ABSTRACT

Title of Thesis: THE ANTIMICROBIAL EFFECT OF BENZOIC ACID OR PROPYL PARABEN TREATMENT COMBINED WITH UV-A LIGHT ON *ESCHERICHIA COLI* O157:H7

Qiao Ding, Master of Science, 2017

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The antimicrobial effect of the combined UV-A light and benzoic acid (BA) or propyl paraben (PP) treatment was evaluated using Escherichia Coli O157:H7. Factors affecting the efficacy of antimicrobial treatments were examined through various microbial and biochemical approaches. A combined 15 mM BA and UV-A treatment exhibited more than 5 log (CFU/mL) reduction in antimicrobial activity via production of reactive oxygen species (ROS), membrane damage and decreasing intracellular pH. Similarly, the combined 3 mM PP and UV-A treatment also caused more than 5 log reduction contributed by membrane damage. UV-A and BA treatment was also found to be effective in a scaled-up, continuous system, while a combined UV-A and PP was able to significantly reduce the likelihood of cross-contamination in simulated fresh produce washing study. The findings from this study

have revealed the potential for the combined treatments that help to improve the safety and quality of fresh produce.

THE ANTIMICROBIAL EFFECT OF BENZOIC ACID OR PROPYL PARABEN TREATMENT COMBINED WITH UV-A LIGHT ON *ESCHERICHIA COLI* O157:H7

by

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Dedication

This thesis is dedicated to my parents, Zunian Ding and Haiying Xiao, who raised me with all the affections and love. I also want to dedicate it to Dr. Rohan Tikekar, who has showed me how to become a dedicated researcher. I also want to give special thanks and dedicate this work to Dr. Solmaz Alborzi and Dr. Luis Bastarrachea for selflessly providing crucial assistance and enthusiastic encouragement during my study. And finally, to all of my friends, without whom none of this would be possible.

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List of Abbreviations

BA: Benzoic Acid

PP: Propyl Paraben

UV light: Ultraviolet Light

EDTA: Ethylenediaminetetraacetic acid

ROS: Reactive Oxygen Species

CFU: Colony Forming Unit

pH_i: Intracellular pH

Chapter 1: Literature review

1.1 Significance, rationale, and hypothesis

Escherichia coli O157:H7 has become a global concern to public health since its first known hemorrhagic colitis outbreak took place at 1982 (Griffin & Tauxe, 1991a). This pathogen is well known for its low infectious dose, unusual acid tolerance, ability to affect all age groups, and their severe infection consequences (Robert L. Buchanan & Doyle, 1997). It may produce Shiga toxins (Stx1 and /or Stx2), leading to hemolytic uremic syndrome in human (Robert L. Buchanan & Doyle, 1997). *E coli* O157:H7 has been identified in a broad spectrum of food products including both processed and raw materials (Burt & Reinders, 2003)(Robert L. Buchanan & Doyle, 1997). Frequent presence of *E. coli* in irrigation water used for growing fresh produce has resulted in several fresh produce related outbreaks (M.-L. Ackers et al., 1998; Gelting, Baloch, Zarate-Bermudez, & Selman, 2011; Solomon, Pang, & Matthews, 2003) and prompted U.S. Food and Drug Administration (FDA) to include stricter requirements for the microbiological quality of irrigation water through its produce safety rule (U.S. Food and Drug Administration, 2016b). Thus, there is now a need to develop a scalable, low-cost intervention technique that can lower the *E. coli* levels in irrigation water. In diverse liquid food products such as juices where *E. coli* is commonly found, it is inactivated through pasteurization (C. S. Chen, Shaw, & Parish, 1993). Although effective, these methods typically involve the use of heat, which may induce quality defects in some products including loss of nutrients and change of flavor profile (Vega-Mercado et al., 1997). Traditionally, it is inactivated through pasteurization or cooking, but these methods all involve the use

of heat, which may induce quality defects in some products including loss of nutrients and changing of flavor profile. Thus, there is also a need to develop an effective, non-thermal technique that can address the *E. coli* issue in liquid food products while retaining its quality.

Ultraviolet (UV) light has been defined as the radiation with wavelength at the invisible region from 200 to 400 nm on the electromagnetic spectrum, which can be further categorized into UV-C (200–280 nm), UV-B (280–320 nm) and UV-A (320–400 nm) (Pattison & Davies, 2006). Among them, the use of UV-C irradiation as a non-thermal disinfection method has long been established (Char, Mitilinaki, Guerrero, & Alzamora, 2010; Franz, Specht, Cho, Graef, & Stahl, 2009; Sutton, Yu, Grodzinski, & Johnstone, 2000), which has been proved to be effective on inactivating pathogens including *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* through the formation of pyrimidine dimer between adjacent pyrimidine molecules on the same strand of DNA, leading to interruption on both DNA transcription and translation (Duffy, Churey, Worobo, & Schaffner, 2000; Franz et al., 2009; Krishnamurthy, Demirci, & Irudayaraj, 2007; Matak & Churey, 2005). More applications of UV-C treatment has been implemented on the production of fresh produce and multiple liquid products including fruit juice and milk after the recognition of UV-C light as an alternative technology to the traditional thermal pasteurization of fresh juice products by the FDA (Duffy et al., 2000; Escalona, Aguayo, Martínez-Hernández, & Artés, 2010; Koutchma, 2008; Matak & Churey, 2005; U.S. Food and Drug Administration, 2000a). However, the performance efficiency of ultraviolet treatment is highly correlated to the UV

transmittance of the liquid (Koutchma, 2008). According to the Beer-Lambert law, the absorption of light depends on the wavelength of the light source and the concentration of the absorbing substance in juice. Except for clean water, UV-C light can only reach a very short penetration depth through the surface of liquids (Shama, 1999). It has been suggested that 90% of the UV-C light was absorbed while penetrating the first millimeter of depth in juice (Sizer & Balasubramaniam, 1999). Therefore, the efficacy for UV-C treatment can sometimes be limited by its high absorbance in liquids. However, due to its long wavelength nature, the transmittance of UV-A light in juice is much higher than other shorter wavelength lights. UV-A light is also the dominant component (95%) of the solar radiation on Earth' surface atmosphere (Pattison & Davies, 2006), which makes the irradiation source readily available.

One of the major drawback of UV-A is that it cannot inactivate microorganism effectively by itself (Guerrero-Beltran & Barbosa-Canovas, 2004). It has been reported that the germicidal effect of UV light becomes negated above 300 nm (Bachmann, 1975). Therefore, finding another treatment to apply additional to target microorganism may be able to increase the potential for UV-A to become an applicable non-thermal treatment. Benzoic acid (BA) is a lipophilic organic compound that is used widely as a major food preservatives in the food industry since its first approval by the FDA at 2000 (Chipley, 2005) (Liu, Yousef, & Chism, 1996). Being classified as a GRAS preservative, BA has been permitted to be used in food production for a maximum level of 0.1% (U.S. Food and Drug Administration, 2016a).

