics of UV-C light irradiated GA. (a) Logarithmic reduction in *E. coli* O157:H7 population by UVC-GA solution (15 mM), GA (15 mM), and water as control. (b) Inactivation kinetics of *E. coli* O157:H7 by UVC-GA solution. The duration of irradiation of GA was constant at 30 min. (c) GA was irradiated with UV-C for various duration (0-60 min) and subsequently incubated with bacteria for 30 min to evaluate the effect of duration of UV-C exposure on the antimicrobial activity of GA. (d) Effect of light wavelength (UV-A: 365 nm, UV-B: 312 nm, UV-C: 245 nm) used for irradiation of GA (15 mM) on the inactivation of *E. coli* O157:H7. Initial bacterial concentration was 7 Log CFU/mL. Mean ± SD.

reduction in *E. coli* O157:H7 population in 30 min and was not significantly different from that achieved from a freshly prepared UVC-GA solution ($P > 0.05$).

The kinetics of microbial inactivation by UV-C exposed GA is shown in **Figure 3-1b**. The inactivation followed typical first-order kinetics with a D-value of 12.6 min and a R^2 value of 0.978. The effect of duration of prior UV-C exposure of GA on the antimicrobial effect was also evaluated in **Figure 3-1c** where GA exposed to UV-C for various durations were subsequently incubated with the bacteria for 30 min. The antimicrobial activity of UVC-GA increased with increase in its duration of exposure to UV-C for up to 60 min. The comparison between antimicrobial activity of UV-A (365 nm) and UV-B (312 nm) and UV-C (254 nm) irradiated GA is shown in **Figure 3-1d**. At the same dose level (4 J/cm^2), the antimicrobial effect of UVC-GA was strongest followed by UV-B irradiated GA and was lowest for UV-A irradiated GA ($P < 0.05$) against *E. coli* O157:H7.

To investigate whether solution pH affects the antimicrobial activity of UVC-GA, a 15 mM GA solution was adjusted from its naturally acidic pH (pH=3.1) to neutral (pH=7) or alkaline (pH=11) pH conditions. UVC-GA solution did not show significant antimicrobial activity ($P > 0.05$) at either pH 7 or pH 11 (**Figure 3-2a**). In addition, bacteria treated by sodium phosphate buffer or HCl solution with the same pH (pH=3.1) as 15 mM GA did not show significant ($P > 0.05$) antimicrobial activity compared to control. To evaluate whether UVC-GA inactivated the bacteria by causing membrane damage that could significantly increase its uptake, fluorescent based indicators diphenylboric acid 2-aminoethyl ester (DPBA) and propidium iodide (PI) were used to measure the level of GA uptaken by cell and bacteria membrane damage, respectively. **Figure 3-2b** shows the corrected fluorescence observed within the bacterial cells from binding of DPBA with taken up GA (Schwalm et al., 2003). **Figure 3-2c** shows the level of membrane

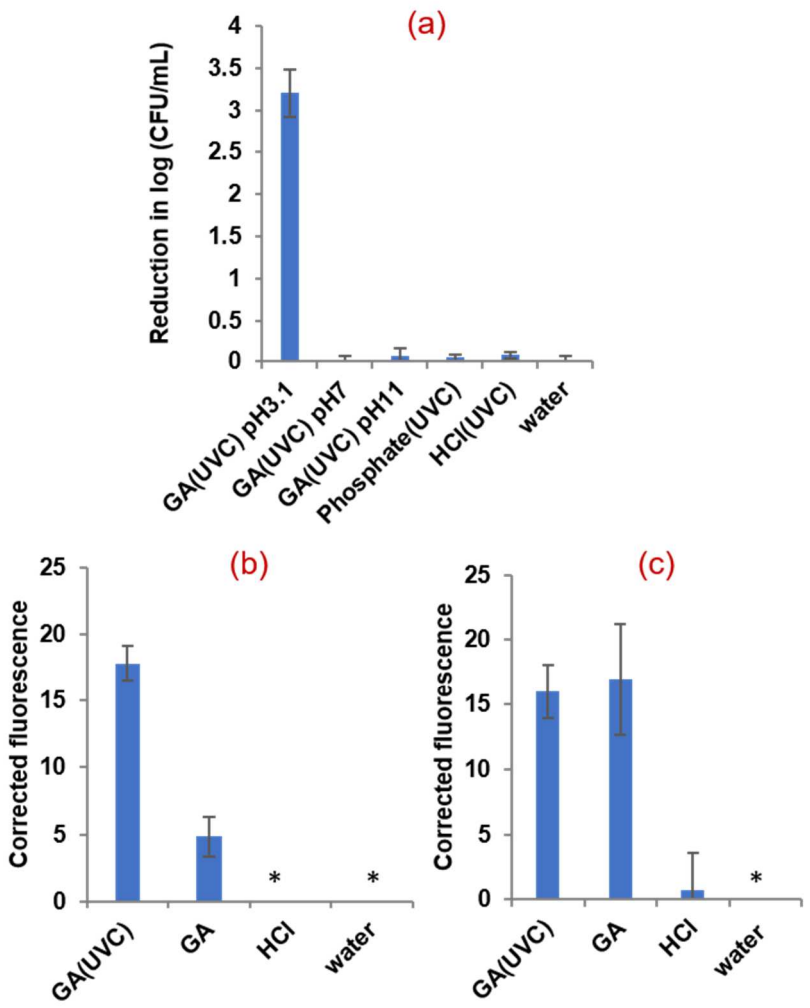

Figure 3-2. (a) *E. coli* O157:H7 inactivation by UVC-GA with adjusted solution pH (pH=3.1, 7.0, and 11.0, respectively), UV-C irradiated phosphate buffer (pH=3.1), and UV-C irradiated HCl solution (pH=3.1). (b) Measurement of the uptake of GA with or without UV-C light irradiation in *E. coli* O157:H7 as measured by binding with DPBA. HCl (pH=3.1) and water were controls. (c) Analysis of membrane damage of *E. coli* O157:H7 by propidium iodide after treatment by UVC-GA and GA. HCl (pH=3.1) and water were controls. GA concentration was 15 mM. Duration of GA treatment was 30 min. Duration of bacterial treatment was 30 min. Initial bacterial concentration was 7 Log CFU/mL. Absolute fluorescence values were corrected

by subtracting the fluorescence values for samples incubated in water and in dark. * indicates that corrected fluorescence value was zero. Mean ± SD.

damage observed within the bacteria exposed to UVC-GA and GA. While there was a significant difference in the corrected fluorescence values for DPBA-GA complex ($P < 0.05$) between UV-C treated GA and GA, there was no significant difference between the fluorescence for PI between the two treatments ($P > 0.05$).

3.3.2 Investigation of the mechanism of the antimicrobial activity of UVC-GA

3.3.2.1 Evaluation of the contribution of quinone formation towards the bactericidal activity of UVC-GA

After UV-C exposure, the color of GA solution became yellow and persisted for at least 12 months, indicating the possibility of formation of a new compound. To determine this possibility, UV-Vis spectroscopy was used (**Figure 3-3a**). However, the spectra for GA with or without UV-C exposure overlapped and no new peak was identified. HPLC results showed that, following 60 min of UV-C treatment, the concentration of GA decreased by a mere 1%, and subsequent HPLC analysis was unable to provide evidence of formation of any new degradation compound indicating that the concentration of the by-product formed may be too low to be directly detected. Electron paramagnetic resonance (EPR) was used in an attempt to directly detect a GA-derived semiquinone radical (**Figure 3-3b**) from UVC-GA solution. While the GA semiquinone radical could be successfully detected in GA solution under alkaline conditions (pH 11.8, positive control), no such EPR signal could be observed in UV-C treated GA under acidic conditions (pH 3.1). Since phenolic compounds like GA are less oxidatively stable at alkaline pH, it is much easier to detect the primary oxidation product such as semiquinone under those conditions (Eslami, Pasanphan, Wagner, & Buettner, 2010). However, under acidic conditions, the oxidation products produced by GA is far fewer than that in the alkaline condition, and semiquinone radicals have proved difficult to be detected directly due to the instability in acidic

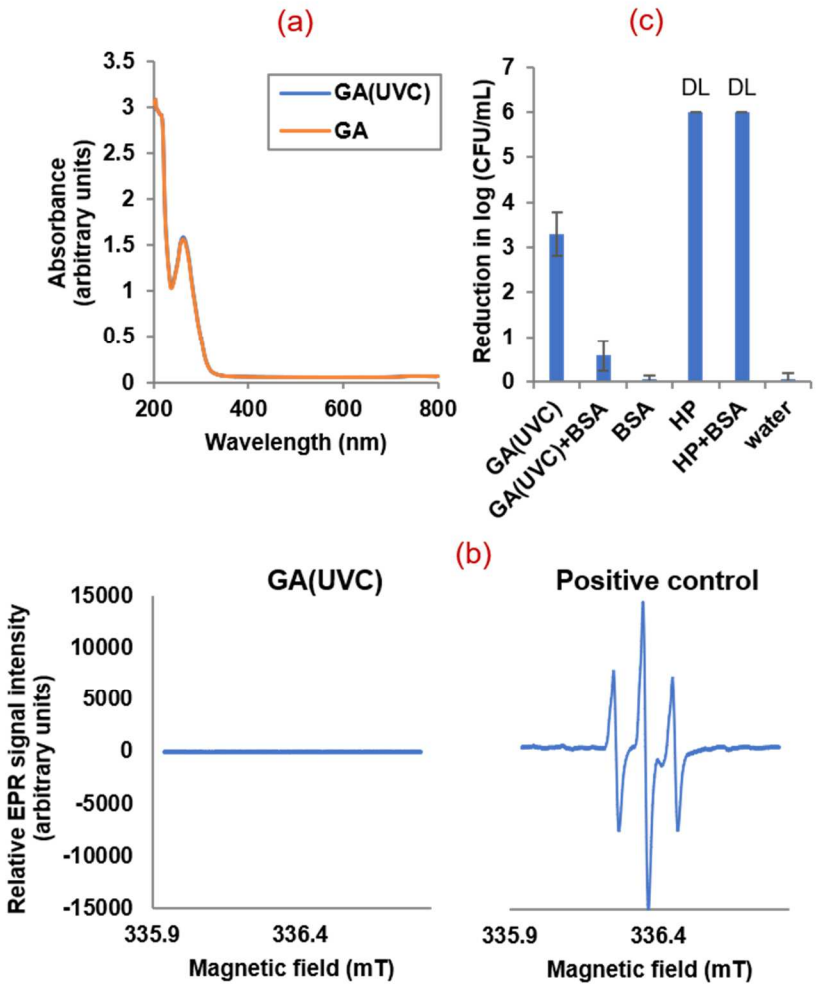

Figure 3-3. Analysis of the role of quinone formation in the antimicrobial activity of UVC-GA. (a) UV-vis spectrum of GA with or without UV-C light exposure for 30 min. (b) EPR spin trapping analysis of GA semi-quinone radical from UVC-GA solution. Duration of GA irradiation was 30 min. Positive control was prepared by adjusting the pH of GA solution to pH 11 using sodium hydroxide (10 N), without UV-C treatment. (c) Logarithmic reduction in *E. coli* O157:H7 treated by UVC-GA, UVC-GA in the presence of BSA, hydrogen peroxide, and hydrogen peroxide with BSA. Water was used as control. GA and BSA concentrations were 15 mM. Hydrogen peroxide concentration was 0.3%. Duration of GA irradiation was 30 min.

Duration of bacterial treatment was 30 min. Initial bacteria concentration was 7 Log CFU/mL. DL indicates the reduction of bacterial population reached detection limitation. Mean ± SD.

conditions (Narayanan, M., Leung, S. A., Inaba, Y., Elguindy, M. M., & Nakamaru-Ogiso, 2015; Song, Wagner, Lehmler, & Buettner, 2008). Therefore, the reason that GA semiquinone radical was not detected in the current experiment could be that the products was below the limit of detection of the instrument.

Benzenesulfinic acid (BSA), a well-known nucleophile that readily forms adduct with electrophiles such as quinones, was added to UVC-GA solution and its effect on the inactivation of *E. coli* O157:H7 was evaluated (**Figure 3-3c**). After addition of 15 mM BSA to 15 mM UVC-GA solution, the reduction of *E. coli* O157:H7 in CFU/mL declined from 3.3±0.5 to 0.6±0.3 log CFU/mL ($P < 0.05$). BSA by itself did not show any ($P > 0.05$) antimicrobial activity. To test whether BSA can also react with electrophiles other than quinones, such as ROS, and thus affect their antimicrobial activity, a 0.3% hydrogen peroxide solution with or without the presence of BSA was tested against *E. coli* O157:H7. Interestingly, the antibacterial activity of 0.3% hydrogen peroxide was not significantly affected by the presence of BSA ($P < 0.05$) and more than 6 log CFU/mL reduction in bacterial population was achieved in the presence or absence of BSA.

3.3.2.2 Evaluation of role of ROS in the observed antimicrobial activity

Figure 3-4a shows the effect of ROS scavengers (300 U/mL catalase, 0.7 M DMSO, and 0.1 M mannitol) on the antimicrobial effect of UVC-GA. The presence of catalase in the UVC-GA significantly ($P < 0.05$) lowered its amicrobial activity in that only less than 1±0.3 log CFU/mL reduction was achieved. The presence of either DMSO or mannitol as hydroxyl radical scavengers also significantly ($P < 0.05$) reduced the antimicrobial effect of UVC-GA. In the presence of DMSO, less than 1±0.1 log CFU/mL reduction was achieved and in the presence of mannitol 1.0±0.2 log CFU/mL reduction was achieved.

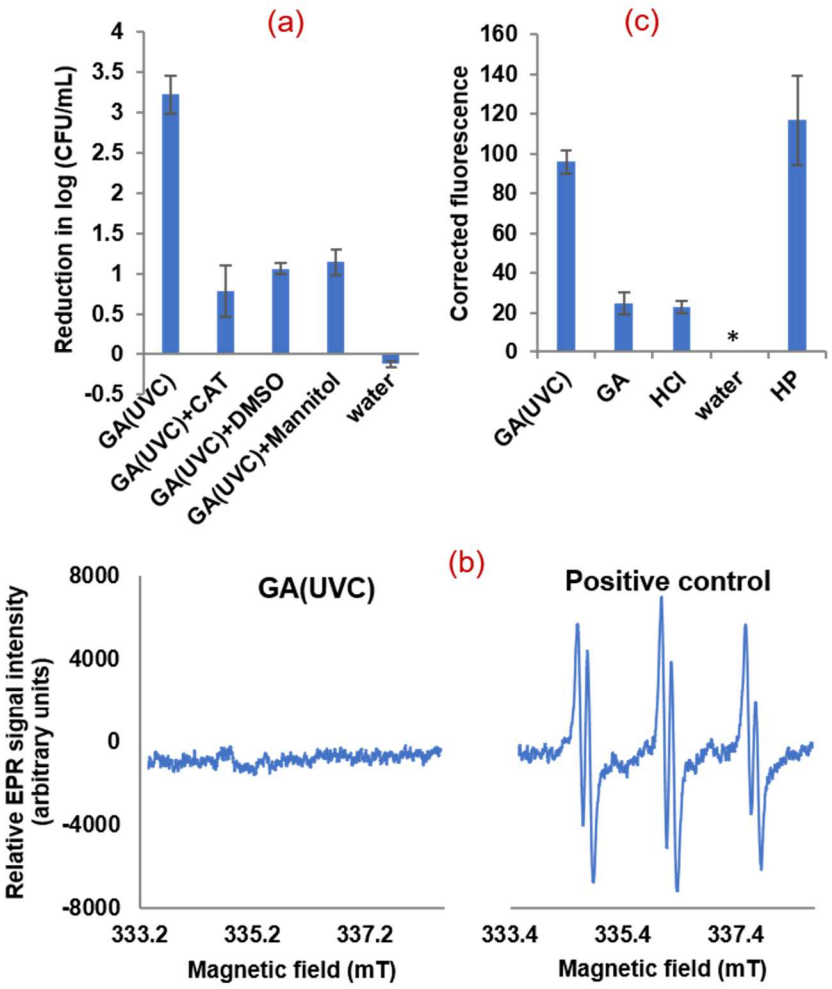

Figure 3-4. Analysis of the role of ROS in the antimicrobial activity of UVC-GA solution. (a) Logarithmic reduction in *E. coli* O157:H7 by UVC-GA in the presence of ROS quenchers (catalase, DMSO, and mannitol). Catalase: 300 U/mL. DMSO: 0.7 M. Mannitol: 0.1 M. GA was 15 mM. (b) EPR spin trapping analysis of hydroxyl radical from UVC-GA solution. Duration of GA irradiation was 30 min. Positive control was prepared by using hydrogen peroxide solution diluted in DI water containing 20 mM POBN and 50 mM Fe(II). (c) Measurement of intracellular oxidative stress experienced by *E. coli* O157:H7 upon select treatments: UVC-GA (15 mM), GA (15 mM), HCl (pH=3.1), water, and hydrogen peroxide (HP, 1.5%). Duration of

GA irradiation was 30 min. Duration of bacterial treatment was 30 min. Initial bacteria concentration was 7 Log CFU/mL. Absolute fluorescence values were corrected by subtracting the fluorescence values for samples incubated in water and in dark. * indicates that corrected fluorescence value was zero. Mean ± SD.

Hydrogen peroxide of 7.2±0.4 μM was detected from freshly made UVC-GA solution by the FOX assay and verified by the Amplex red assay. After storing in dark at room temperature for 4 consecutive weeks, the hydrogen peroxide concentration remained close to the initial concentration. When catalase (300 U/mL) was added to the testing solution (either freshly made or after storing for 1-4 weeks), the hydrogen peroxide concentration fell below the limitation of detection and was not significantly ($P < 0.05$) different to DI water as control. Hydrogen peroxide was not detected from freshly made GA (15 mM) solution, catalase (300 U/mL), HCl (pH=3.1) solution, or DI water under the experimental condition. In addition, EPR spin trapping was used in an attempt to detect hydroxyl radicals that might form as a consequence of UV-C irradiation of GA solutions. A positive control was prepared by mixing ferrous ions with hydrogen peroxide to initiate the Fenton reaction, and resulted in a characteristic signal of hydroxyl radical (**Figure 3-4b**). However, hydroxyl radical from UVC-GA was not detected.

Intracellular oxidative stress was also measured using CellROX assay **(Figure 3-4c).** Cells treated by UVC-GA exhibited about 2.5-fold higher ($P < 0.05$) level of intracellular oxidative stress than that treated by 15 mM GA solution without UV-C exposure, and about 5.1-fold higher ($P < 0.05$) than that of bacteria incubated in DI water as control. There was no significant difference ($P > 0.05$) in the fluorescence intensity between bacteria treated by 15 mM pure GA solution and HCl (pH=3.1), suggesting no difference in intracellular oxidative stress.

3.4. Discussion

Consistent with our experiments where 15 mM GA did not show significant ($P > 0.05$) antimicrobial activity against stationary phase *E. coli* O157:H7 in 60 min, previous studies have

also found that the efficacy of bactericidal activity of GA is limited against pathogens such as *E. coli* O157:H7 (C. Xu et al., 2014; Zhang & Yang, 2017). The membrane damaging effect (Borges et al., 2013) and the generation of ROS due to the autoxidation of GA (K. Nakamura et al., 2012) have been shown to be the reasons for its antimicrobial activity. Our current study demonstrates a new approach to enhance the antimicrobial activity of GA by UV-C irradiation. UVC-GA (15 mM) solution exerted significantly ($P < 0.05$) higher (> 3 log CFU/mL in 30 min) antimicrobial activity against *E. coli* O157: H7 compared to untreated GA. Moreover, the antimicrobial effect increased with increased irradiation time of GA, as well as increased contact duration of irradiated GA with the bacteria. The wavelength dependence of antimicrobial activity of UV irradiated GA might be due to different levels of absorbance of the incident UV light by GA. UV light intensity and wavelength can greatly influence the productions of photo and hydroxyl radical generated, which in turn affect the photochemical oxidation of GA and the production of degradation intermediates, and the subsequent antimicrobial activity (Son, Choi, Zoh, & Khan, 2007). Previously, antimicrobial techniques based on combined treatment of selected natural compounds and UV or visible light have been established. The mechanisms of those antimicrobial techniques are primarily attributed to the generation of ROS of photo-oxidized compound upon light exposure (Cossu et al., 2016; F. Xu et al., 2018), or to the synergistic hurdle effect of the combination of two sublethal antimicrobial approaches (Corbo et al., 2009; Raso & Barbosa-Cánovas, 2003). Our previous study showed that the simultaneous treatment of UV-A and GA exerted a synergistic antimicrobial activity against *E. coli* O157:H7 while either UV-A or GA by itself did not have significant bactericidal effect (Ensafi, Jamei, Heydari-Bafrooei, & Rezaei, 2016). In the current study, a novel finding was that enhanced antimicrobial activity of UVC-GA continued to persist even when the UV-C light source was

removed. Moreover, the antimicrobial activity of UV-C exposed GA was stable in that the bactericidal effect did not decrease significantly after the irradiated GA solution was stored for up to 4 weeks. These attributes suggest that the enhanced antimicrobial activity of GA after UV-C exposure was not due to a cumulative effect of GA and UV light as two hurdles.

Since GA solution without previous UV light exposure did not show significant ($P > 0.05$) antimicrobial activity in the current experimental setting, acidic pH of GA solution was likely to be not a reason for its bactericidal effect. Furthermore, bacteria treated by UV-C exposed HCl or phosphate buffer solution with the same pH (pH=3.1) as 15 mM GA, did not show significant ($P > 0.05$) antimicrobial activity (**Figure 3-2a**). However, it was interesting to observe that acidic pH was required for UV-irradiated GA to maintain its antimicrobial activity. When the pH of GA solution was adjusted to 7 or 11 either before or after UV-C exposure, bactericidal effect was attenuated ($P > 0.05$). Therefore, it was a specific interaction between GA and UV-C that exerted the antimicrobial activity.

Since acidic pH might be necessary for the UVC-GA to maintain the undissociated form of GA, it is plausible that UVC-GA needs to permeate cell membrane to exert its antimicrobial activity. It is also plausible that UVC-GA may cause higher membrane damage than GA and thereby increase its uptake within the cells and show higher antimicrobial effect compared to GA. Based on results in **Figure 3-2b**, it is apparent that UVC-GA was uptaken. Based on results in **Figure 3-2c**, it is also apparent that UV-C treated GA caused some membrane damage compared to water. However, while there was a significant difference in the corrected fluorescence values for DPBA-GA complex in UVC-GA and GA, indicating a potentially higher level of uptake of UV-C treated GA compared to GA, we argue that this difference is not a driving force behind the higher antimicrobial activity observed for UVC-GA. This is based on our previously published

study where we observed that increase in GA uptake was correlated with and possibly a result of correspondingly higher membrane damage within the bacteria (Q. Wang et al., 2017). Since we did not observe a significant difference in the extent of membrane damage by GA and UVC-GA, we postulate that enhanced uptake or membrane damage were not responsible for the enhanced antimicrobial activity observed for UV-C treated GA. One hypothesis for the mechanism of the antimicrobial activity of UVC-GA is the generation of stable compounds during UV exposure, such as quinone. Another hypothesis is the generation of ROS and the subsequent oxidative stress to bacteria.

The yellow color observed of UVC-GA was indicative of formation of quinone in the solution. GA can be oxidized to quinone or hydroxyl related derivatives with the formation of hydrogen peroxide, due to the oxidation tendency of the three aromatic hydroxyl groups of GA (Eslami et al., 2010). By using UV as the oxidizing source accompanied by oxidation catalyst such as oxygen, quinone can be produced as an intermediate of GA oxidation by either the electrophilic attack of hydroxyl radical, or direct oxidation by oxygen dissolved in the water (Quici & Litter, 2009; Wysocka et al., 2018). Therefore, it was assumed that GA oxidized compound such as quinone by UV light irradiation could be responsible for the antimicrobial effect. To verify this hypothesis, benzenesulfinic acid (BSA) was added to UVC-GA solution for antimicrobial evaluation. Due to the high electrophilic character of quinone, it can react with nucleophiles like BSA to form adduct (C. M. Oliveira, Ferreira, De Freitas, & Silva, 2011; Sanhueza et al., 2017). A significant ($P < 0.05$) reduction in antimicrobial activity by adding BSA to UVC-GA solution supports a hypothesis that quinone may be generated during the exposure of GA to UV-C and is responsible for the antimicrobial activity (**Figure 3-3c**). The hypothesis of forming quinone-like compound in the UVC-GA supports the relative stability of its antimicrobial activity. Although

the mechanism by which quinone causes toxicity is complex, it has been shown that all quinones generate ROS through redox cycling with their semiquinone radical (Ensafi et al., 2016; Vaughan et al., 2010). Once quinone permeates through cell membrane, it reacts rapidly with respiratory electron-transfer chains, leading to intracellular oxidative stress (Yang, Sau, Lai, Cichon, & Li, 2014; Yasukawa, Uchida, & Matsue, 1998). This is consistent with the CellROX result in **Figure 3-4c** that significantly increased intracellular oxidative stress was detected in the UVC-GA solution.

Previous literature has shown that phenolic compounds including GA have the ability to generate ROS such as hydrogen peroxide and hydroxyl radical under UV/Vis light exposure, which contributed to antimicrobial effect (17, 30). A study showed that GA simultaneously exposed to blue light of LED at 400 nm for 15 min inactivated more than 5 log CFU/mL of *Staphylococcus aureus*, of which antimicrobial activity was attributed to the generation of hydrogen peroxide and hydroxyl radical (K. Nakamura et al., 2012). Similarly, other phenolic compounds such as caffeic acid, chlorogenic acid, epigallocatechin, epigallocatechin gallate, and proanthocyanidin are found to exert antimicrobial activities against selected bacteria to some extent when exposed by light in the wavelength range of UV-A or blue light (Keisuke Nakamura et al., 2015). Our previous study also found that GA generated hydrogen peroxide during UV-A exposure. Therefore, it is plausible that ROS could be one of the antimicrobial factors for UVC-GA. In the current study, catalase (hydrogen peroxide scavenger), DMSO and mannitol (hydroxyl radical scavengers) were added along with GA for UV-C exposure, and the significantly reduced antimicrobial activity indicated that hydrogen peroxide and hydroxyl radical were involved in the bactericidal effect of UVC-GA. The detection of hydrogen peroxide in the GA solution irradiated by UV-C verified the generation of ROS. However, the amount of hydrogen peroxide was too

low (approximately 7 μM) to exert an effective antimicrobial activity by itself. Also, hydroxyl radical was not successfully detected from GA solution after UV-C exposure. The reason might be that the concentration of hydroxyl radical was too low to be detected. In addition to the limited detection of ROS, the relatively long lasting (at least 4 weeks) of the antimicrobial activity of UVC-GA could not be explained by the effect of ROS, which has a short life. Therefore, although the generation of ROS was involved in the antimicrobial activity of UVC-GA, it cannot fully explain the mechanism of its antimicrobial activity.

3.5 Conclusion

We demonstrated that UVC-GA exhibits enhanced antimicrobial activity against *E. coli* O157:H7 compared to gallic acid that was persistent for at least 4 weeks of storage at room temperature. The antimicrobial activity was affected by solution pH and the wavelength of UV-C exposure. The generation of ROS during UV light exposure, and photo-oxidized compound of GA such as quinone contributed to the antimicrobial activity of the post-irradiated GA solution. UVC-GA has the potential to be applied as a novel antimicrobial in food systems, especially when sustained antimicrobial activity is required.

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Chapter 4: Application of GA and UV light-based treatments for decontamination of fresh produce

Hypotheses

1) The GA and UV light-based treatments can be applied to decontaminate fresh produce during washing.
2) The two GA and UV light-based treatments will not have a significant effect on the texture and color quality of fresh produce being washed.

Abstract

This study evaluated the antimicrobial efficacies of two newly developed, non-thermal techniques: the simultaneous interaction of UV-A light and gallic acid, and gallic acid post-exposed by UV-C light in fresh produce. Baby spinach leaves and cherry tomatoes were used as model produce and *E. coli* O157:H7 was inoculated on the surface of produce to mimic the contamination condition. Results showed that UVA+GA treatment at 15 mM in 30 min resulted in 3.50±1.04 log CFU/g reduction of *E. coli* O157:H7 inoculated on the surface of baby spinach. Cherry tomatoes and baby spinach washed by UVC-GA treatment at 15 mM in 15 min showed a significantly ($p< 0.05$) lower bacterial load than that by GA alone, and > 4 log and 1.5±0.2 CFU/g of the bacterial population were inactivated in 15 min, respectively. Furthermore, no significant ($p>0.05$) change of color of spinach and tomato samples after treatment was observed. Also, The UVC-GA treatment did not ($p>0.05$) change the firmness of tomato samples. Results of this study demonstrated that both UVA+GA and UVC-GA treatments are potential alternatives as novel non-thermal inactivation techniques to traditional disinfection methods in fresh produce industry. Further research are needed to optimize the process of the two treatments for industry use.

4.1 Introduction

A large portion of the fresh produce is consumed raw and the number of foodborne outbreaks associated with these produce products has increased in recent years (Herman, K. M., Hall, A. J., & Gould, 2015). For the United States, the number of foodborne pathogen outbreaks due to fresh produce ranged from 23 to 60 per years during 2004-2012, and there were substantial increases in 2006 (57 outbreaks), 2008 (51 outbreaks), and 2011 (60 outbreaks) (Callejón et al., 2015). About 13% of those produce associated outbreaks were multistate outbreaks. The number of produce-associated outbreaks remains high and represents a significant health and financial issue. Since produce can be contaminated at many points from farm to table, the occurrence of these outbreaks calls for the need to improve prevention strategies (Lynch, Tauxe, & Hedberg, 2009).

A wide spectrum of pathogens have been documented in produce associated outbreaks, including *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., enteropathogenic strains of *E. coli* and *hepatitis A virus.* A significant number of foodborne outbreaks and gastrointestinal illnesses occur annually in the United States owing to *E. coli* O157:H7 (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). *E. coli* O157:H7 is a Shiga toxin-producing serotype responsible for a variety of intestinal and extra-intestinal diseases, such as diarrhea, urinary tract infections, septicemia, and neonatal meningitis. It is considered to be a major cause of hemorrhagic colitis and haemolytic uremic syndrome. They are resistant to environmental stresses and can survive the gastrointestinal conditions (Chung, Bang, & Drake, 2006). Dairy cattle are the natural reservoirs of enteropathogenic strains of *E. coli*. Some of the food vehicles that have been sources of foodborne infections are fresh and fresh-cut vegetables such as spinach, lettuce, sprouts, and ground meat (United States Centers for Disease Control and Prevention, 2006).

Annual estimates of over 17600 illness and 20 fatalities due to *E. coli* toxins have been recorded in the nation (Scallan et al., 2011). Among them, a spinach-associated *E. coli* O157:H7 outbreak in 2006 resulted in 204 illness and 3 deaths in US and Canada (World Health Organisation, 2007).