The antimicrobial activity of these acids is related to the transfer of their undissociated forms into the microbial cell (Salmond, Kroll, & Booth, 1984). As a weak organic acid preservative, the disinfection effect of BA can be determined by its capacity to lower intracellular pH as well as the delocalization of negative charged ions inside bacterial cells that increases membrane mobility (Chipley, 2005) (Salmond et al., 1984). However, in most studies, the antimicrobial effect of BA was observed over a long period (1 to 15 days) (Ceylan, Fung, & Sabah, 2004; T Zhao, Doyle, & Besser, 1993a), which makes BA less practical to be used as an effective antimicrobial agent by itself during food production process. Moreover, although the undissociated form of BA can inhibit yeast and fungi at concentrations of 0.05% and 0.1%, which makes it an excellent antifungal agent (Davidson, Sofos, & Branen, 2005) (Rusul & Marth, 1988), the effectiveness for BA by itself as an antimicrobial treatment against some pathogenic bacteria in food is questionable. Studies conducted on *Listeria monocytogenes* have led to a finding that BA at concentration of around 1000 to 3000 ppm expressed strong bacteriostatic, but low bactericidal activities against the pathogen (El-Shenawy & Marth, 1988; Yousef, El-Shenawy, & Marth, 1989).

Another antimicrobial preservative, parabens, have also been known for its antimicrobial activity. As an ester of p-hydroxybenzoic acid, the alkyl chain of paraben makes it less dependent on the environment pH when compared to BA, which can be a huge advantage for it to be used in food and cosmetic products where the pH in the product is relatively higher than the working pH range of BA (Davidson et al., 2005). It has been proved to be fungicidal but less effective against pathogenic

bacteria species (Davidson et al., 2005). The antimicrobial activity of parabens have not been well explored, although some previous studies have suggested that it may induce membrane damage in treated cells (Bargiota, Rico-Munoz, & Davidson, 1987; Fukahori, Akatsu, Sato, & Yotsuyanagi, 1996). The long alkyl chain also limited the solubility of parabens in water, which serve as another major drawbacks for parabens to be incorporated in food products as a antimicrobial agent (Davidson et al., 2005).

By combining two or more antimicrobial factors, it has been proved that a synergistic antimicrobial effect could be generated with less requirement for energy input and treatment intensities (Ross, Griffiths, Mittal, & Deeth, 2003). The finding leads to the concept of hurdle technology, which uses a combined preservation factors to achieve synergistic interactions between traditional and advanced preservation treatments to produce additive or even synergistic antimicrobial effects with minimal impact on the texture, flavor, and nutrient profile of foods (Leistner, 1992; Ross et al., 2003). When using the combination between non thermal processing and acidification with antimicrobial organic compound, it has been found that bacterial cells are likely to suffer from stresses from the loss of membrane and other cell functionality combined with intracellular pH changes (Vega-Mercado, Pothakamury, Chang, Barbosa-Cánovas, & Swanson, 1996). Therefore, it has been hypothesized in this study that the combined treatment between UV-A light and benzoic acid or propyl paraben treatment could induce significant antimicrobial effect at an optimized condition by generating a combination of stresses inside pathogenic bacteria. *Escherichia coli* 0157:H7 was the target pathogen in this study.

1.2 Escherichia coli O157:H7

During the last 20 years, the risk of foodborne illness has increased remarkably, causing nearly a quarter of the population at higher risk for illness today (Oliver, Jayarao, & Almeida, 2005). There are currently more than 200 known diseases are transmitted through food contaminated with variety of agents including bacteria, fungi, viruses, and parasites (Oliver et al., 2005). Although the food supply in the United States is one of the safest in the world, foodborne pathogens are still responsible for about 9.4 million illness cases took place in the United States each year (CDC, 2016). According to the Centers for Disease Control and Prevention (CDC), in the year of 2014, 864 foodborne disease outbreaks were reported, resulting in 13,246 illnesses, 712 hospitalizations, 21 deaths, and 21 food recalls (CDC, 2016). Therefore, the prevention of illness and death induced by foodborne pathogens will still remain as a priority task in the foreseeing future.

Escherichia coli O157:H7 is an unusually virulent foodborne pathogen with low infectious dose, wide range of target age group, severe consequences of infection, unusual acid tolerance, and apparent special but inexplicable association with ruminants that are used for food. All of which make it more significant than other well-recognized foodborne pathogens, forcing food microbiologists to rewrite the rule book on food safety (Robert L. Buchanan & Doyle, 1997). It was first recognized as a human pathogen in 1982 after an investigation on two hemorrhagic colitis outbreaks caused by consumption of undercooked hamburger contaminated with *E. coli* O157:H7 (Riley et al., 1983). It was later discovered that it was a member of a group of *E. coli* strains that shared the similar pathogenic potential, known as the

enterohemorrhagic (EHEC) *E. coli* (Griffin & Tauxe, 1991b). Unfortunately, it did not catch enough attention until another massive outbreak caused by the same reason took place in 1993, when it was finally recognized as a threatening pathogen species (Bell et al., 1994). *E. coli* O157:H7 has now become the most common and most studied member among all EHEC *E. coli* strains (Griffin & Tauxe, 1991b). The disease-defining symptom of EHEC is hemorrhagic colitis (HC), also known as bloody diarrhea, which is induced partially by the two toxins produced by EHEC strains: Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2) (Robert L. Buchanan & Doyle, 1997). The toxin contains two subunits: Subunit A can inactivate globotriaosylceramide (Gb_3) receptors on the surface of endothelial cells; subunit B can inactivate the 28S ribosome to block protein synthesis. With the presence of other virulence markers like the *eae* chromosomal gene, the inactivation of Gb_3 receptors by the toxin is likely to induce HC and hemolytic uremic syndrome (HUS) (Robert L. Buchanan & Doyle, 1997).

The growth and survival of *E. coli* O157:H7 are dependent on various environmental factors including temperature and pH (Robert L. Buchanan & Doyle, 1997). The minimum growth temperature for *E. coli* O157:H7 under otherwise optimal conditions was observed at 8–10 °C (Rajkowski & Marmer, 1995), while the maximum tolerable growth temperature was below 44 °C (Doyle & Schoeni, 1984). The most suitable pH range for *E. coli* O157:H7 to grow is at 5.5 to 7.5, while the minimum pH range was identified to be around 4.5-4.5 (R.L Buchanan & Klawitter, 1992; R L Buchanan & Bagi, 1994). There have been reports for the observation of *E. coli* O157:H7 growth in acidic foods, including mayonnaise (Zhao & Doyle, 1994)

and apple cider (T Zhao, Doyle, & Besser, 1993b). Studies have found that *E. coli* O157:H7 has high degree of acid tolerance, making it able to survive for 2 to 7 hours in a pH2.5 environment at 37 °C (Benjamin & Datta, 1995), which may contribute to its survival in acidic foods.

According to a summary made by the CDC (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005), in the United States from 1982 to 2002, *E. coli* O157:H7 has caused a total of 350 outbreaks, which breaks down to 8,598 cases, including 1,493 (17.4%) hospitalization cases, 354 (4.1%) HUS cases, and 40 (0.5%) death cases. The number of reported outbreaks began rising in 1993, and peaked in the year of 2000. Possible reservoirs of *E. coli* O157:H7 have been identified by different studies including cattle (Meng, Zhao, Zhao, & Doyle, 1995; T Zhao, Doyle, Shere, & Garber, 1995), sheep (Kudva, Hatfield, & Hovde, 1996), and water (Faith et al., 1996; Keene et al., 1994). Major sources responsible for *E. coli* O157:H7 outbreaks include beef, fresh produce, dairy products, person-to-person route (fecal-oral route), and water (including recreational water and drinking water) (Rangel et al., 2005).