Cross-contamination of leafy green with *E. coli* O157:H7 may occur both pre-harvest such as irrigation water, soil and the environment, as well as post-harvest via cutting, washing and packaging (Jung, Jang, & Matthews, 2014). In order to address foodborne outbreaks, antimicrobial washing treatments are used to eliminate pathogenic bacteria that inhabit fresh produce surfaces and to prevent spread in the fresh produce processing environment (López-Gálvez et al., 2010). Currently, chlorine is the sanitizing agent most used by the fresh produce industry, mainly due to its antimicrobial activity and low cost. However, the effectiveness of chlorinated water against some pathogens such as *E. coli* O157:H7 is limited (Beuchat, 1999). A higher concentration of chlorinated water for increased effectiveness may cause product tainting, and result in residues on the products (Adams, M. R., Hartley, A. D., & Cox, 1989). In addition, increasing public health concerns about the possible formation of chlorinated organic compounds and the emergence of new, more tolerant pathogens have called for the need for alternatives to the use of chlorine (Singh, Singh, Bhunia, & Stroshine, 2002). The safety and efficacy of chlorine might eventually lead to the implementation of restrictions by regulatory agencies (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008). Therefore, there is an increasing need to investigate the efficacy of new sanitizers and other alternative technologies.

There is no single sanitization treatment so far that has been shown to completely eliminate pathogens on fresh produce without affecting the produce quality (Issa-Zacharia, Kamitani, Miwa, Muhimbula, & Iwasaki, 2011). Organic acids and plant-derived compounds have gained

interest due to their antimicrobial activity and their consumer-friendly nature. Those compounds are Generally Recognized As Safe (GRAS) and their bactericidal efficacy against *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* on fresh produce has been previously investigated (Akbas & Ölmez, 2007; Ganesh, Hettiarachchy, Griffis, Martin, & Ricke, 2012). Phenolic compounds present in plants, herbs and spices are known to have antimicrobial properties (Balasundram, Sundram, & Samman, 2006). However, the concept of combined decontamination treatments with natural antimicrobial agents might be a more effective strategy for pathogen reduction than the application of single treatments (Ho, Luzuriaga, Rodde, & Tang, 2011).

Previously, we have developed two non-thermal inactivation treatments based on the interaction between GA, a naturally present phenolic compound, and ultraviolet light, where bacteria are (a) simultaneously exposed to combined UV-A light and gallic acid treatment (UVA+GA) or (b) exposed to gallic acid solution that has been pre-exposed by UV-C light (UVC-GA). The objective of this study is to evaluate the inactivation efficacy of the two newly developed treatments on fresh produce against *E. coli* O157:H7, using baby spinach and cherry tomato as model produce. The texture and color quality of the treated produce were also evaluated.

4.2 Methods

4.2.1 Bacterial strain, culture condition

The bacterial strain used in this study was Shiga toxin negative *E. coli* O157:H7 (ATCC #700728, Manassas, VA) provided by Prof. N. Nitin at University of California-Davis. This strain is non-pathogenic but it possesses similar phenotypic characteristics as the toxigenic strain of *E. coli* O157:H7. This bacterium is also rifampicin resistant. Stock cultures were maintained

at -80 °C in tryptic soy broth (TSB) supplemented with 20% glycerol. The culture was re-activated by transferring to a plate of tryptic soy agar (TSA) and stored at 4 °C for four weeks. Prior to the experiments, a colony from the TSA plate was transferred into 10 mL TSB and incubated at 37 °C overnight for 20-24h to reach stationary phase with a concentration of approximately 9 log CFU/mL.

4.2.2 Antibacterial activity of UVA+ GA on E. coli O157:H7 inoculated on spinach leaves

Organic baby spinach leaves were bought in a local retail market. Four leaves were soaked in 2% (w/v) bleach for 10 min followed by washing in sterilized water and drying before use. *E. coli* O157:H7 was cultured in LB added with rifampicin 50 μg/mL and incubated as already described. The culture was then diluted 100-fold in water to achieve 6 log CFU/mL. One hundred microliters of the diluted bacterial suspension were spotted on the surface of each of the four leaves and 900 μL of a sterile 11.1 mM gallic acid solution or DI water were added to the bacteria droplet on each of the leaf. One leaf was then exposed to UV-A light for 30 min. One leaf was used for the determination of initial load of bacteria on the surface of leaf without treatment. Other two leaves were controls that incubated in the dark with gallic acid or in the dark with water. After treatment, leaves were carefully transferred into plastic bags (Whirl-Pak, Fisher Scientific Inc., Pittsburgh PA, USA) where 9 mL of water was added to resuspend bacteria from the leaves surface. The bags were then rubbed by hand to mix the sample and an aliquot of 100 μL of the liquid was serially diluted in PBS and plated on LB agar added with rifampicin 50 μg/mL. Plates were incubated at 37 °C for 24 h and the population of *E. coli* O157:H7 was interpreted as log (CFU/g) by plate counting. Each treatment was performed in 4 replicates.

4.2.3 Antibacterial activity of UVC-GA treatment on E. coli O157:H7 inoculated on the surface of cherry tomatoes and baby spinach

Organic cherry tomatoes and baby spinaches were purchased from a local supermarket. Four tomatoes or spinaches were rinsed in DI water and air dried under a biological fume hood until no water drop can be observed on the surface before immersed in the suspension of *E. coli* O157:H7 of which the initial concentration was approximately 7 log CFU/g for 30 min. Then, tomato or spinach samples were air dried under a biological fume hood for another 30 min. One of the inoculated tomatoes and spinach leaves was used to determine the initial bacteria load, while the others were used for washing treatment. GA (15 mM) solution that was exposed to UV-C light for 60 min was used as treatment solution. GA without UV-C irradiation and water were controls. Samples with inoculated bacteria were immersed in UV-C irradiated GA, GA solution, and water, respectively, for 15 min. After that, each sample was immediately placed in a sterile stomacher bag with 4.5 mL BPW and were rubbed by hand for 5 min separately. The solutions were then serially diluted in BPW, plated on EMB agar, and counted following incubation at 37 °C for 24 h.

4.2.4 Color analysis

A Hunter color analyzer (HunterLab colorimeter model EZ-45/0 CX2405, Hunter Associates Laboratory, Reston, VA, USA) was used for the instrumental color evaluation. Measurements were taken from treated and untreated grape tomatoes surface without inoculation of *E. coli* O157:H7. The values were expressed as color in terms of L*, a*, b* values, where: L* defines lightness (the maximum for L* is 100, which represents white and the minimum is 0, which represents black). A positive a* value indicates redness (−a* is greenness) and a positive b* values yellowness (−b* is blueness) on the hue-circle. To compare color changes, the total color

difference was expressed as $\Delta E^* = \sqrt{(L_0^* - L_t^*)^2 + (a_0^* - a_t^*)^2 + (b_0^* - b_t^*)^2}$, where "0" was the untreated control and "t" the treated sample. The instrument is calibrated to a standard white ceramic disk and a black trap before taking any measurements. Results are expressed as an average of triplicates.

4.2.5 Texture analysis

Changes in texture of grape tomato surface were evaluated with a TA-XT2i texture analyzer (Texture Technologies Corp, Hamilton, MA) texture analyzer with a flat-tipped cylindrical stainless probe (3 mm). After selected treatment, the tomato samples were cut in half and were placed onto the press holder with the skin up in contact with the probe at room temperature. The samples were compressed, and data were expressed as maximum force (grams) to cause a deformation of 3 mm in a single compression-decompression cycle at a steady speed of 5 mm min^{-1}.

4.2.6 Statistical analysis

All experiments were performed in triplicate. Data were subjected to one-way analysis of variance (ANOVA) and the two-tailed unpaired *t*-test ($\alpha = 0.05$) by Microsoft Excel 2016 (Microsoft Inc., Redmond WA, USA).

4.3 Results and discussion

4.3.1 Inactivation of Escherichia coli *O157:H7 on surface of fresh produce*

Studies have shown that washing contaminated fresh-cut vegetables in standardized washing water without sanitizer results in a high contamination level in the washing water with *E. coli* form the washed product, regardless of the large quantities of water used (Petri, Rodríguez, &

García, 2015). Therefore, the use of effective disinfection sanitizers is strongly recommended to inactive pathogens in the water used in the fresh produce processing. **Figure 4-1** shows the remaining bacteria on spinach samples after the treatments. Although GA in absence of UVA light did not cause a significant bacterial inactivation, a simultaneous treatment of UVA + GA resulted in at least 3.50 ± 1.04 log CFU/g reduction without any visible color or texture change in leaves. This result demonstrates that the synergistic interaction of UV-A light and GA treatment was effective in inactivating *E. coli* inoculated on the surface of the spinach leaves. The magnitude of inactivation was lower than that observed in the model wash water. This can be attributed to a higher variability in the extent of inactivation on spinach surface compared to that in the solution. Nevertheless, the leaf surface bacterial inactivation assay offers a proof-of-concept of the synergistic effect in inactivating the bacteria on the surface of spinach. It is to be noticed that one reason spinach leaves were chosen as model produce for UVA+GA treatment was due to the plate shape of the leaves so that the whole surface of the leaves are accessible to UV-A exposure. It is difficult to provide a uniform exposure to the produce samples with a cubic or spherical shape (e.g. tomato), thus lowering the antimicrobial activity of UVA+GA treatment. For this reason, tomatoes were not used as the samples for UVA+GA treatment but were applied for UVC-GA treatment, which does not require an on-site exposure of UV light.

The antimicrobial activity of UVC-GA was evaluated on the surface of both fresh organic cherry tomatoes and leaves of baby spinach (**Figure 4-2**). The initial load of *E. coli* O157:H7 inoculated on the surface of sample was approximately 4.5 log CFU/g. After 15 min, tomatoes washed by water reduced 0.8±0.2 log CFU/g of population. Tomatoes washed by GA achieved a reduction of 2.1±0.5 log CFU/g, while tomatoes washed by UVC-GA showed a significantly ($P < 0.05$) lower bacterial load and more than 4 log CFU/g of the bacterial population were inactivated. It is

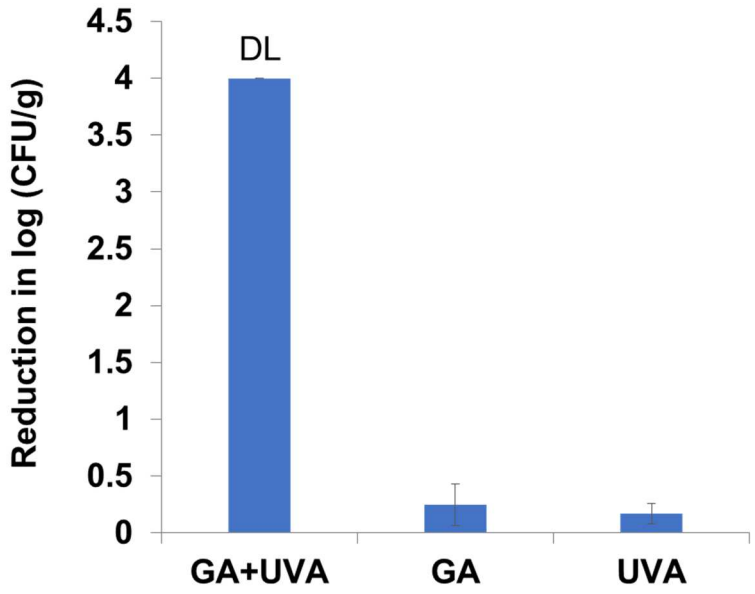

Figure 4-1. Antimicrobial activity of UV-A+GA treatment on *E. coli* O157:H7 inoculated on the surface of spinach (UVA+GA). Controls consisted of incubation with GA in dark and incubation of bacteria without GA. DL indicates the reduction of bacterial population reached detection limitation. Values are means ± standard deviation, n=3.

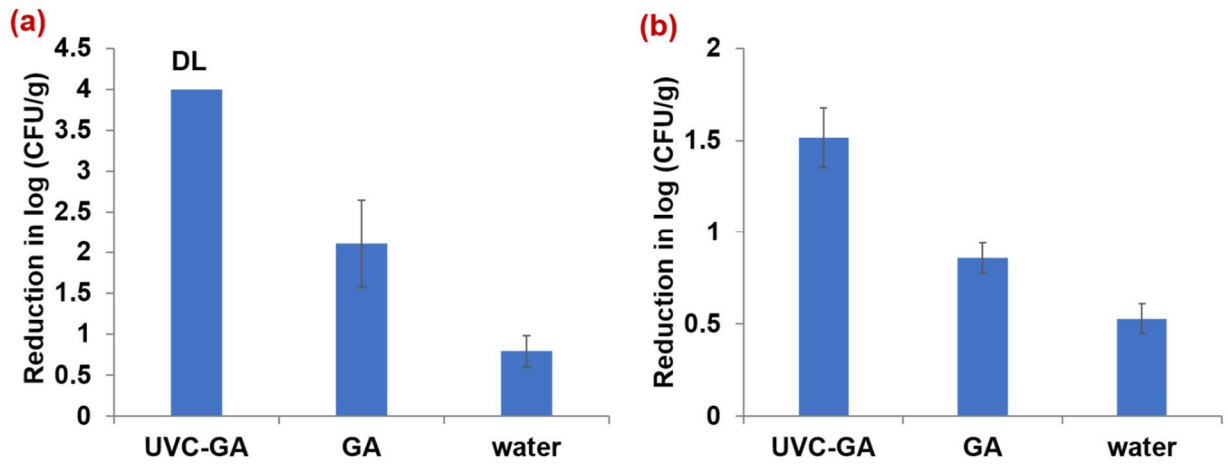

Figure 4-2. Inactivation of *E. coli* O157:H7 on the surface of (a) cherry tomatoes and (b) baby spinach leave. Logarithmic reduction in *E. coli* O157:H7 on the surface of tomatoes treated by UV-C irradiated GA, GA, and water. The initial concentration of bacteria for inoculation was 7 log CFU/g and the concentration of bacteria inoculated on the surface of tomatoes before the treatment was 4.5±0.5 log CFU/g. Duration of GA treatment was 60 min. Duration for washing tomatoes was 15 min. DL indicates the reduction of bacterial population reached detection limitation. Values are means ± standard deviation, n=3.

important to highlight that when the incubation time was prolonged to more than 1 h, both GA and UVC-GA were able to achieve more than 4 log CFU/g reduction in bacterial count on the surface of tomatoes highlighting that they both may be an effective antimicrobial treatment. Although the behavior of UVC-GA treatment on spinach leaves was less effective as the reduction in microbial load by UVC-GA was 1.5±1.2 log CFU/g, similar to the tomato samples, the inactivation efficacy of UVC-GA treatment was significantly ($p<0.05$) higher than that of GA alone (0.9±0.1 log CFU/g) and water (0.5±0.1 log CFU/g) as control. Eosin methylene blue (EMB) agar was used in this experiment instead of TSA to identify the inoculated *E. coli* O157:H7 while inhibiting the growth of other microorganism which may be naturally present on the surface of tomatoes. Since EMB is a selective and differential media, it could affect the recovery of sublethal injured bacteria. Therefore, the effectiveness of the tested antimicrobial was not completely comparable to previous results obtained from using TSA. Nevertheless, it was evident that UVC-GA is a promising antimicrobial agent on the application of fresh produce decontamination.

4.3.2 Color and texture analysis of fresh produce after inactivation treatment

Most fresh produce such as lettuce and spinach are delicate products and therefore, the textual quality and appearance can be easily affected by the application of physical or chemical methods of processing. To evaluate the effect of UVA+GA/UVC-GA treatment on the quality of produce, color change of both spinach and tomato samples were measured before and after the treatment. The firmness of tomato samples was also analyzed. However, the firmness of spinach leaves was not measured due to the lack of a specific probe in our texture analyzer for leafy materials. The change in the surface color of baby spinach and tomatoes of selected treatment was shown in **Table 4-1**. The results were displayed by lightness (L^*), redness (a^*),

Table 4-1. The Hunter's color analysis of the surface (**a**) baby spinach and (**b**) grape tomatoes after selected treatments measured by lightness (*L**), redness (*a**), yellowness (*b**) and change in total color difference (*E**). Values are means ± standard deviation, n=3.

(a)

Sample	**ΔL***	**Δa***	**Δb***	**ΔE***	**Corresponding RGB color**
UVA+GA	-1.22±1.24	-0.63±1.13	0.09±0.46	2.48±0.54	
UVC-GA	0.15±0.72	-0.01±0.35	0.22±1.07	2.24±0.41	
UVA	-0.69±0.96	-0.33±0.36	0.79±1.49	1.86±0.64	
GA	-0.72±0.30	0.07±0.52	0.60±0.78	2.97±2.04	
Water (control)	-0.52±0.69	-0.50±0.24	0.98±0.32	2.83±1.43	

(b)

Sample	**ΔL***	**Δa***	**Δb***	**ΔE***	**Corresponding RGB color**
UVC-GA	-0.39±0.93	0.63±1.30	0.37±0.81	1.53±0.85	
GA	0.01±1.33	-0.14±1.47	-0.84±1.24	1.77±1.37	
water	-0.15±0.88	-1.00±1.52	-0.98±0.69	1.78±1.32	

yellowness (b^*) and change in total color difference (E^*). For both baby spinach and tomato, no significant differences ($p>0.05$) in color values (L^* a^* b^*) as well as the total color differences (E^*) were found among the applied washing treatments. Difference in visual appearance of treated and untreated tomatoes and spinaches was also not observed. The firmness of fresh-cut tomatoes with or without selected treatments was expressed as the maximum force to deform 3 mm (**Figure 4-3)**. Although the firmness values of UVA+GA and UVA treated tomatoes were higher than the water rinsed tomato and untreated control, the difference was not significant ($p> 0.05$). Tomato became softer after UVC-GA treatment, but the change in firmness was not significant ($p>0.05$) compared with control. Treatment by only GA (15 mM) solution did not ($p> 0.05$) change the firmness of tomatoes as compared with water rinsed samples and untreated controls. In general, the results here show that unlike conventional sanitizing treatment that can adversely affect the texture of the produce, the treatments used in this study did not alter the appearance of spinach or tomato.

4.4 Conclusion

Decontamination of fresh produce and wash water with low-cost sanitizers such as peroxides and chlorine-based compounds is still a common practice within the industry. However, there is growing need for new sanitation treatments that are not only low-cost and effective but also environmentally friendly and safe for the workers at the processing industry. Results in the current study shows that the simultaneous interaction between UV-A light and GA can enhance the inactivation of inoculated bacteria on baby spinach leaves as compared with controls. GA solution after UV-C light exposure also showed inactivation efficacy against *E. coli* O157:H7 inoculated on the surface of grape tomato and spinach leaves. In addition, UVA+GA and UVC-GA treatments used in this study did not affect the color, firmness, and visual quality of baby

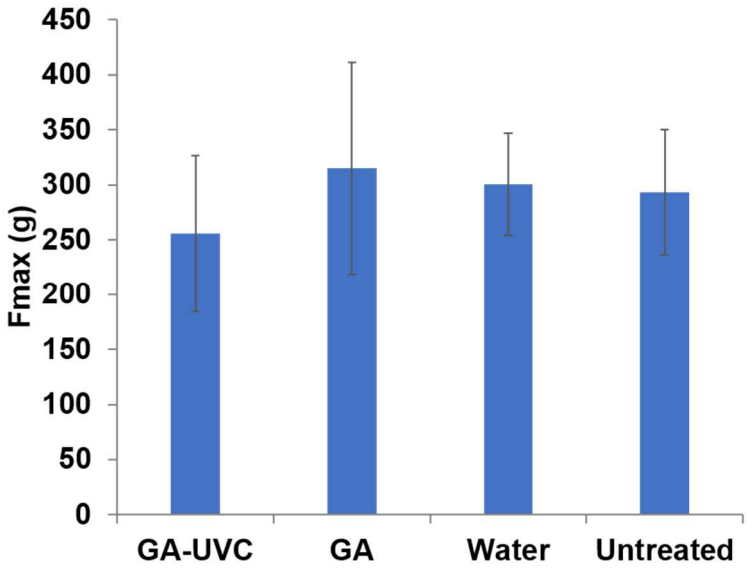

Figure 4-3. Change in texture of grape tomatoes with or without selected treatment by texture analyzer. Data were expressed as maximum force (gram) to cause a deformation of 3 mm. Values are means ± standard deviation, n=3.

spinaches and grape tomatoes. Based on the relatively low cost of GA, its efficacy and its GRAS status, the two GA and UV light-based treatments may be a good natural alternative to chlorine based antimicrobial agents. Further research is needed to determine the optimal processing using these novel inactivation treatments.

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Chapter 5: The development of adaptive response in *E. coli* O157:H7 associated with UV and GA-based antimicrobial treatments

Hypotheses

1) Prior exposure to sublethal stress (heat, acidity, osmotic stress, and oxidative stress) will increase the resistance of *E. coli* O157:H7 towards GA and UV-based treatments
2) Exposure to mild GA and UV light-based treatments selects for cross-resistant *E. coli* O157:H7 towards heat, acid, and oxidative challenge

Abstract

Foodborne pathogens can develop adaptive responses towards sublethal stresses encountered in the environment, affecting the inactivation efficacy of the following food processing interventions. Previously, we have developed two non-thermal treatments based on the interaction between GA and UV light against *E. coli* O157:H7, where bacteria are, (a) simultaneously exposed to UV-A light and gallic acid (UVA+GA) or (b) gallic acid prior exposed to UV-C light (UVC-GA). The objectives of this study were to investigate the development of adaptive response of *E. coli* O157:H7 towards the two GA and UV light-based treatments on exposure to environmental stresses at sublethal levels. In addition, the effect of repetitive exposure to mild GA and UV light-based treatments on the acquisition of resistance towards stresses such as heat, acid and hydrogen peroxide was evaluated.

Results showed that prior exposure to heat and acid shocks increased ($p<0.05$) the resistance of *E. coli* O157:H7 towards subsequent UVA+GA treatment, while sodium chloride shock increased ($p<0.05$) their sensitivity of cells to that treatment. Interestingly, the same prior shocks resulted in a different response to UVC-GA treatment where only heat shock showed protective ($p<0.05$) effect to the following UVC-GA treatment, while acid and sodium chloride increased ($p<0.05$) the sensitivity of bacteria to UVC-GA. Exposure of bacteria to prior hydrogen peroxide shock had no effect ($p>0.05$) on the sensitivity of the bacteria to either UVA+ GA or UVC-GA treatments. In addition, repeated exposure to UVA+GA or UVC-GA treatments selected for sub-population that demonstrated higher ($p<0.05$) resistance towards these treatments (<1 log CFU/mL reduction). This sub-population subsequently showed higher ($p<0.05$) cross-resistance to heat and acid treatments as well. Subsequent experiments revealed that production of enzymes such as superoxide dismutase and general stress response regulator, RpoS, were upregulated and

were highly likely to be associated with the development adaptive response. Thus, sublethal conditions encountered in the food processing environment should be considered for the development and optimization of novel non-thermal technologies.

5.1 Introduction

Foodborne bacteria are exposed to a variety of stresses in the environment. They have the ability to survive the environmental stresses, multiply in food and cause spoilage as well as illness (Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015). If the stress is mild, it causes injury to the bacteria. If the stress is severe, it causes inactivation. Injured bacteria in food are of concern since thy can survive and even grow when favorable conditions are encountered (Roszak, D. B., & Colwell, 1987). Such mild stresses are very often encountered by the bacteria in food as well as in the food processing environment. For instance, more and more minimal food processing is done to meet the increasing demand of consumer for fresh-like food products with good nutritional quality. In such minimal processing, only mild treatments are given to the food product (Shankar, Jeevitha, & Shadeesh, 2017). Examples of the different environmental stresses bacteria can experience during the processing chain are heat (e.g. cooking), osmosis (e.g. pickling), acid (e.g. fermentation), chemical sanitizers (e.g. chlorine and hydrogen peroxide), and cold (e.g. refrigeration). Once bacteria are exposed to a mild stress, they can adapt and are able to tolerate further severe stresses (Heinrich et al., 2016). Apart from single stress response, it has been found that exposure to one stress can confer advantages or disadvantage to bacteria to adapt to another stress. This phenomenon is also called cross-protection or cross-stress phenomenon (Rowe & Kirk, 1999). For example, salt stressing of *Listeria monocytogenes* by exposure to 6% (w/v) NaCl in brain heart infusion resulted in significant increased resistance toward the following 50 ppm nisin challenge in that there was 2.1 log CFU/mL difference in inactivation between cultures with or without prior exposure to NaCl (Bergholz, Tang, Wiedmann, & Boor, 2013). Another study showed that stationary phase *S. aureus* pretreated with alkaline and heat stresses for 2 h induced cross-protective effect in the following pulsed electric field treatment

and the D-values increased 6.3 and 2.8 fold respectively, compared with control (Liao et al., 2018). The cross-protection might help bacteria to survive throughout food processing, resulting in serious risk to food safety and public health. Most studies on cross-protection emphasized on the effects of conventional stress agents such as heat, cold, and acid used in food processing. However, only limited investigations have been conducted to explore the effect of various stresses on the resistance or sensitivity to emerging non-thermal technologies (Calvo, Alvarez-Ordóñez, Prieto, Bernardo, & López, 2017). Since the current trend toward the application of mild, minimal food processing techniques may result in more stress responses in foodborne pathogens, understanding of microbial stress response toward a novel technology is of importance for further optimization and development. Cross-protection of food borne pathogens should be taken into consideration when assessing the effectiveness of different combination of novel non-thermal processing techniques (Chen, 2017).

E. coli O157:H7 has been associated with a large number of food-borne pathogens with a low infectious dose (Lim, J. Y., Yoon, J. W., & Hovde, 2010). It also exhibits a stress response to sublethal environmental stresses. These stress responses can result in resistance to variety of environmental or processing parameters. Previous studies have shown that *E. coli* O157:H7 has the adaptive potential towards environmental stresses or processing factors such as high temperature, high ethanol, carbon source starvation, and UV irradiation (Chung, Bang, & Drake, 2006). Cross-protection of *E. coli* O157:H7 acquired to a specific stress after pre-treatment with another stressor has also been observed for various stress combinations. For example, *E. coli* O157:H7 pre-adaptation to glucose or nitrogen limitation increases survival rates after a heat shock or oxidative stress. This phenomenon has been suggested to be linked to RpoH, a heat shock or oxidative stress factor and an important role in protein synthesis under some stress

conditions (Jenkins, Schultz, & Matin, 1988). Transcriptional profiling in *E. coli* O157:H7 revealed a high degree of overlap between different stresses, such as starvation, high temperature, osmatic and acidic stresses, and it was found that some of the stresses induce the oxidative stress regulons that can at least partially explain the cross-protection between these stresses (Dragosits, M., Mozhayskiy, V., Quinones‐Soto, S., Park, J., & Tagkopoulos, 2013). In addition, *E. coli* O157:H7 have been found to be acquire resistance towards non-thermal treatment such as high hydrostatic pressure (Robey et al., 2001).

In our previous studies, we have developed two non-thermal treatments based on the interaction between GA and UV light and their effectiveness against *E. coli* O157:H7 have been demonstrated. The first treatment is the simultaneous application of UV-A light and GA (UVA+GA), of which the mechanism of the amicrobial effect was associated with the cellular uptake of GA, the generation of reactive oxidative species (ROS), the inactivation of enzymes, and the damage of bacterial membrane. The second treatment involves UV-C light exposed GA that shows enhanced antimicrobial effect compared to GA alone. The mode of action behind this enhanced activity is attributed to the formation of oxidized compounds of GA such as quinone, as well as the generation of ROS during UV-C light exposure. In the current study, we focused on the adaptive response of *E. coli* O157:H7 towards two newly developed non-thermal inactivation treatments with or without prior exposure to different environmental stresses that are sublethal to bacteria. The objectives of this study is to investigate the effect of 1) previous exposure to sublethal stress (heat, acidity, osmotic stress, and oxidative stress) on the development of resistance towards GA and UV based treatments, and 2) exposure to GA and UV light-based treatments on the development of cross-resistance to other stresses such as heat, acid and hydrogen peroxide, using *E. coli* O157:H7 as a model microorganism.

5.2 Methods

5.2.1 Bacterial strain, preparation, and environmental stress exposure

Rifampicin resistant, shiga toxin negative *E. coli* O157:H7 (ATCC #700728, Manassas, VA) was selected as a model Gram-negative pathogen. The bacteria were cultured in Tryptic Soy Broth (TSB) at 37 °C for 20 h to obtain the bacterial population in stationary phase before each experiment. Stock cultures were maintained at -80 °C in tryptic soy broth (TSB) supplemented with 20 % glycerol. The working stock was transferred to a plate of tryptic soy agar (TSA) at 4 °C and renewed monthly. Prior to the experiments, a colony from the TSA plate was transferred into 10 mL TSB and incubated at 37 °C overnight for 20-24 h.

Heat, acid, osmotic stress, and oxidative stress were selected as pre-treatments to investigate the adaptive response of *E. coli* O157:H7. For heat stress, a colony of *E. coli* O157:H7 stored at 4 °C was transferred to a TSB broth and incubated at 42 ℃ for 20 h (Cebrián, Sagarzazu, Pagán, Condón, & Mañas, 2008), while the control was incubated at 37 °C for the same duration. Acid shock was induced by transferring the stock culture of *E. coli* O157:H7 to a TSB broth with 1% added glucose and incubated at 37 °C for 20 h. The control was grown in TSB without any glucose. The use of TSB + glucose is known to be an effective approach of inducing acid tolerance in enterohemorrhagic bacteria (Buchanan, 1996). After overnight incubation, the pH of bacterial culture with added 1% glucose became 4.5±0.4, indicating a successful adaptation to mild acidic environment, while the control culture was 6.5±0.5. For osmotic and oxidative shock, *E. coli* O157:H7 stock culture was transferred into 10 mL TSB and incubated at 37 °C overnight for 20 h, followed by centrifugation at 4500 g for 10 min. Then, the pellet was resuspended in 4.5% NaCl, or 0.2 mM hydrogen peroxide, or water as control and incubated at room temperature (22 °C) for 3 h. The concentrations of NaCl (McMahon, Xu, Moore, Blair, &

McDowell, 2007) and hydrogen peroxide (Semchyshyn, Bagnyukova, Storey, & Lushchak, 2005) were chosen based on previous studies that could induce corresponding resistances without rendering significant reduction in cells. The concentration of the resuspended culture was approximately 9 log CFU/mL. After stress exposure, the culture of pre-treated *E. coli* O157:H7 and controls were subjected to UVA+GA and UVC-GA treatments.

5.2.2 UV Light source

Both UV-A and UV-C light sources (Spectroline ^{TM}, Westbury NY, USA) were bench-top, batch type chambers with a peak wavelength of 365 nm and 245 nm, and an average intensity of 3425 $\mu W/cm^2$ and 4550 $\mu W/cm^2$, respectively. In both cases, the treatments were applied at the surface from a distance of 17 cm.