It is worth to notice that outbreaks caused by the consumption of fresh produce have become increasingly common, half of which were caused by contamination on produce that took place prior to any kitchen processing (Rangel et al., 2005). This contamination are likely to come from multiple sources including soil or improperly composted manure, contaminated irrigation or postharvest washing water, or infected food handlers (Beuchat, 1997). *E. coli* O157:H7 has been isolated from irrigation water implicated in the growth of contaminated lettuce during an outbreak in Montana at 1995 (M. L. Ackers et al., 1998), which indicated that

contaminated irrigation water might be a source for *E. coli* O157:H7 contamination on fruit and vegetables. It has also been found that *E. coli* O157:H7 may effectively transmit from contaminated irrigation water to lettuce plants with no contact with soil, which suggested that the pathogen was taken up through the root system within the water (Solomon, Yaron, & Matthews, 2002; Steele & Odumeru, 2004; Wachtel, Whitehand, & Mandrell, 2002). The above studies also found existence of pathogen in lettuce tissues, which means some bacteria cells have made their way inside the plants where they are inaccessible to water on plant surface, making them become resistant to postharvest washing (Solomon et al., 2002; Steele & Odumeru, 2004). Therefore, using irrigation water that either come from selected and monitored good-quality water source or received appropriate treatment in the production of fresh produce is highly recommended.

1.3 Chemical preservative

1.3.1 Benzoic acid

Benzoic acid (C_6H_5COOH) has the chemical structure shown in Figure I. Also known as benzencarboxylic acid, BA has a molecular weight of 122.1 and is soluble for a certain amount in water (0.27 g in 100 mL water at 18 C) (Davidson et al., 2005). The physical appearance of BA is colorless/ white, glossy monoclinic flakes or needles in its pure form (Davidson et al., 2005; Davidson, Taylor, & Schmidt, 2013).

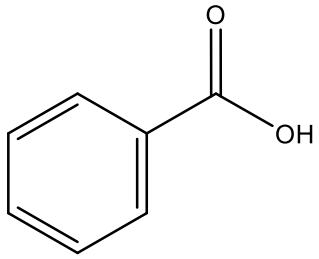


Figure I: Benzoic Acid Structure

BA occurs naturally in a variety of foods and organisms. Besides being recognized as a major content in black berry extracts, mushrooms and fresh tomatoes. BA has also been found in different types of cultured dairy products and chesses, which could come from bacterial fermentation of hippuric acid or phenylalanine in those products. In places where benzoic acid is not allowed to be used as food preservative (like Switzerland), the levels of naturally existed BA in food products varies from 10 to 1000 parts per million. (Abdullah, Young, & Gamed, 1994; Humpf & Schreier, 1991; Marlatt, Ho, & Chien, 1992) (Chipley, 2005).

Benzoic Acid has been recognized as a chemical preservative to be used in cosmetic, drug and food industries for a long time. Its antimicrobial effect was first described in 1875 by H. Fleck, and was introduced as a food preservative twenty years later when massive production of BA has become available through catalytic oxidation or air oxidation of toluene (Lück & Jager, 1997). It has been widely accepted by the food industry since its approval to be used as a food preservative by the Food and Drug Administration (FDA) at 1977 (Chipley, 2005). BA has been classified as a Generally Recognized As Safe (GRAS) preservative with a maximum permitted level to be used in food production at 0.1% in the United States (U.S. Food and Drug Administration, 2016a). Benzoic acid has been proved to have relatively

low toxicity. The LD_{50} of benzoic acid for rats after oral administration is 1.7-3.7 g/kg body weight (Lück & Jager, 1997; Sado, 1973). Another study found that no damage was detected after oral administration of 4 % benzoate acid for 90 days in test animals (Deuel, Alfin-Slatee, Weil, & Smyth, 1954; Lück & Jager, 1997). Due to its low cost, colorless, relatively low toxicity characteristics, BA is one of the most popular preservative being used in the world (Davidson et al., 2013).

Just like most weak organic acids, the diffusion of acid molecules through the bacteria cytoplasmic membrane is a crucial step for the inactivation activity of BA (Stratford & Rose, 1986)(Lambert & Stratford, 1999). It has been reported in a study conducted on *Saccharomyces cerevisiae*, only undissociated BA can be taken up by the cells (Macris, 1975). Therefore, the antimicrobial activity of BA mainly comes from the undissociated molecules (Davidson et al., 2005). The internalized BA molecules will then start to dissociate into protons and acid anions due to the fact that the intracellular pH in the cytoplasm is close to neutral, which cannot transfer back to the extracellular environment through the cytoplasmic membrane, leading to an increased concentration of charged ions inside the cell (Lambert & Stratford, 1999). The accumulation of protons will make the cytoplasm become acidic, which may have inhibition effects on the growth of microorganisms through the disruption of acidic intracellular pH towards multiple cellular activities including glycolysis, cell signaling or active transportation (Krebs, Wiggins, Stubbs, Sols, & Bedoya, 1983)(Freese, Sheu, & Galliers, 1973)(Thevelein, 1994). BA may also intervene and cause damage to the enzymatic structure of microorganism (Lück & Jager, 1997),

including the enzyme systems controlling acetic acid metabolism, oxidative phosphorylation, and citric acid cycle (Bosund, 1963).

The undissociated form of BA can inhibit yeast and fungi at concentrations of 0.05% and 0.1%, which makes it an excellent antifungal agent (Davidson et al., 2005) (Rusul & Marth, 1988). Although some food poisoning pathogen can be inhibited by 0.02% BA, many spore forming bacteria turn out to be more resistant towards BA (Davidson et al., 2005). Studies conducted on *Listeria monocytogenes* have led to a finding that BA at concentration of around 1000 to 3000 ppm expressed strong bacteriostatic, but low bactericidal activities against the pathogen (El-Shenawy & Marth, 1988; Yousef, El-Shenawy, & Marth, 1989). Therefore, BA by itself as an effective antimicrobial treatment against some pathogenic bacteria in food is questionable.

Due to its low pKa value of 4.19 (Hollingsworth, Seybold, & Hadad, 2002), BA is usually used only for preserving strongly acidic products like pickled foods (Lück & Jager, 1997). It is mostly effective when added as preservative in foods and beverages with a natural or acidified pH below 4.5 (Davidson et al., 2005). Benzoic acid can be employed at a concentration of around 0.1% for preserving pickled vegetables, which has a high acid content that helps to prevent the adverse effect of benzoic acid on the flavor (Lück & Jager, 1997).