5.2.3 UVA+GA or UVC-GA treatments and repetitive inactivation cycles

To conduct the UVA+GA treatment, an overnight grown culture of *E. coli* O157:H7 was diluted in sterilized 10 mM GA solution prepared in DI water to reach a final concentration of approximately 7 log CFU/mL. Following that, 2 mL of the bacterial suspension was transferred to a well of a 6-well flat bottom polystyrene plate and exposed to UV-A light for 30 min. Bacterial suspensions incubated with select compounds and stored in the dark for 30 min were used as controls. UVC-GA solution used in the current study was prepared by diluting GA in DI water to achieve a final concentration of 15 mM. The solution was then filtered through a 0.25 μm sterile filter and transferred to crystal petri dish (KIMAX®, NJ, USA) and exposed to UV-C light for 30 min. The, bacteria was diluted (approximately 7 log CFU/mL) and incubated in this UV-C exposed GA for another 30 min. After UVA+GA or UVC-GA treatment, the populations were aseptically retrieved from the previous treatment and the survival was determined.

To evaluate whether a more resistant sub-population can be identified following repeated exposure to these treatments. *E. coli* O157:H7 cultures were subjected to repetitive cycles of exposure to either UVA+GA or UVC-GA treatments and survivors were re-grown to the original concentration. Specifically, after each cycle, survivors of UVA+GA or UVA-GA treated populations were inoculated 1:100 into fresh TSB, and regrown for 20 h at 37 °C prior to a next cycle of treatment. This was repeated for 16 cycles, when the inactivation from the treatment was less than 1 log CFU/mL. The 16^{th} cycle cultures, which were UVA+GA or UVC-GA resistant, were stocked at 4 °C on TSA to be used for subsequent experiments. Control of the selection experiment was conducted by serial passaging of the parental culture in the absence of the UVA+GA or UVA-GA treatment. Stationary cultures were repeatedly diluted 1:10,000 in fresh TSB and grown for 20 h to stationary phase again, until successive growth for 16 growth cycles.

5.2.4 Heat, acid, and oxidative challenge

Bacterial cultures after 16 cycles of either GA+UVA or UVC-GA treatment were used to determine the resistance to stresses including heat (56 □C), acid (pH 3, HCl), NaCl (25% w/v), and hydrogen peroxide (0.1%). In the case of heat challenge, the cultures were first diluted in DI water to reach a concentration of approximately 7 log CFU/mL, then heated by submerge coil apparatus (ICA, Sherwood Instruments, Lynnfield, Mass.) For acid, NaCl, and hydrogen peroxide challenge, cultures were diluted in medium containing the lethal factor to reach a concentration of approximately 7 log CFU/mL. During the inactivation by the lethal factors, samples were removed at selected intervals and plated onto TSA. The survival populations were determined as described above.

5.2.5 Microbial determination

Plate count method was used for determination of bacterial cultivability. The samples of bacteria suspensions were serially diluted in 0.2 % (w/v) buffered peptone water, and an aliquot of 100 µL suspensions from each dilution was transferred and plated onto Tryptic Soy Agar (TSA, Difco™, Detroit MI USA) plates. The plates were incubated at 37 °C for 24 h under atmospheric condition before enumeration.

5.2.6 Superoxide dismutase (SOD) activity assay

Activity of superoxide dismutase (SOD) within *E. coli* O157:H7 was analyzed using Superoxide dismutase assay kit according to the manufacturer's protocol (Cayman Chemical, MI). Samples were homogenized in 20 mM HEPES buffer (pH = 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose), followed by centrifugation at 1,500 ×g for 5 min at 4 °C, and recovery of the supernatant. To quantify SOD activity, 200 µL of diluted SOD radical detector (included in the assay kit), 10 µL of sample, and 20 µL of diluted xanthine oxidase (included in the assay kit) were successively added to a 96-well plate, covered with the plate's lid, and incubated on a shaker for 30 min to mix at room temperature. The absorbance was analyzed using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at a wavelength of 440 nm. The SOD activity was determined by referring to a SOD activity standard curve established using the same assay kit.

5.2.7 Acid phosphatase assay

Acid phosphatase activity was determined by Acid Phosphatase Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI). 500 µL of 25 mM p-nitrophenylphosphate in 250 mM glycine hydrochloride (pH 2.5) was added to 50 µL of stationary phase cells of

overnight cultures and after 10 min of incubation at 37 °C the reaction was terminated by the addition of 1 mL of 1 N NaOH. Then, the absorbance at 410 nm was measured. Differences in acid phosphatase activity was expressed as fold change with respect to control.

5.2.8 Determination of membrane damage by propidium iodide (PI)

Cellular membrane damage was determined by PI as previously described in Chapter 3. Samples were centrifuged for 2 min at 10,000 ×g, washed with DI water, and to which PI was added to reach a final concentration of 5 μM. Next, samples were incubated for 15 min in dark, followed by washing with DI water and resuspended in 500 μL phosphate buffered saline solution Finally, each of the samples was transferred to a 96-well plate and fluorescence intensity was measured using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA) at excitation/emission wavelength of 490/635 nm using a SpectraMax M5e plate reader (Molecular Devices LLC, Sunnyvale CA). The fluorescence intensity was corrected using the following equation.

$$Corrected\ fluorescence = fluorescence\ intensity_{\ treated\ sample} - fluorescence\ intensity_{\ control}$$

5.2.9 Statistical analysis

Experiments were performed in triplicates. Statistical analysis of the triplicate data was performed using the two-tailed unpaired *t*-test ($\alpha = 0.05$) by Microsoft Excel 2016 (Microsoft Inc., Redmond WA, USA).

5.3 Results

5.3.1 Effect of prior heat, osmotic and oxidative stresses on sensitivity of the bacteria towards UVA+GA and UVC-GA treatments

Reductions in *E. coli* O157:H7 after prior exposure to sublethal heat, acid, osmotic or oxidative stress followed by UVA+GA or UVC-GA treatment are shown in **Figure 5-1**. *E. coli* O157:H7 with prior heat shock significantly ($p< 0.05$) increased the resistance to subsequent antimicrobial treatment of both UVA+GA and UVC-GA. With prior heat shock, the level of inactivation in *E. coli* O157:H7 from UVA+GA treatment lowered from 3.3±0.3 to 2.6±0.2 log CFU/mL compared with controls, and was reduced from 2.8±0.9 to 0.8±0.3 log CFU/mL with regards to UVC-GA treatment. On the contrary, bacteria pre-exposed to NaCl showed increased sensitivity towards both GA+UVA and UVC-GA treatments. Interestingly, pre-exposure to acid stress due to overnight fermentation of glucose induced resistance of bacteria towards GA+UVA treatment (reduction in population reduced from 4.4±0.4 to 2.2±0.3 log CFU/mL, $p< 0.05$), but sensitivity towards UVC-GA treatment (reduction in population increased from 2.1±0.1 to 2.9±0.5 log CFU/mL, $p> 0.05$). No significant change in sensitivity was observed for hydrogen peroxide pre-treated cells ($p>0.05$) after either UVA+GA or UVC-GA treatment.

5.3.2 Creation of E. coli O157:H7 sub-population that is resistant towards UVA+GA or UVC-GA treatments

E. coli O157:H7 were subjected to repetitive inactivation cycles of either UVA+GA or UVC-GA treatment for up to 16th of cycle. As shown in **Figure 5-2**, during the repetitive cycles of treatments, the survivors progressively showed increased resistance to the inactivation from the treatment of UVA+GA and UVC-GA, respectively. The initial parental culture treated by

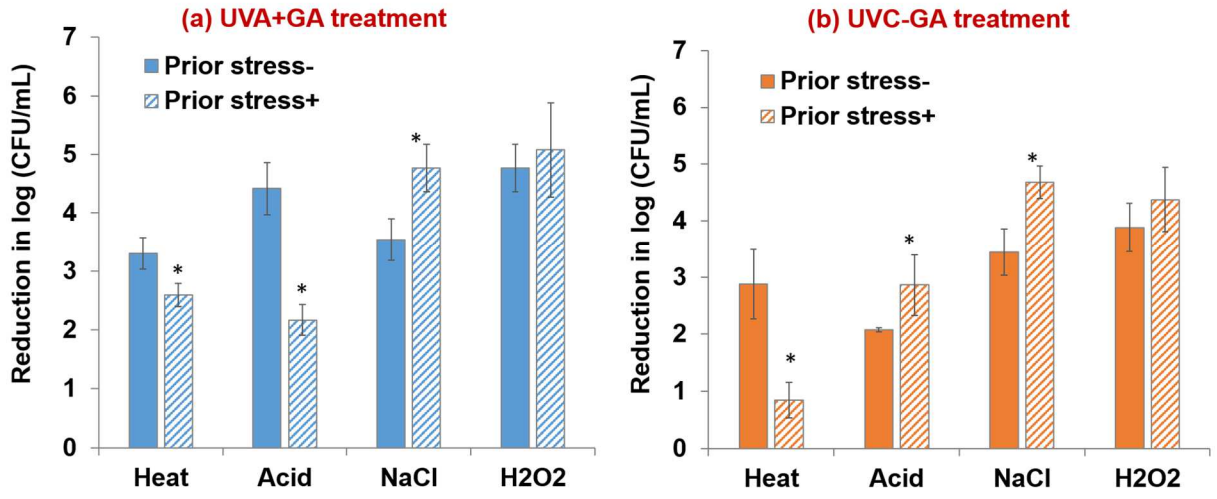

Figure 5-1. Effects of environmental stresses on microbial adaptive responses. Reduction in *E. coli* O157:H7 after prior exposure to heat, acid, osmosis, or oxidation sublethal stress, followed by (a) UVA+GA treatment and (b) UVC-GA treatment. Mean ± SD.

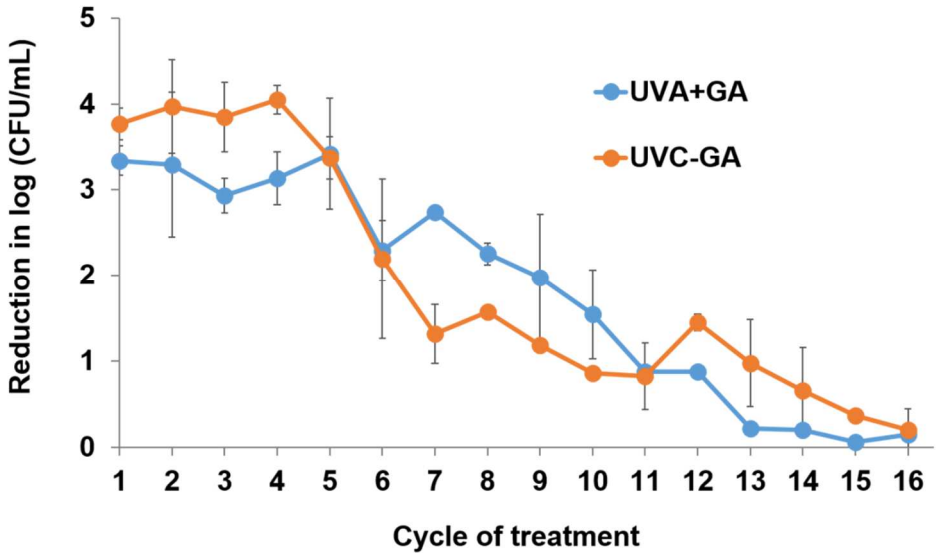

Figure 5-2. Selective enrichment of *E. coli* O157:H7 towards UVA+GA or UVC-GA treatments. Wild-type *E.coli* O157:H7 were exposed to either UVA+GA or UVC-GA shocks. After each treatment, survivors were determined and was expressed as reduction in log CFU/mL. Mean ± SD.

UVA+GA and UVC-GA treatment were reduced by 3.34±0.17 and 3.77±0.19 log CFU/mL, respectively. After the 6th cycle of treatment, the reduction in *E. coli* O157:H7 population was 2.29±0.35 and 2.19±0.93, respectively, which were significantly different from that of their parental culture. After the 11th cycle of UVA+GA treatment and 13th cycle of UVC-GA treatment, the reduction in population became less than 1 log CFU/mL. The repeated exposure to UVA+GA and UVC-GA treatment were stopped at their 16th cycle, when the reduction in population of *E. coli* O157:H7 reached 0.15±0.04 and 0.20±0.25 CFU/mL, respectively. Compared with the parental culture, the reduction in *E. coli* O157:H7 population were reduced from 3.3±0.2 to 0.2±0.1 CFU/mL towards UVA+GA treatment, and 3.8±0.2 to 0.2±0.2 CFU/mL towards UVC-GA treatments, respectively, after the 16 inactivation cycles.

5.3.3 Resistance of wild-type E. coli O157:H7, UVA+GA resistant culture, and UVC-GA resistant culture towards heat, acid, and oxidative challenge

Figure 5-3 shows the profiles of the survival curves of wild type *E. coli* O157:H7, UVA+GA resistant and UVC-GA resistant culture exposed to thermal (**Figure 5-3a**), acid (**Figure 5-3b**), and oxidative (**Figure 5-3c**) challenges. As the figure shows, the inactivation of *E. coli* O157:H7 followed different profiles depending on the type of culture and the challenge. During thermal challenge, the inactivation of *E. coli* O157:H7 keep increasing in the 20 min, when the parental culture was completely inactivated at the end of challenge. The D-values for wild type, UVA+GA and UVC-GA resistant populations by thermal challenge were 4.4±0.7, 9.3±0.3, and 7.5±0.2 min, respectively, which are significantly ($p<0.05$) different from each other. Both UVA+GA and UVC-GA resistant cultures showed increased ($p<0.05$) D-value, indicating increased resistance towards heat challenge. Similar trend was found in the acid challenge experiment, where the D value of wild type *E. coli* O157:H7 treated by acid challenge (13.2±4.4

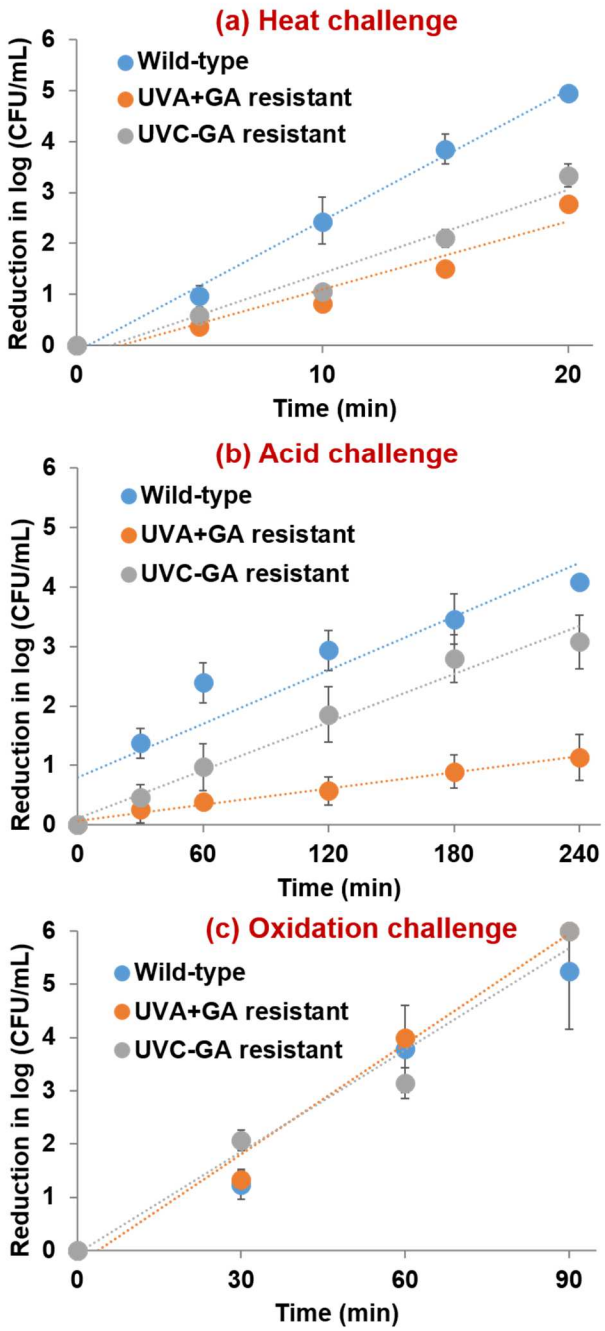

Figure 5-3. Survival of the wild-type *E. coli* O157:H7, UVA+GA resistant culture, and UVC-GA resistant culture after exposure to (a) heat (58 □C), (b) acid (GA, pH 3.1), and (c) oxidation (H_2O_2, 0.1%) challenge. Survival was determined by plate counts prior and after treatment, respectively. Mean ± SD.

min) was that was significantly ($p<0.05$) lower than that of UVA+GA (222.7±77.4 min) or UVC-GA (66.9±13.0 min) resistant cultures. However, there was no significant difference ($p>0.05$) in D-values among the three cultures towards the hydrogen peroxide challenge during the 90 min treatment.

5.3.4 Analysis of SOD activity and acid phosphatase activity

The activity of SOD within wild type, UVA+GA resistant and UVC-GA resistant *E. coli* O157:H7 cultures is shown in **Figure 5-4a**. The UVA+GA resistant culture showed a 1.42±0.07 ($P < 0.05$) higher fold of SOD activity than the wild type, while the UVC-GA culture was 1.34±0.16 fold ($P < 0.05$) higher than the wild type control. There was no significant difference between UVA+GA and UVC-GA resistant cultures in terms of SOD activity. **Figure 5-4b** shows the activity of acid phosphatase of wild type, UVA+GA resistant and UVC-GA resistant *E. coli* O157:H7. Similar to the trend of SOD activity among the three types of cultures, UVA+GA resistant culture showed the highest enzyme activity that it was 1.27±0.03 fold higher than the wild type control. However, there was no significant difference of acid phosphatase activity between wild type and UVC-GA resistant culture (1.06±0.09 fold compared with wild type control).

5.3.5 Membrane integrity

Membrane integrity of *E. coli* O157:H7 of wild type, UVA+GA and UVC-GA resistant cultures before and after corresponding UVA+GA and UVC-GA treatments were analyzed by PI, which is a fluorescent dye can penetrate cell with a damaged membrane (**Figure 5-5**). Before the treatment, the fluorescence intensities were in the same level ($P> 0.05$) among the three types of cultures. After the wild type control and UVA+GA resistant culture received UVA+GA

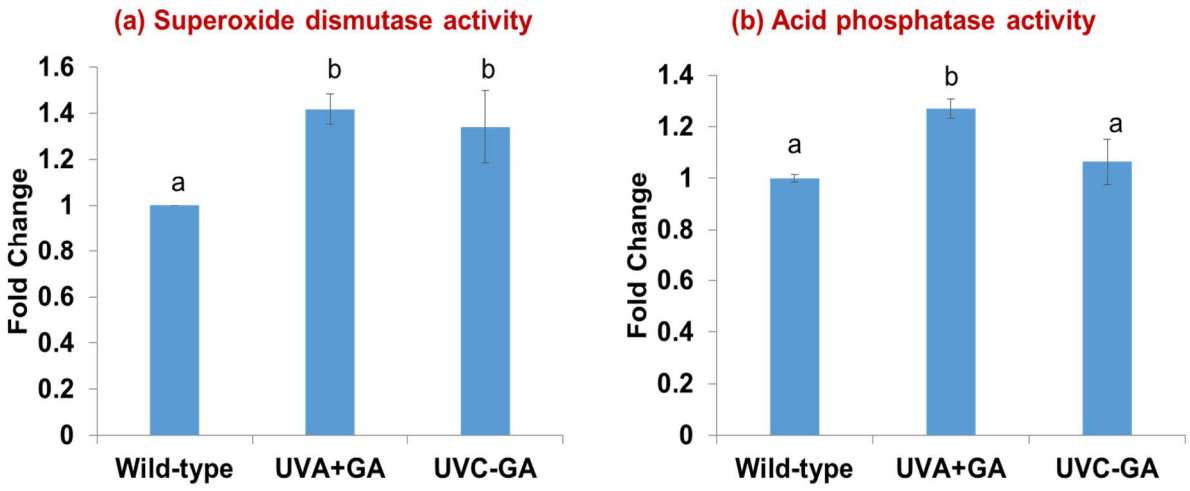

Figure 5-4. Activity of superoxide dismutase (a) and acid phosphatase (b) of the wild-type *E. coli* O157:H7, UVA+GA resistant culture, and UVC-GA resistant culture. Mean ± SD.

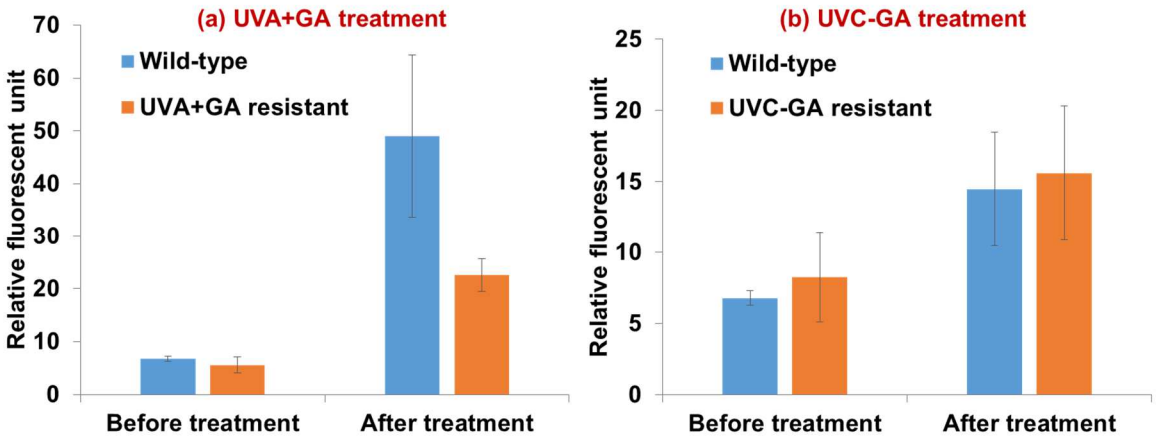

Figure 5-5. Membrane damage as indicated by the fluorescence level of propidium iodide in wild-type *E. coli* O157:H7, UVA+GA resistant culture, and UVC-GA resistant culture, with or without the correspondent (a) UVA+GA and (b) UVC-GA treatments. Mean ± SD.

treatment, the corrected fluorescence of PI of wild type control increased from 6.78±0.52 to 48.97±15.34, while the UVA+GA resistant culture increased from 5.57±1.49 to 22.55±3.11. The difference of relative fluorescence between wild type and UVA+GA resistant culture after the treatment was significant (P <0.05). Increased relative fluorescence intensities were also observed in wild type (from 6.78±0.52 to 14.48±4.02) and UVC-GA resistant culture (from 8.26±3.17 to 15.59±4.72) after treating by UVC-GA. However, the intensity levels were not significantly different (P > 0.05).

5.4 Discussion

Stresses that are either applied during the growth of microorganisms or during processing may trigger adaptive responses in the microorganisms that enhance their survival ability to food processing methods applied subsequently (Mattick, Jorgensen, Legan, Lappin-Scott, & Humphrey, 2000). The characterization of stress adaptation responses in microorganisms is important both scientifically and with respect of food safety. Although the development of cross-resistant considered quite frequent, there is limited information available about the effect of prior shock to microbial cells on their sensitivity to subsequent non-thermal techniques (Scheyhing, Hörmann, Ehrmann, & Vogel, 2004). In the current study, *E. coli* O157:H7 was used as a model Gram-negative bacteria to investigate the stress adaptation responses related to the two recently developed non-thermal inactivation treatment: UVA+GA and UVC-GA. The varying levels of inactivation from UVA+GA and UVC-GA treatments under different pre-adaptation shocks suggest that the cross-stress response was induced in the bacterial cells and the effect was stress specific. Both resistance and sensitivity can be induced depending on the type of prior shock bacteria experienced and the following inactivation treatment (UVA+GA or UVC-GA). This phenomenon has also been observed in other non-thermal technologies when the targeted

microorganisms experienced previous sublethal stresses. For example, one study showed that acidic, oxidative, hyperosmotic and cold pre-adaptation treatment increased the susceptibility of *S. aureus* to pulsed electric field, while heat and alkaline shocks helped to develop the resistance to this treatment (Cebrián, Raso, Condón, & Mañas, 2012).

Results (**Figure 5-1**) in this study demonstrated that previous exposure of *E. coli* O157:H7 to heat and acid resulted in an increase ($p<0.05$) in resistance to UVA+GA inactivation process. This result indicated that both heat and acid shock proteins might be involved in building up UVA+GA tolerance. On the contrary, prior exposure to sodium chloride increased the vulnerability of the bacteria to the UVA+GA treatment. Hydrogen peroxide shock did not provoke any significant change in resistance to the following treatment. In UVC-GA inactivation treatment, previous acid shock actually induced sensitivity ($p< 0.05$) towards following UVC-GA. The similarities and differences of cross-stress responses developed towards UVA+GA and UVC-GA treatment suggest that there might be differences in the mechanism of inactivation of the two inactivation treatments, although they might share some similar targets that can be affected by sublethal stresses such as heat and sodium chloride shocks.

The development of stress tolerance has been generally attributed to the induction of specific groups of proteins. In the cases of cross-stress responses, the development of resistance has been linked either to the induction of the general stress responses, controlled by alternative sigma factor σ^s (RpoS) for Gram-negative bacteria, or to the existence of an overlap between the responses triggered by different stresses (Cebrián et al., 2012; Flahaut, Hartke, Giard, & Auffray, 1997). Heat stress has been found to induce a universal protective response and the cross-protection provided by heat stress has been widely observed (Richter, Haslbeck, & Buchner, 2010). For example, Lou and Yousef examined the effect of sublethal heat (45 °C for 1 h) on the

resistance of *L. monocytogenes* to certain environmental stresses and found that this greatly increased resistance of the pathogen to normally lethal doses of hydrogen peroxide, ethanol, and NaCl, etc. *E. coli* cells exposed to a 42 °C exhibited resistance to a subsequent osmotic shock at 2.5 M NaCl (Lou & Yousef, 1997). As the development of cross-protection responses in *E. coli* has been generally associated with the activation of RpoS and heat shock sigma factor σ^{32} (RpoH), it is possible to assume that the synthesis of heat shock proteins were involved in the stress response development towards UVA+GA and UVC-GA treatments. Our previous studies have found that the generation of ROS and oxidative stress contribute to the antimicrobial activities of UVA+GA and UVC-GA treatments (Wang, Q., Leong, W., Elias, R. J., & Tikekar, n.d.; Wang, Q., Oliveira, E. F., Alborzi, S., Bastarrachea, L. J., & Tikekar, 2017). In addition, it has been suggested that heat shock and oxidative stress are correlated to each other, as heat shock response can be induced by a variety of oxidizing agent such as hydrogen peroxide, while oxidative stress response can be observed in heat shocked cells (Aertsen, De Spiegeleer, Vanoirbeek, Lavilla, & Michiels, 2005; Christman, Morgan, Jacobson, & Ames, 1985). Therefore, this phenomenon indicated that the increased resistance of bacteria towards UVA+GA and UVC-GA treatments after heat shock might be partially due to the generation of heat shock proteins that upregulate the cell protection against oxidative stress exerted by UVA+GA and UVC-GA treatments. The role of heat shock proteins in protecting oxidative stress damage has also been reported in eukaryotic cells (Kalmar & Greensmith, 2009; Liu, Fu, Xu, Wang, & Li, 2015), which support our hypothesis. Nevertheless, it has also been suggested that in some cases the development of stress resistance can occur without the synthesis of proteins but by the changes in the properties of cell components (Scheyhing et al., 2004). The mechanism of the development of cross-resistance of a specific pair of stresses needs to be further investigated. In

contrast to heat shock, acid shock induced resistance and sensitization towards subsequent UVA+GA and UVC-GA treatment, respectively. It might be due to the fact that UVA+GA and UVC-GA treatment had different inactivation targets of the bacterial cell. For example, the exposure to acid shock can induce the change of bacterial membrane such as the composition of fatty acid, which leads to the modified permeability of the cell to the environment. As a result, the uptake of GA by the bacterial cell, which was identified as a necessity for UVA+GA treatment to be effective, decreased. On the contrary, the uptake of GA does not a significant factor in the effectiveness of UVC-GA treatment. Therefore, the physiological changes induced by acid shock may lead to damage or protection of the targets and result in the opposite effect towards UVA+GA and UVC-GA treatment. Further work is required to verify this hypothesis and to elucidate the mechanisms behind the cross-resistance.

Not only resistance but also sensitization can be induced by the exposure to prior sublethal treatment. Results in this study found that sodium chloride shock induced sensitivity of *E. coli* O157:H7 towards UVA+GA and UVC-GA subsequent treatment. Our previous studies found that membrane damage to bacterial cell is highly associated with the inactivation process for UVA+GA treatment. It is also known that cytoplasmic membranes are a primary site of injury during an osmotic stress such as induced by sodium chloride (Mille, Beney, & Gervais, 2002). Therefore, it might be inferred that the increased vulnerability of *E. coli* O157:H7 towards UVA+GA treatment was due to the enhanced level of membrane injury caused by the previous sodium chloride shock. The increased sensitivity towards non-thermal treatment has also been reported in previous study. For example, *E. coli* was found to be more vulnerable to non-thermal plasma with prior adaptation to osmosis and oxidation (Liao et al., 2018). Such stress

combinations could be applied for the achievement of more effective inactivation in in hurdle technologies.

When bacteria were treated by UVA+GA or UVC-UV treatment with a low intensity, after many cycles of process the survivors progressively developed resistance towards the two treatments that were selectively exerted on them (**Figure 5-2**). The cross-protection phenomenon was also observed in such *E. coli* O157:H7 that have developed resistance to UVA+GA or UVC-GA treatment by the selective enrichment towards the two treatments, respectively (**Figure 5-3**). Consistent with previous observation, cells that acquired resistance to UVA+GA or UVC-GA treatment also showed increased resistance to heat at lethal level (**Figure 5-3a**). This phenomenon supports the assumption that *E. coli* O157:H7 might share some of the defense response pathways towards heat and the UVA+GA/UVC-GA treatment and similar heat response proteins might be produced. Population that are resistant to UVA+GA/UVC-GA treatment also increased the resistance to challenge by organic acid (GA solution, pH 3.1) (**Figure 5-3b**). UVA+GA/UVC-GA resistant cultures did not show adaptive response towards oxidative stress (0.1% hydrogen peroxide) in 90 min (**Fig 5-3c**). However, the consequences of adaptive responses towards oxidative stress vary with types and concentrations of ROS species that bacteria exposed to (Fang, Frawley, Tapscott, & Vázquez-Torres, 2016). Therefore, we may not be able to simply conclude that UVA+GA/UVC-GA treatment has no effect on stress response of oxidative challenge.