For the preservation of fresh fruit juices, benzoic acid is mainly used at the concentration at around 0.05 % to 0.1 % to preserve fruit juices intended for further processing including pasteurization to achieve inactivation on the enzymes and other spoilage microorganisms (Lück & Jager, 1997). Benzoic acid added in fresh juice

products can help protect against enzymatic spoilage and bacterial spoilage. An investigation conducted on the preservative effect of multiple food preservatives against *E.coli* O157:H7 in apple cider suggested that BA can effectively reduce the heat resistance of the target pathogen and is two times more effective than sorbate (Dock, Floros, & Linton, 2000). When combining with other weak organic acids like fumaric acid, benzoic acid has been shown to have promising antimicrobial effect towards *E.coli* O157:H7 in apple cider at a concentration of 0.05% during a 6 hour treatment at 25 C°, achieving the FDA requirements for a 5-log reduction (Comes & Beelman, 2002). Benzoic acid were also used in soft drinks at a dosage of 0.02 % as an inexpensive yet efficient preservative against spoilage by yeasts (Page, Conacher, Weber, & Lacroix, 1992).

Due to its excellent antifungal activity, benzoic acid has also been applied in fresh produce industry to control postharvest disease among fruits and vegetables as a fungicide, especially against pathogenic strains of *Aspergillus flavus* in peanuts (Uraih & Offonry, 1981). Benzoic acid-based polymer coating/ film made with polysaccharide/ protein (Baldwin, Nisperos, Chen, & Hagenmaier, 1996) and methylcellulose mixed with chitosan (Chen, Yeh, & Chiang, 1996) have been developed and applied on the surface of different fruits including apples slices and bananas. Benzoic acid has also been used as a fungicide in animal feeds at a concentration of 0.1% (Davidson et al., 2005).

1.3.2 Paraben

The antimicrobial effect of phenolic compounds (chemical compounds consisting an aromatic hydrocarbon ring bonded directly with hydroxyl group(s))

have been recognized since Joseph Lister first started to use phenol to sanitize surgical equipment in 1867 (Davidson et al., 2005). Although the use of phenol has been declined later for its toxicity, other phenolic compounds, including parabens, have been recognized for their antimicrobial effect and potential to be used as preservatives in foods.

Parabens, also known as esters of *p*-hydroxybenzoic acid, have a general structure as shown in Figure II. The molecular mass for various esters are 152.15 for *p*-Hydroxybenzoic acid methyl ester, 166.18 for *p*-Hydroxybenzoic acid ethyl ester, 180.21 for *p*-Hydroxybenzoic acid propyl ester, 194.23 for *p*-Hydroxybenzoic acid butyl ester, and 236.21 for *p*-Hydroxybenzoic acid heptyl ester (Davidson et al., 2005). Based on their chemical structure, the solubility for parabens in water is inversely related to the alkyl chain length. Methyl paraben has water solubility of 0.25 g/100 g at room temperature; ethyl paraben has water solubility of 0.17 g/100 g; propyl paraben has water solubility of 0.05 g/100 g (Lück & Jager, 1997). Owing to its hydrophobicity, parabens, especially those with high alkyl chain length, have few application in aqueous system and relatively unfavorable distribution within oil in water emulsions (Lück & Jager, 1997). Although the sodium salts of *p*-hydroxybenzoic acid esters are readily water soluble, they are not considered to be stable enough in water because their strong alkalinity will induce rapid hydrolysis (Lück & Jager, 1997). In the United States, methyl (U.S. Food and Drug Administration, 2016c) and propyl paraben (U.S. Food and Drug Administration, 2016c) are classified as generally recognized as safe (GRAS) products at maximum concentration of 0.1% each.

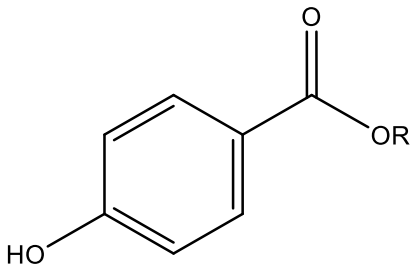


Figure II: General Structure for Parabens

The antimicrobial effect of parabens was first investigated in 1920s. Thanks to the esterification of the carboxylic group on the benzene ring of parabens, their antimicrobial activity is relatively independent of the medium pH (remain undisscoiate at pH up to 8.5) compared with benzoic acid that normally dissociate at around pH 5.0 (Davidson et al., 2005). Parabens have effective antimicrobial activity at pH range around 3 to 8, which makes them superior to the organic preservative acids (such as benzoic acid) in food systems with low to neutral pH (Aalto, Firman, & Rigler, 1953). The antimicrobial action of the parabens is proportional to the length of their alkyl chain (in the alcohol component) (Aalto et al., 1953) (Davidson et al., 2005). Investigations on the antimicrobial effect of parabens with different alkyl chain length against *E. coli* revealed that the uptake of parabens was logarithmically proportional to the chain length from methyl to butyl (Fukahori et al., 1996). Studies conducted on *E. coli* and *Bacillus subtilis* (Eklund, 1980) showed that the parabens with long alkyl chain length, as non-polar phenolic compounds, are generally more effective against Gram-positive bacteria than Gram-negative bacteria. It has been suggested that the lipopolysaccharide layer on the cell wall of Gram-negative bacteria generated a screening effect that contribute to their resistance towards non-polar

phenolic compounds like parabens (Eklund, 1980)(Freese et al., 1973). The effectiveness of parabens' inhibition activity against fungi is much higher than bacteria, which also increases as proportion to the increase of alkyl chain length (Thompson, 1994) (Davidson et al., 2005).

The mechanism of action for the antimicrobial activity of parabens has not been well understood, but studies have already indicated that the destruction on cytoplasmic membrane structure might be one of the main reasons (Tatsuguchi, Kuwamoto, Ogomori, Ide, & Watanabe, 1991)(Davidson et al., 2005)(Lück & Jager, 1997). Leakage of intracellular compounds such as ribonucleic acid (RNA) (Furr & Russell, 1972), and inhibition of amino acids and essential nutrients uptake including alanine, serine, phenylalanine (Eklund, 1980), and glucose (Tatsuguchi et al., 1991), have been detected in different bacteria species including *E. coli* and *B. subtilis*, indicating cytoplasmic membrane disruption. It has been postulated that parabens may neutralize chemical and electrical forces that are responsible for establishing a normal membrane gradient (Eklund, 1980). *Staphylococcus aureus* strains with different lipid compositions on cytoplasmic membrane have been identified to have different resistance to parabens (higher total lipid and phospholipid percentage, lower fatty acids percentage for paraben-resistant strain), suggesting that the absorption of parabens may depend on membrane fluidity, which contributes to the source for paraben resistance on pathogens (Bargiota et al., 1987).

Currently, instead of being used in a wide variety of foods, parabens are used predominately in the preservation of pharmaceutical and cosmetic products (Davidson et al., 2005). It has been reported that the major drawbacks that prevent

parabens from being used in food industry include problems with its unfavorable taste and low solubility in water (Lück & Jager, 1997). However, the use of parabens as preservatives have been tested in a wide variety of food products including bakery products, soft drinks, jams, pickles, and alcohol drinks, where they were incorporated into food mixtures by dissolving in water, ethanol, or food products itself (Davidson et al., 2005). Parabens can also be used in active antimicrobial packaging by incorporation into polymeric films. Ethyl and propyl parabens has been incorporated into a low-density polyethylene film, which can release parabens into simulated food system (Dobiás, Chudackova, Voldrich, & Marek, 2000). In another study (Chung, Chikindas, & Yam, 2001) where propyl paraben was mixed into a styrene-acrylate copolymer coating, the observed inhibition effect against *Saccharomyces cerevisiae* of the slow release coating was proved to be better than the direct addition of the paraben compound.