The increased enzyme activities of UVA+GA and UVC-GA resistant cultures (**Figure 5-4**) may be explained by the activation of intracellular response under stress conditions. Increased activity of SOD in both UVA+GA and UVC-GA resistant cultures indicated the involvement of ROS in triggering the adaptation response towards UVA+GA and UVC-GA treatments. It is known that

SOD helps to catalyze the intracellular superoxide radical into oxygen and water, which can prevent oxidative damage (Rani & Singh Yadav, 2015). In our previous studies, it has been found that ROS plays an important role in both of the UV and GA based treatments. In addition, SOD activity was observed to be inhibited by UVA+GA simultaneous treatment, probably due to the large amount of ROS generated during the inactivation process. During the selective adaptation towards the UV and GA based stresses, bacteria may have developed adaptive response to defend the ROS generated by the UV and GA interactions. Thus, the increased SOD activity may be one of the defense mechanisms towards oxidative stress. A study by Bore et al. founded that acidic treatment (pH 4.5) induced *S. aureus* with the upregulation of a serial of significant genes for oxidative stress response, including SOD gene *soda* (1.78 fold), the catalase gene *kata* (2.41 fold), and alkyl hydroperoxide reductase gene *ahpC* (2.31 fold) (Bore, Langsrud, Langsrud, Rode, & Holck, 2007). Therefore, genes related to ROS defense enzyme might be involved in the development of resistance towards UVA+GA and UVC-GA treatments. Acid phosphatase activity is an dependent phenotypical marker of RpoS, which is the master regulator of general stress responses (Dong, Yu, & Schellhorn, 2011). In Gram-negative bacteria such as *E. coli*, RpoS controls the expression of more than 50 genes involved in the general stress response, and can be induced by several different stresses including nutrient starvation, osmotic stress, heat, acid stress, and oxidative stress (Rowbury, 2003). Increased activity of acid phosphatase indicated that the gene *rpoS* also contributed to the adaptive response towards UVA+GA treatment, which is consistent with our previous assumption. This result was also consistent with one previous study that both elevated level (1.65 fold) of acid phosphatase and gene *rpoS* activity was found in selected high pressure and heat resistant culture of *E. coli* O157:H7 (Vanlint, Rutten, Govers, Michiels, & Aertsen, 2013).

5.5 Conclusion

In this study, we found that common stresses encountered during food processing and environment could confer cross-stress response in *E. coli* O157:H7 towards heat, acid, and osmotic challenge. Results showed complexity of adaptive responses in that both resistance and sensitization can be induced depending on the stress applied and the treatment studied. Repeated exposure to UVA+GA or UVC-GA treatments can also select for sub-population that demonstrates higher resistance towards these treatments as well as higher cross-resistance to other lethal stress such as heat and acid. During the development of resistance towards UV and GA based treatments, enzymes that scavenge ROS and their corresponding genes might be involved. In addition, gene *rpoS*, the master regulator of the general stress response, is highly likely to be associated with the adaptive response process. Results of this study also imply that different cross-protection adaptations of pathogens should be considered when assessing the microbial safety of different combinations of non-thermal food processing technologies.

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Chapter 6: Overall conclusions and future work

6.1 Overall conclusions

The synergistic interaction of UV-A light and GA was affective against *E. coli* O157:H7, and the antimicrobial mechanism of this UVA+GA treatment is as follows: GA by itself is a mild antimicrobial and has a pro-oxidant ability. The presence of UV-A light increases the uptake of GA. Once GA is internalized, the interaction between GA and UV-A directly or indirectly induces intracellular ROS formation, leading to oxidative damage. Concurrently, the activity of ROS defending enzyme, such as SOD, is also inhibited, magnifying the oxidative damage to *E. coli* O157:H7. Other than oxidative stress, the acidification effect of GA and membrane damage of UV-A is also associated with the inactivation of *E. coli* O157:H7. It is also plausible that these combinations of stresses may have an impact on the bacterial DNA and metabolism. These complimentary stresses affect various aspects of cell metabolism and structure, and ultimately lead to the death of bacteria. PG showed a stronger antimicrobial activity in the presence of UV-A light than UVA+GA. In addition to the generation of oxidative stress, a higher level of bacterial membrane damage was responsible for the antimicrobial effect of UVA+PG treatment.

UV-C exposed GA exhibits enhanced antimicrobial activity against *E. coli* O157:H7 compared to GA that was persistent for at least 4 weeks of storage at room temperature. The antimicrobial activity was affected by solution pH and the wavelength of UV-C exposure. The generation of ROS during UV light exposure, and photo-oxidized compound of GA such as quinone contributed to the antimicrobial activity of the post-irradiated GA solution. The effectiveness of inactivating bacteria on the surface of tomatoes showed that this UVC-GA has the potential to be

applied as a novel antimicrobial in food systems, especially when sustained antimicrobial activity is required.

The UVA+GA treatment can enhance the inactivation of inoculated bacteria on baby spinach leaves as compared with controls. UVC-GA treatment also showed inactivation efficacy against *E. coli* O157:H7 inoculated on the surface of grape tomato and spinach leaves. In addition, UVA+GA and UVC-GA treatments affect the color, firmness, and visual quality of baby spinaches and grape tomatoes. Based on the relatively low cost of GA, its efficacy and its GRAS status, the two GA and UV light-based treatments may be good natural alternative to chlorine based antimicrobial agents.

Common stresses encountered during food processing and environment could confer complex cross-stress response in *E. coli* O157:H7 towards UVA+GA and UVC-GA treatments, that both resistance and sensitization can be induced depending on the stress applied and the technology studied. Repeated exposure to UVA+GA or UVC-GA treatments can also select for sub-population that demonstrates higher resistance towards these treatments as well as higher cross-resistance to other lethal stress such as heat and acid. During the development of resistance towards UV and GA based treatments, enzymes that scavenge ROS and their corresponding genes might be involved. In addition, gene *rpoS*, the master regulator of the general stress response, is highly likely to be associated with the adaptive response process. Results of this study also imply that different cross-protection adaptations of pathogens should be considered when assessing the microbial safety of different combinations of non-thermal food processing technologies.

6.2 Future work

For future work, a better understanding of inactivation mechanism of both UVA+GA and UVC-GA treatments to further enhance the efficacy of these two novel techniques is necessary. Also, the fate of GA after UV exposure is still not clear. Further research needs to be conducted to analyze the degradation compounds of UV light exposed GA using higher sensitivity instrumentation and tie the finding to the mechanism of action of UVC-GA treatment.

In addition, further research is required to determine the optimal processing parameter of UVA+GA and UVC-GA treatments to reduce the treatment duration of fresh produce. More types of food samples need to be investigated for the two treatments. More studies are also required to assess the impact of our findings in industrial settings.

Lastly, the development of adaptive response in bacteria related to UVA+GA and UVC-GA needs further investigation. Correlating microbial stress responses to key genes, regulators and pathways at molecular levels through genome-wide transcriptome analysis to reveal the potential mechanisms of stress responses is necessary. The identified molecular targets during a specific stress response can then be useful for developing strategies for impeding adaptive response and increasing the effectiveness of antimicrobial treatment.

Appendix A: Evaluating the generation of hydrogen peroxide from GA on exposure to UV-A light

Methods

The generation of hydrogen peroxide was investigated by ferrous ion oxidation xylenol orange (FOX) method with some modifications (Jiang, Woollard, & Wolff, 1990). It is based on the ability of hydrogen peroxide to convert ferrous ions into ferric ions which can form a complex with xylenol orange (XO), the concentration of which is determined using spectrophotometry. GA solutions were prepared by dissolving 0.5 mM GA in distilled water (pH 3.3) or phosphate buffer (100 mM, pH 7.4). DI water without GA was used as control. An aliquot (5 mL) of each test solution was transferred to a crystallizing dish (KIMAX®, NJ, USA) and exposed to UV-A light or incubated in dark for 15 min. Samples were obtained periodically for hydrogen peroxide measurement. FOX assay reagent of 50 µL containing 1 mM xylenol orange, 2.5 mM ferrous sulfate, 1 M sorbitol,
and 250 mM sulfuric acid was added to 350 µL samples, followed by incubation at room temperature for 30 min. After that, the absorbance of solutions was measured at 560 nm using a Spectroscopy M5e plate reader (Molecular Devices LLC, Sunnyvale CA). Hydrogen peroxide concentrations were determined based on an external standard curve (Supplementary figure 2).

Results and Discussion

Figure A1 shows the generation of hydrogen peroxide from GA solution in natural or modified neutral pH, in the presence or absence of UV-A light. In 5 min, GA in pH 7.4 solution in presence UV-A light generated significantly ($P<0.05$) higher amount of hydrogen peroxide than other samples did, followed by GA in neutral pH incubated in dark. GA in pH 3.3 with UV-A

light exposure also generated more hydrogen peroxide than GA without UV-A. Water by itself did not generate hydrogen peroxide with or without UV-A. This result suggests neutral pH environment enhance autoxidation process of GA and more hydrogen peroxide is generated. This is consistent with previous studies that autoxidation of GA proceeds and behaves differently with higher pH values (Eslami, Pasanphan, Wagner, & Buettner, 2010; Nikolić, Veselinović, Mitić, & Živanović, 2011). Our result also suggests that UV-A light increased the amount of hydrogen peroxide generated by GA, probably due to oxidation.

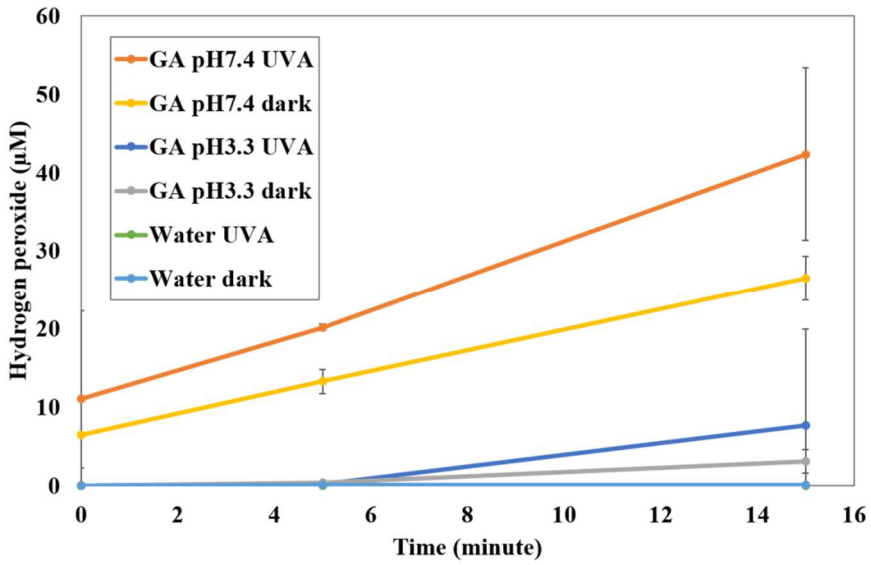

Figure A1. Generation of hydrogen peroxide from GA (0.5 mM) in DI water or phosphate buffer (100 mM, pH 7.4), in the presence or absence of UV-A light exposure. Mean ± SD.

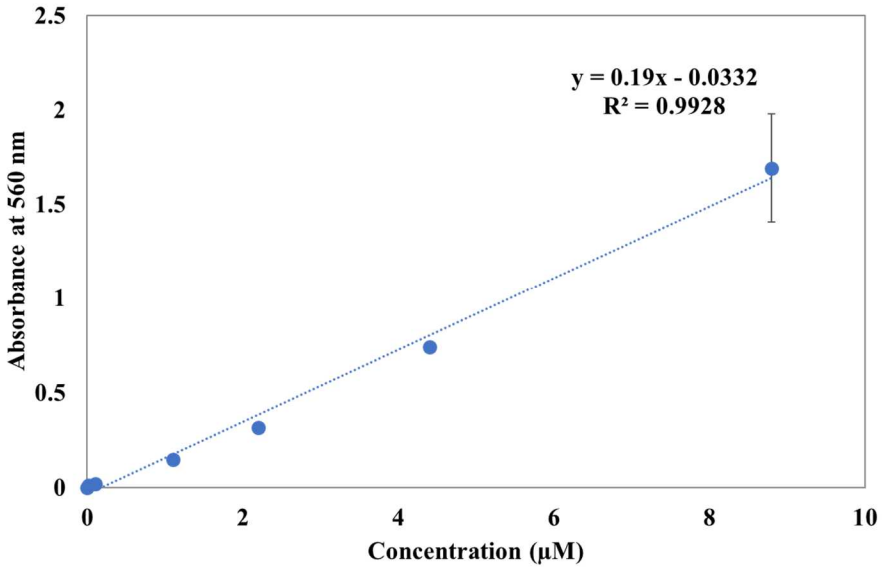

Figure A2. FOX assay standard curve for hydrogen peroxide measurement. Hydrogen peroxide was serial diluted at room temperature (24 °C) and the absorbance was measured by UV-Vis spectrophotometer.

Appendix B: Effect of metal ions on the antimicrobial activity of GA and Py solution

Methods

The antimicrobial activity of GA or Py with or without the presence of metal ions was evaluated. Ferric chloride was added into 15 mM GA or Py water solution to reach a concentration of 2.5 mM. This GA+Fe or Py+Fe solution was then treated against stationary phase culture *E. coli* O157:H7 with an initial concentration of approximately 7 log CFU/mL for 30 min. To evaluate if UV light exposure enhance the antimicrobial activity of GA+Fe, the freshly made GA+Fe solution was also under exposure of UV-C light for 30 min before treating against bacteria. Bacteria was also exposed to Ferric chloride (2.5 mM) alone as control.

Results and Discussion

When treated against *E. coli* O157:H7 by itself, GA did not show antimicrobial activity (< 1 log CFU/mL). However, the presence of 2.5 mM ferric ions in GA solution resulted in 1.8 log CFU/mL reduction in population, while ferric ions by themselves at the same concentration did not show any effect (< 1 log CFU/mL). Interestingly, when the solution pH of GA was adjusted from its natural pH (pH 3.1) to pH 8, the antimicrobial activity was not observed (< 1 log CFU/mL). Since it has been founded that UV-C light enhances the antimicrobial activity of GA, it was assumed that the UV-C exposure have similar effect on the GA+Fe solution. However, UV-C post-irradiated GA+Fe solution only inactivated 0.8 log CFU/mL of population, which was less effective than that of GA+Fe solution without UV-C light exposure. It was also interesting to notice that Py+Fe solution resulted in more than 5 log CFU/mL reduction of bacterial population in 30 min, while Py solution did not show antimicrobial activity by itself. This observed enhanced antimicrobial activity of GA/Py with ferric ions might be due to the

Fenton reaction in the system that generates ROS, which caused oxidative damage to the bacteria. It has been reported that GA forms complex with ferric ions under acidic conditions with blue color (Hynes & Ó Coinceanainn, 2001). The color change of the solution was also observed in the current experiments. Previous studies have also shown that the presence of metal ions such as Ferric ions speeds up the oxidation reaction of phenolic compounds such as pyrogallol, catechol, and gallic acid to the corresponding semiquinones and *o*-quinone by the dissolved oxygen (Al-Abadleh, 2015). The formation of quinone species is also accompanied by the redox cycle in that ferric ions was reduced to ferrous ions, which undergoes autoxidation back to ferric ions by the dissolved oxygen in water and stabilized by complex formation with phenolic molecules. The generation of quinone might also contribute to the antimicrobial effect of GA/Py+Fe solution.

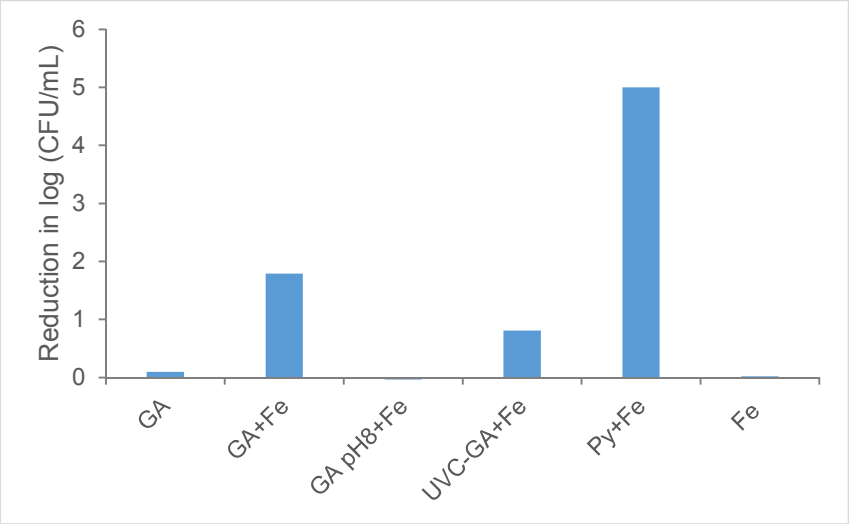

Figure B: Effect of ferric ions on the antimicrobial activity of GA and Py against *E. coli* O157:H7

Appendix C: Antimicrobial activity of Py and PG after UV-C light exposure

Methods

GA and its two derivatives, Py and PG was evaluated for their antimicrobial activities after the exposure under UV-C light for 30 min against *E. coli* O157:H7. The bacterial culture was stationary phase with an initial concentration of approximately 7 log CFU/mL. The treatment duration of bacteria in the selected solutions was 30 min. The reduction in population was calculated based on using DI water as a control. BSA was added into the solution as a quinone scavenger to evaluate if such photo-oxidation compounds was formed during the exposure of UV-C light and was involved in the antimicrobial activity.

Results and Discussion

GA after UV-C light exposure showed enhanced antimicrobial activity as previously reported. UV-C light exposed PG showed a higher antimicrobial activity than that of GA that more than 5 log CFU/mL reduction in population was achieved. Py did not show any antimicrobial activity after UV-C exposure. Unlike UVC-GA, adding BSA into UVC-PG solution did not quench the antimicrobial activity of UVC-PG that the difference in the bacterial reduction with or without BSA was less than 1 log CFU/mL. Therefore, it was inferred that the formation of quinone contributed to the antimicrobial activity of UVC-GA, while it was not a driving force for UVC-PG solution. UVC-PG might has a different antimicrobial mechanism than that of UVC-GA.

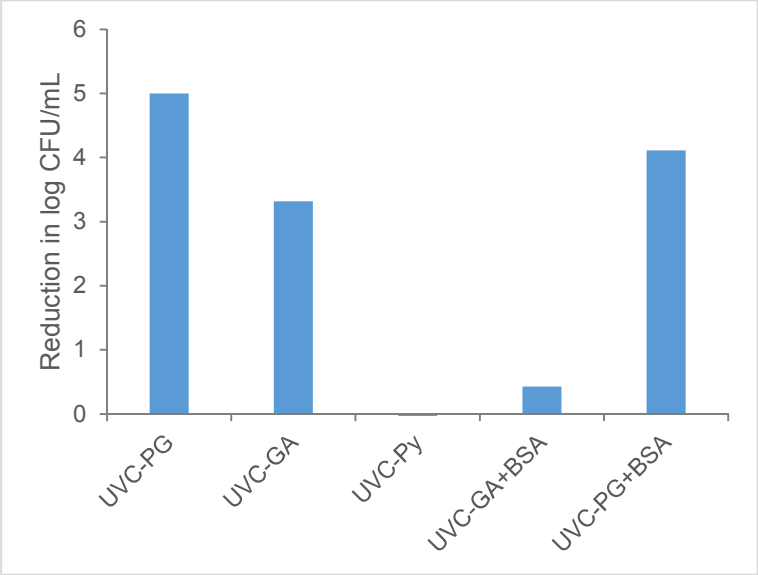

Figure C: Antimicrobial activity of GA, Py and PG after UV-C light exposure against *E. coli* O157:H7.

Appendix D: Effect of polyphenol oxidase on antimicrobial activity of UVC-GA

Methods

To investigate if the enhanced antimicrobial effect of GA under UV-C treated was a result of oxidation to quinone, we incubate GA with tyrosinase, a type of polyphenol oxidase. Tyrosinase (Worthington Biochemical ™) was added to GA solution of 15 mM to reach a concentration of 268 U/mL. The solution was incubated overnight at room temperature and centrifuged to remove tyrosinase to stop the reaction. The supernatant was transferred and treated against *E. coli* O157:H7 for 30 min.

Results and Discussion

Figure D shows that GA oxidized by tyrosinase caused 0.75±0.20 log CFU/mL reduction, while UV-C treated GA caused 2.81±0.28 log CFU/mL reduction of *E. coli* O157:H7. Therefore, the mechanism of antimicrobial activity of GA under UV-C cannot be merely attributed to the oxidation of GA to quinone. Other transformation products of GA during UV-C exposure might be responsible for the enhanced antimicrobial. In addition, the optimal pH range for tyrosinase activity is pH 6-7. It might be that the activity of tyrosinase in the GA solution (pH 3.1) was too low to be effectively oxidizing GA to quinone.

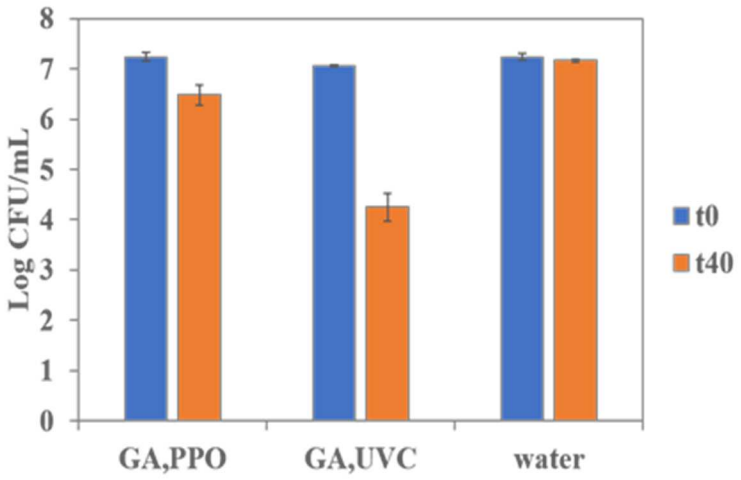

Figure D: Antimicrobial activity of GA (15 mM) treated by polyphenol oxidase or UV-C light against *E. col*i O157:H7.

Appendix E: Antimicrobial activities of UV light treated GA against *E. coli* O157:H7 or *Listeria innocua* at exponential phase

Methods

Shiga toxin negative *E. coli* O157:H7 (ATCC #700728, Manassas, VA) and *Listeria innocua* were model bacteria representing Gram-negative and Gram-positive pathogens respectively. To achieve exponential phase cultures, the bacteria were cultured overnight for 15 h in TSB at 37 °C. Then, the culture was inoculated into a new TSB broth, incubated at 37 °C for 2 h (*E. coli* O157:H7) or 5 h (*Listeria innocua*) to reach mid-exponential phase of each bacteria. To determine the antimicrobial efficacy of selected treatment against bacteria, the bacteria were diluted and the suspensions were incubated in solutions with selected compounds. After incubation, the bacterial suspension was serially diluted in buffer peptone water and plated on TSA. The number of CFU was determined by plate counts after incubation at 37 °C for 24 h (*E. coli* O157:H7) or 48 h (*Listeria innocua*) in triplicates.

Results and Discussion

Figure E shows the amicrobial activity of UV-C irradiated GA (5 mM) against mid-log phase *E. coli* O157: H7 and *Listeria Innocua*. After incubating for 30 min in UV-C irradiated GA solution, mid-log phase *E. coli* reached a reduction of 3.9±0.5 log CFU/mL in population, while GA solution without UV-C exposure achieved 1.1±0.2 log CFU/mL of reduction. On the contrary, *Listeria Innocua* at mid-log phase has only less than 1 log CFU/mL of reduction, regardless of the UV light exposure on GA. DI water as a control did not have any ($P > 0.05$) antimicrobial

activity towards either *E. coli* O157:H7 or *Listeria Innocua.* Results indicated that *E. coli* O157:H7 at exponential phase were more sensitive than at stationary phase to antimicrobials. In addition, it is inferred that *Listeria innocua* was more resistant to UV-C exposed GA than that of *E. coli* O157:H7.

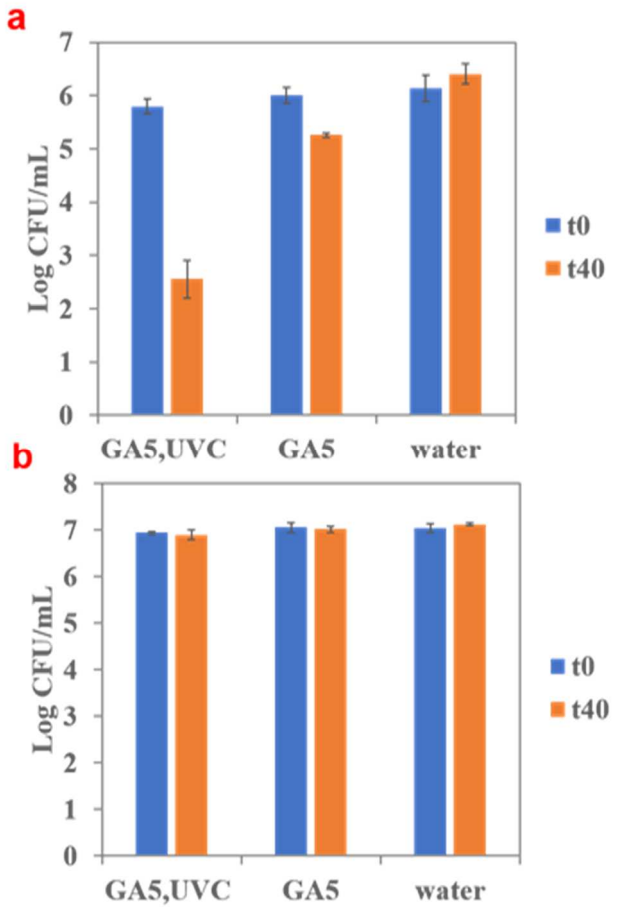

Figure E: Antimicrobial activities of UV-C light exposed GA (5 mM) against (a) *E. coli* O157:H7 and (b) *Listeria innocua* at exponential phase.

Appendix F: The possibility of reversion of resistance of UVA+GA/UVC-GA resistant culture

Methods

Cultures of UVA+GA and UVC-GA resistant *E. coli* O157:H7 as described previously were cultured overnight for 20 h in fresh TSB at 37 °C. Then, the two types of cultures were evaluated for their resistance towards UVA+GA or UVC-GA treatments, respectively. If the resistance towards the UVA+GA or UVC-GA treatments remained after overnight incubation in TSB, the culture was sub-cultured again into fresh TSB and incubated in the same condition overnight. This incubation was repetitively performed until the resistance of the cultures were reverted back to the wild-type culture.

Results and Discussion

It was observed that both UVA+GA and UVC-GA resistant cultures reverted back to original culture in regards of their resistance towards UVA+GA or UVC-GA treatment within 5 cycles of passages in fresh TSB medium. This observation indicated that the acquired resistance towards repetitive exposure to moderate UVA+GA and UVC-GA treatments was not due to the genetic mutation of bacterial DNA. It might be the temporary change in gene expression during the exposure of UVA+GA or UVC-GA treatment that leads to the development of resistance towards the two treatments.

Appendix G: SDS-PAGA analysis of cellular protein of original, UVA+GA, and UVC-GA resistant cultures of *E. coli* O157:H7

Methods

The cell extract was prepared by transferring 1 mL sample to an Eppendorf tube, centrifuged at 10,000 ×g for 2 min and washed with DI water, and resuspended in 1 mL HEPES buffer (20 mM, pH = 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). Then, 500 µL of suspension was transferred to a new Eppendorf tube containing approximately 400 µL of silica beads, followed by centrifugation at 1,500 ×g for 5 min at 4 °C. An amount of 20 µL of each supernatant was collected and mixed with 5 µL of bromophenol blue. Then, the sample was heated at 100 °C for 2 min and cooled down to room temperature. The same quantity of extracted protein (10 mg) was loaded onto a 10% polyacrylamide gel gradient in standard Tris/glycine chamber buffer (0.025 M Tris-HCl, 0.129 M glycine, 0.1% SDS) and separated with a mini-vertical electrophoresis (Bio-Rad) at 100 V for 2 h. Broad Range (6.5–200 kDa; Bio-Rad) was used as a marker for molecular mass. The gels were stained with Coomassie blue (2 mM Coomassie Brilliant R-250, 45% methanol, 10% acetic acid) for 1 h and destained with a solution containing 20% methanol and 10% acetic acid.

Results and Discussion

The image of the gel (**Figure G**) shows the SDS-PAGE pattern of cellular proteins from original *E. coli* O157:H7 and UVA+GA/UVC-GA resistant cultures. Compared with the protein expression in the original culture, the two types of resistant cultures exhibited an increased

expression level of protein in the molecular weight range of 52-79 kDa, indicating the presence of heat shock proteins (Hsps) induced in the resistant bacteria in response to the UVA+GA and UVC-GA treatments. The expression of Hsps in the resistant cultures were consistent with our previous finding that cross-protection was observed in UVA+GA and UVC-GA resistant cultures. The generation of Hsps could be a way that the cell adapt to the unfavorable UVA+GA/ UVC-GA stress. This phenomenon is linked to the activation of the genes responsible for encoding these proteins. However, further analysis such as Western blot are needed to identify the Hsps induced by UVA+GA and UVC-GA treatments.

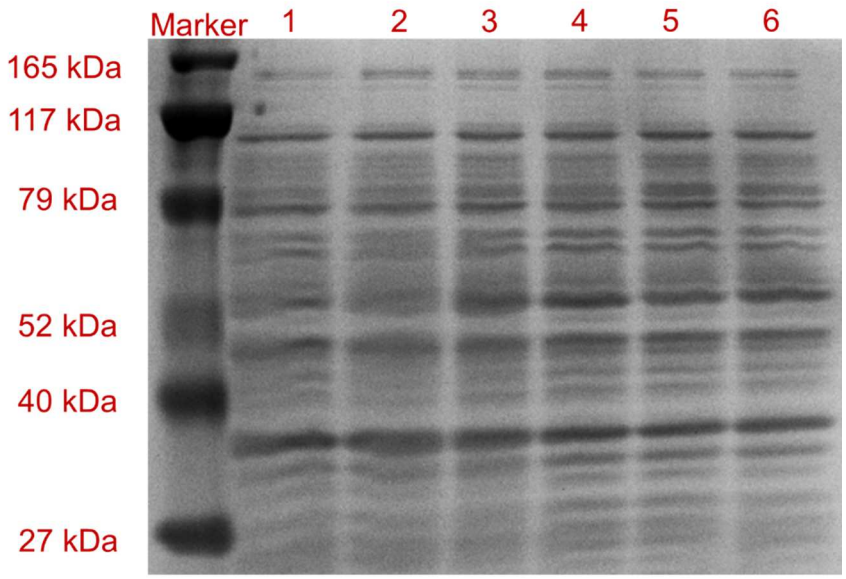

Figure G: SDS-PAGA analysis of cellular protein of original, UVA+GA, and UVC-GA resistant cultures of *E. coli* O157:H7. Lanes 1&2, original culture; lanes 3&4, UVA+GA resistant cultures; lanes 5&6. UVC-GA resistant cultures.

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