1.4 Ultraviolet (UV) light processing

Traditional processing technics, such as heating, smoking and salting, have long been applied in food industry for food processing and preservation. Among them, thermal treatment has been recognized as one of the most extensively available method for the inactivation of pathogenic microorganisms in food systems to achieve the required 5-log reduction in number of the most resistant pathogens (U.S. Food and Drug Administration, 2000b). However, thermal treatment also comes with some major side effects including potential damage on the flavor profile and nutritional quality of food products. Novel processing technologies is therefore being developed to minimize these unfavorable changes and to improve shelf life, which is especially

preferable for heat sensitive products such as fresh produce and fruit juice (Koutchma, 2014).

The concept of non-thermal processing technology has gained an increasing recognition in recent years as an alternative method to replace the traditional thermal processing method. Some of the most widely studied methods include high pressure processing (batch or continuous), pulsed electric fields, and ultraviolet light (Guerrero-Beltran & Barbosa-Canovas, 2004) (Tran & Farid, 2004). While they can inactivate foodborne pathogens just like other processing methods involving the use of heat, non-thermal processing methods also come with advantages such as low energy utilization and the preservation of sensory attributes and nutrition value due to the low processing temperature (Vega-Mercado et al., 1997).

Among all the novel processing technology that has been developed, the ultraviolet (UV) light technology is recognized as one of the most promising and innovative antimicrobial treatment technology being adopted by the food industry. UV) light is defined as the radiation with wavelength at the invisible region from 200 to 400 nm on the electromagnetic spectrum, which can be further categorized into UV-C (200–280 nm), UV-B (280–320 nm) and UV-A (320–400 nm). (Pattison & Davies, 2006). UV light range below 200 nm, also known as extreme UV, is readily absorbed by almost all substances, which makes it only transmittable within vacuum and is impractical to be used in real life situation (Koutchma, 2014). Although it is sometimes described as irradiation, UV treatment should be separated from ionizing radiation sources that deliver residue radioactivity such as gamma or x-ray irradiation to avoid potential consumer confusion. UV lamps are usually used as the illumination

source, where UV light photons will be emitted from the atoms and ions in gas discharge, serving as the direct energy source for all the photochemical reactions happened during the treatment time.

The antimicrobial of UV-C light treatment has been studied extensively for years and have been proved to be germicidal against microbial organisms including bacteria, viruses, protozoa, molds and yeasts, and algae (Guerrero-Beltran & Barbosa-Canovas, 2004)(Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). The mechanism behind the antimicrobial activity of UV-C is mainly at the nucleic acid level. The photons in UV-C light will energize electrons in the pyrimidine nucleoside base of DNA, which will lead to the formation of covalent bonds between adjacent thymine and cytosine bases and forming cyclobutane thymine dimmers in DNA (Pattison & Davies, 2006)(Koutchma, 2014)(Guerrero-Beltran & Barbosa-Canovas, 2004). The generation of dimmers will cause structure damage to the DNA and inhibit DNA regeneration, leading to inactivation of bacteria cells. Because the DNA molecules have maximum absorbance at 260 nm (Koutchma, 2014), the antimicrobial activity decreases as the wavelength deviate from its optimum range, which will become neglected at wavelength above 300 nm (Guerrero-Beltran & Barbosa-Canovas, 2004). UV-B radiation may also induce damage to cellular structure upon direct exposure to UV light. UV-B light primarily targeted at intracellular DNA and proteins, as well as some small molecules in cells such as carotenoids and eumelanin (Pattison & Davies, 2006). Studies have proved both UV-B and UV-C to be genotoxic and mutagenic, contributed by their ability to cause DNA damage.

Treatment with UV-A light, however, has been shown to have less antimicrobial activity in previous studies due to its relatively low absorbance by DNA and proteins inside bacteria cells (Fluhr & Gloor, 1997; Peak, Peak, Moehring, & Webs, 1984). However, UV-A radiation has long been confirmed for its ability to induce free radical and singlet oxygen formation through photosensitization and Type I- and II- mediated mechanism, which may induce excessive intracellular oxidative stress and causing indirect damage to important biomolecules including DNA, protein and lipids (Pattison & Davies, 2006). Briefly, photosensitizer inside cells like Flavin or NAD (P) H will enter triplet excited state through its exposure to UV-A light, which will then form a pair of charged radicals through electron abstraction (Cadet, Douki, Ravanat, & Di Mascio, 2009). In Type I photosensitization mechanism, the photosensitizer radical anions may react with molecular oxygen, leading to the production of superoxide anion radical, which later will be transferred into H_2O_2 through dismutation and undergoes Fenton reaction with the presence of Fe^{2+}, resulting in the production of ROS (Cadet et al., 2009; Winterbourn, 1995). The charged radicals may also form neutral radicals via deprotonation and hydration reactions, leading to the production of peroxyl radicals through the reaction with O_2 or superoxide anion radical (Cadet et al., 2009). Both end products can cause oxidation damage on essential cellular components including DNA, proteins and lipids (Pattison & Davies, 2006). In Type II photosensitization mechanism, the energy transfer between triplet state photosensitizer and molecular oxygen lead to the production of singlet oxygen ($^1O_2$), which may oxidize biomolecules consisting double bonds, including guanine and tryptophan (Greer, 2006) (Cadet et al., 2009).

In food industry, continuous flow system have been widely employed in the production for liquid drinks and beverages, which brings up the importance for designing a UV treatment system that fits into a continuous flow system while still retain the same scale of antimicrobial activity. There have been many UV processing systems that have been designed to work on continuous flow environment being developed, some of which have even been commercialized (Koutchma, 2014). The annular UV processing system (as shown in Figure III) is designed to have treatment fluid pumped through the gap between two stationary cylinders. The inner cylinder is usually made with quartz sleeve that protect the UV lamp irradiated inside, which is surrounded by a metal cylinder as the reactor wall (Ye, Forney, Koutchma, Giorges, & Pierson, 2008). (In some designs, the location of quartz tube may vary to allow for the installation of more UV lamps.) The absorption and flow rate of the treated fluid products are considered to determine the geometric length and size of the gap for an optimum antimicrobial effect. UV processing systems designed for laminar flow condition are usually equipped with thin film reactors, which has short gap width to reduce the penetration depth to provide a throughout exposure to UV light for microorganisms presented at different locations of the liquid when passing through the reactor (Koutchma, 2014; Ye et al., 2008). Turbulent flow reactors generally have larger gap width and are operated at a much higher speed. They utilize the fluid turbulence generated by high flow rate to facilitate better mixing, which can improve the equality of distribution of UV light exposure in liquid flowing through the gap while processing a fairly high volume of product at limited time (Koutchma, 2014). The design of a turbulent systems allows for a more evenly distributed UV dose than

a laminar system, but when the absorption coefficient of the treated product is high, under irradiated sublayer may still exist, which could lead to incomplete UV treatment (Koutchma, 2009, 2014). In that case, the gap size for turbulent flow reactor will need to be reduced to lower the penetration depth for UV. There are more advanced designs for continuous flow UV reactors that are being developed to overcome the limitations posed in laminar and turbulent UV reactors, including static mixer system and dynamic mixer system (Koutchma, 2009, 2014; Koutchma, Parisi, & Patazca, 2007).

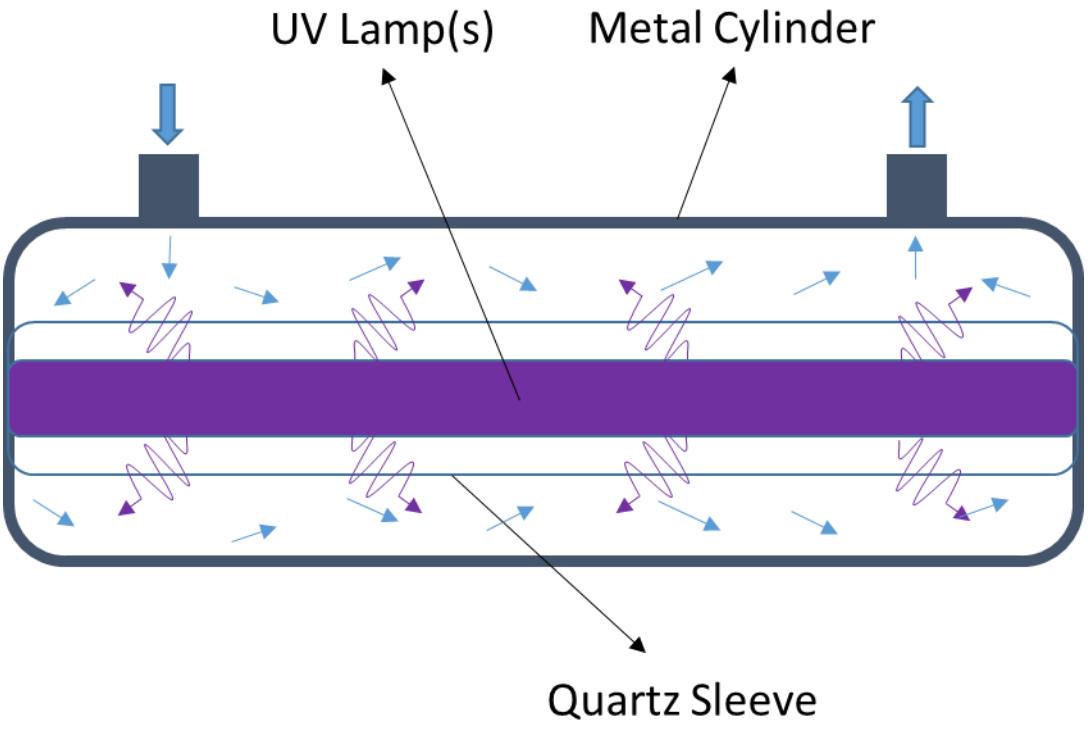


Figure III Annular UV processing system general layout

1.5 EDTA and Gram-negative cell membrane

Ethylenediaminetetraacetic acid (EDTA) is a colorless crystalline solid with a molar mass at 292.244 g/mol, which is slightly soluble in water (0.05 g/100 g at room temperature). It has been categorized as generally recognized as safe (GRAS) product to be used in foods for enhancing color and flavor retention and preservative effect for up to 365 parts per million (U.S. Food and Drug Administration, 2016d). It has been used in many experiment methods to increase outer membrane permeability of a wide range of gram-negative bacteria to extracellular compounds (Beacham, Kahana, Levy, & Yagil, 1973; R. E. Hancock, 1984; Muschel & Gustafson, 1968).

The gram-negative cell is protected a cell wall consist of two membranes separated by a layer of peptidoglycan located inside a cellular compartment known as the periplasm (Brown, Wolf, Prados-Rosales, & Casadevall, 2015). The inner cytoplasmic membrane is composed with phospholipid bilayer liberally studded with a wide variety of polypeptides, helping with bacterial metabolic activities including generation of energy, active transport of nutrients and export of byproducts, and enzymatic synthesis (DiRienzo, Nakamura, & Inouye, 1978). The inner cytoplasmic membrane also serves as a major barrier for hydrophilic or charged molecules. The peptidoglycan generally serves as stabilizer for maintaining cellular shape and osmotic pressure (R. E. Hancock, 1984). The outer membrane of gram negative bacteria is an unusual monolayer composed with primarily lipopolysaccharides (LPS), which is an amphiphilic molecule containing a hydrophobic region (Lipid A) that has 5 or 6 fatty acids linked to diglucosamine phosphate (DiRienzo et al., 1978; R. E. Hancock, 1984). Lipid A is covalently attached to an oligosaccharide core, which sometimes could also be a repeated polysaccharide unit known as O-antigen

(DiRienzo et al., 1978; R. E. Hancock, 1984; Palva & Makela, 1980). The strong negative charge on the surface of gram-negative bacteria cells is contributed by the net negative charge carried by LPS, which is an important function for the protection of gram-negative bacteria. LPS is localized on bacteria outer membrane through hydrophobic interactions between outer membrane protein and Lipid A and more importantly, noncovalent cross-bridging of adjacent LPS molecules (LPS-LPS interaction) stabilized with divalent cations including Mg^{2+} and Ca^{2+} (R. E. Hancock, 1984; Schweizer, Hindennach, Garten, & Henning, 1978; Yamada & Mizushima, 1980). Together with the strong surface negative charge, a well-structured LPS matrix stabilized by divalent cation cross-bridging can provide gram-negative bacteria cells resistance towards most of the extracellular hydrophobic antibacterial compounds.

The mode of action for EDTA to act as a membrane permeability enhancer is that it can remove stabilizing divalent cations from their binding sites in LPS through its strong chelating function (R. E. Hancock, 1984; Leive, 1974), leading to increase of the electrostatic repulsion between neighboring lipopolysaccharides (LPS) molecules (Nikaido & Vaara, 1985). This process can weaken the LPS-LPS interaction and resulting a significant proportion (around 30% and 67% of total LPS in E. coli (Graham, Treick, & Brunner, 1979)) of LPS being released from the outer membrane (Hazelbauer, 1975; Nikaido & Vaara, 1985). Since LPS is a critical compartment to the outer membrane, the loss of LPS will at least partially induce an injured outer membrane that is permeable for macromolecules (Vaara, 1992). The loss of LPS will also make it possible for phospholipids to appear in the outer leaflet of the outer membrane and fill in the empty spots left by the lost LPS (Nikaido &

Vaara, 1985). Extracellular hydrophobic compounds may use the formed phospholipid bilayer patches as channels to penetrate inside the cytoplasm, leading to the loss of function on cell membrane (Vaara, 1992).

1.6 Intracellular stress

1.6.1 Oxidative stress

Oxidative stress can be defined as an excess of prooxidants within cells, which is usually caused by the accumulation of reactive oxygen species (ROS) generated by the incomplete oxygen reduction during aerobic respiration and enhanced by exposure to environmental stresses including UV radiation, light, metals, or redox active drugs (such as paraquat) (S B Farr & Kogoma, 1991; Gisela Storz, Tartaglia, Farr, & Ames, 1990).

As the starting material of the aerobic respiration reactions, the two outermost electrons of the ground state triplet molecular oxygen occupy separate antibonding orbitals with parallel spins (Apel & Hirt, 2004). The reaction with organic molecules in cells is restricted because molecular oxygen would need to accept a pair of electrons with parallel spins that fit into its free electron to oxidize other molecules, while electron pairs in most molecules have antiparallel spins (Cadenas, 1989). However, the spin restriction of molecular oxygen can be overcome by the production of the much more reactive ROS including singlet oxygen (through energy transfer reaction), or superoxide anion, hydrogen peroxide, and hydroxyl radical (through electron transfer reactions) with help from several respiratory chain enzymes associated to membrane and some intracellular transition metals such as Fe or Cu (Apel & Hirt, 2004; S B Farr & Kogoma, 1991).

In order to cope with the toxic ROS accumulated inside the cells, aerobic (or Facultative anaerobic) bacteria have developed an efficient defense mechanism to protect themselves from undertaking fatal oxidative stress. This mechanism allows bacteria to stabilize the intracellular ROS concentration at an acceptable level. Bacteria regulate its intracellular oxidative stress level by different agents. 1) Constitutively presented non-enzymatic antioxidants such as glutathione (GSH), β-carotene, ascorbic acid, α-tocopherol, and NADPH and NADH pools can help to maintain a suitable intracellular oxidative stress level by scavenging excessive ROS (Cabiscol, Tamarit, & Ros, 2000). For example, high concentration of GSH is presented in bacteria cells as an antioxidant buffer system to prevent the oxidative damage from ROS. Glutathione reductase and NADPH will help to maintain its buffering capacity by reducing it from its oxidative form back into GSH (Cabiscol et al., 2000). 2) Specific enzymes that serve as active scavengers for different ROS can help to decrease the oxidative stress level. For example, and catalase can convert superoxide anion can be converted to hydrogen peroxide with the help of superoxide dismutase (SOD), which will then be removed and broken down to molecular oxygen (O_2) and water. 3) Regulation of intracellular concentration for transition metals/ metal ions also contributes in controlling the oxidative stress. Fe, for example, is a critical component for the generation of ROS. It serves as the catalyst for Fenton reaction that yield hydroxyl radicals (S B Farr & Kogoma, 1991)(Cabiscol et al., 2000); and also modulate the expression of iron-containing enzymes (like SOD) that decreases excessive ROS (Cabiscol et al., 2000; Niederhoffer, Naranjo, Bradley, & Fee, 1990). The intracellular iron concentration is regulated through both specific

membrane-bound receptors that controls the entrance of Ferrous ions and proteins inside cells (like bacterioferritin and ferritin) that capture and store excessive irons inside cells (Cabiscol et al., 2000; Carrondo, 2003).

However the bacteria perfect their ROS-protection systems, damage will still be induced when the degree of oxidative stress exceeds the capacity of the cell defense systems, which will happen to multiple cellular components including nuclear acids, membrane, and protein. Investigations on the mutagenesis frequencies of *E.coli* with compromised ROS-protection system found that the frequency for spontaneous mutagenesis increased by exposure to increased oxygen (Spencer B Farr, D'Ari, & Touati, 1986), which proves that the excessive intracellular ROS may induce oxidative damage to DNA, leading to high mutation rates (Spencer B Farr et al., 1986; Gisela Storz et al., 1990). Other studies also found that mutagenesis happened on bacteria species with impaired oxidative stress adjusting system is more frequent when under aerobic conditions (Greenberg & Demple, 1988; G Storz, Christman, Sies, & Ames, 1987), which again proves the damage from excessive intracellular ROS may cause to cellular DNA. Due to that fact that DNA has high affinity to iron (Rai, Cole, Wemmer, & Linn, 2001), it is likely to be an especially favored site for Fenton reaction to happen. It has therefore been hypothesized that hydroxyl radical, as the product from Fenton reaction, is the major contributor to the oxidative damage on DNA (Imlay, 2003). Hydroxyl radical can pull electrons from either sugar or base moieties, which generates different DNA radicals and producing a broad spectrum of mutations (Dizdaroglu, Rao, Halliwell, & Gajewski, 1991; Hutchinson, 1985) (Imlay, 2003). Oxidative damage may also happen to cell

membrane, where a rapid loss of proton motive force transport was observed on *E.coli* cells treated with hydrogen peroxide for 5 minutes (S B Farr, Touati, & Kogoma, 1988). Oxidative damage on protein has also been observed. It has been pointed out that protein cysteinyl residues can be oxidized by hydrogen peroxide, which produces sulfenic acid adducts that lead to the generation of disulfide cross-links with other cysteines or further oxidation to sulfinic acid moieties (Imlay, 2003). Protein may also be carbonylated by hydrogen peroxide through Metal-catalyzed oxidation reactions (Dukan et al., 2000; Imlay, 2003). Besides that, studies have suggested that excessive superoxide anion may also induce inactivation of enzyme containing iron-sulfur cluster (Imlay, 2003). Hydroxyl radical can be electrostatically attracted to the iron atom due to its localized positive charge, where the cluster will be univalently oxidized and become unstable (Flint, Tuminello, & Emptage, 1993). It will eventually degrade and lose its catalytic iron atom, which leads to the inactivation of enzyme (Flint et al., 1993; Imlay, 2003). Since SOD also contain the iron-sulfur cluster structure, the cellular ROS-protection system is likely to be deactivated by excessive superoxide anion presented inside cells.

1.6.2 Intracellular pH

The intracellular pH (pH_i), or cytoplasmic pH, is a measure of proton concentration inside cells. Microorganisms are constantly exposed to the environment with protons, the level of could sometimes differ by nine orders of magnitude in different habitats where microorganisms live (Padan, Zilberstein, & Schuldiner, 1981). Although it is true that a few microorganisms can live under environment with extreme pH (*Helicobacter pylori* living in stomach milieu at pH of 2), most bacteria

do not have strong acid/ base tolerance (Padan et al., 1981). Therefore, in order to keep their internal pH constant, bacteria have developed different mechanism to maintain proton at different concentrations on either side the cell membrane.

Possible mechanisms for cytoplasmic pH regulation include preexisting cytoplasmic buffers, biochemical production of H^+ or OH^-, and active transport of protons (McLaggan, Stephen, & Booth, 1998). The amino acid side chains of proteins (like aspartic acid or lysine) are the basic components of the cytoplasmic buffer that can offset a limited amount of intracellular acidification or alkalinization (Sanders & Slayman, 1982) (McLaggan et al., 1998). Production of acid or base through metabolic activities has also been observed through the detection of protein decarboxylases and deaminases thesis at different extracellular pH (Gale, 1943). The dominant mechanism for maintaining pH_i is the active transport of protons across cell membrane. The ability of primary proton pumps to regulate proton transportation is constrained by proton motive force (PMF), which is produced by the proton concentration differences across cell membrane and is dependent on the chemical difference in proton concentration (ΔpH) and the electrical difference in charge ($\Delta\Psi$) (Foster, 2004). When the pH outside the cell is lower than inside, protons will be pulled inwardly from extracellular environment to reach chemical equilibrium. Large pH gradient will be formed as a result of bacteria maintaining their pH_i at neutrality through primary proton pumps, which needs to be compensated by reversing charge difference to avoid influx of protons (Foster, 2004). Therefore, it is essential to generate intracellular positive charge by either cation influx or anion efflux to maintain the necessary pH gradient. The cation-proton antiport has been proved to

play a dominant role in preventing intracellular acidification through bulk proton extrusion with cation uptake (Brey, Beck, & Rosen, 1978; Sanders & Slayman, 1982). The antiport exchanges internal protons for external major cellular cations (Na^+ or K^+), resulting in increasing of pH_i (Foster, 2004; Sanders & Slayman, 1982).

Despite the pHi stabilizing mechanisms discussed above, limitation may be reached when the intracellular proton level has overwhelmed the capability of the adjusting system or the bacteria no longer have enough energy to support continuously active transportation of protons and cations. The intracellular pH will eventually deviate from its original range, which could affect many critical cellular physicochemical reactions, including hydrolysis, ionization and oxidoreduction (Padan et al., 1981). Most proteins and other biologically important molecules need to be presented in an environment with a narrow pH range falling around neutrality to remain stable or reach their optimum activity. With loss of all these functionalities, cell death will eventually be induced.

2 Chapter 2: The antimicrobial effect of benzoic acid treatment combined with UV-A light on *Escherichia coli* O157:H7

2.1 Material and methods

2.1.1 UV-A treatment

The UV-A treatment was conducted within a UV crosslinker unit manufactured by Spectronics Corporation (Westbury, NY, USA). This bench top UV processor is equipped with 5 UV-A light bulbs (8 W, peak wavelength 365 nm) that has been installed on the ceiling of the inner light-proof chamber. The UV-A light intensity on the sample surface during exposure was 2015 μW/ cm^2 as measured at the Intensity Mode of the device.

2.1.2 Bacteria culture

A Shiga toxin negative *Escherichia coli* O157:H7 (ATCC #700728, Manassas, VA) was a generous gift from Dr. N. Nitin at University of California-Davis. The bacterium was cultured in Tryptic Soy Broth (TSB) and was grown at 37 °C for 20 hours, indicating it has reached a stationary phase (approximately 10^9 CFU/mL).

2.1.3 Inactivation of *Escherichia coli* O157:H7 with simultaneous treatment of UV-A light and benzoic acid

Benzoic acid (BA) (Fisher Scientific, Waltham, MA, USA) was prepared in deionized (DI) water, which were then sterilized by passing through a 0.2 μm syringe filters. Overnight incubated *E. coli* culture was diluted in sterilized DI water to reach a concentration of approximately 1×10^8 CFU/mL. It was further diluted with sterilized BA solutions to achieve a final concentrations of 0, 8, 10 or 15 mM for BA and approximately 1×10^7 CFU/mL for bacteria. In an experiment studying the role

of EDTA in accelerating microbial inactivation, EDTA solution was added to 10 mM BA solution at a final concentration of 1 mM. The interaction between EDTA and metal ions was investigated by adding 4 mM $CaCl_2$ and 4 mM $MgCl_2$ into the solution. To evaluate the effect of solution pH on the antimicrobial activity of the simultaneous treatment, 15 mM BA was also prepared in phosphate buffer saline (PBS) to reach pH 6.27. 2 mL of the solutions prepared as described above was transferred into a well of a 6-well flat bottom polystyrene plate and exposed to UV-A light for up to 30 minute. Controls for this experiment consist of incubating bacteria and BA in dark and exposing bacteria to UV-A light in the absence of BA for the same amount of time. Samples were obtained periodically during the UV exposure, serially diluted in 0.2% buffered peptone water and 100 µL solution of each dilution was plated on Trypticase™ soy agar (TSA). All agar plates were incubated overnight at 37 °C before counting.

2.1.4 Inactivation of *Escherichia coli* O157:H7 with sequential treatment of UV-A and benzoic acid

To evaluate the contribution of an individual treatment (UV-A or BA) on the observed microbial lethality and to investigate the possibility of an interaction between the two treatments, we performed an experiment where the bacteria were exposed to UV-A light and BA in sequence. An overnight incubated *E. coli* culture was diluted in sterilized DI water to reach a concentration of approximately 1×10^7 CFU/mL. The diluted bacteria solution was then exposed to UV-A light in 6-well plates for 30 minute. The bacterial cells were harvested by filtering 10 mL UV treated sample solution through a sterilized 0.2 µm Express Plus® Polyethersulfone (PES) membrane filter (EMD Millipore, Ireland) inside a sterilized Swinnex™ Filter Holder

(EMD Millipore, Ireland), which were then incubated in 0 or 15 mM BA solutions for 30 minute in dark after transferring the membrane filters into 10 mL of treatment solutions with vigorous vortex. Control experiments were conducted by incubating diluted bacteria solution in dark for the first 30 minute before the treatment of benzoic acid.

A treatment with a reversed sequence was also examined by diluting overnight incubated *E. coli* culture in sterilized 0 or 15 mM BA solutions to reach a final concentration of approximately 1×10^7 CFU/mL and incubating in dark for the first 30 minute. Bacterial cells were then harvested from 10 mL incubated solution and resuspended in 10 mL sterilized DI water following the same method illustrated above. The solutions were then exposed to UV-A light for 30 minute in 6-well plates. Control experiments were conducted by incubating the BA treated bacteria solution in dark during the second 30-minute treatment. Bacterial enumeration was performed using method described earlier.

2.1.5 Investigation of the cell membrane integrity during UV-A and EDTA treatments

Propidium iodide was used as an indicator of the integrity of cell membrane. A treatment sample containing 1×10^9 Log/mL *E. coli* and 0 or 15 mM BA was exposed to UV-A for 30 minute. A control sample was incubated in dark for the same amount of time. 1 mL of the incubated sample was then washed once with sterilized DI water and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was then re-suspended in 5 μM PI solution and incubated at room temperature for 15 minute. The incubated solution was further washed with 1 × PBS and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was resuspended in 500 μL 1

× PBS. 100 μL of the solution was transferred into an opaque 96 well plate separately. The fluorescence intensity was measured at an excitation wavelength of 535 nm and an emission wavelength of 617 nm on a SpectraMax M5e microplate reader (Molecular Devices, CA, USA).

2.1.6 Scanning electron microscopy (SEM)

Scanning electron microscopy was conducted to observe the morphology of the bacteria after the simultaneous treatment. A treatment sample containing 1×10^9 Log (CFU/mL) *E. coli* and 0 or 15 mM BA was incubated under UV-A exposure for 30 minute. A same control sample was incubated in dark for the same amount of time. The incubated samples were filtered through a sterilized 0.2 μm membrane filter. The bacterial cells were then fixed by incubating the membrane filters in glutaraldehyde for 1 hour. After being rinsed in sterilized DI water for three times and ethanol with a serial concentration range for six times, all samples were incubated in a petri dish overnight for dehydration. All dehydrated samples were then mounted on an SEM stub and being sputter coated with a 20 nm thick layer gold. A scanning electron microscope (XEIA3, TESCAN, Kohoutovice, Czech Republic) was used to examine the samples. All images were recorded at an accelerating voltage of 10.0 kV.

2.1.7 Investigation of intracellular oxidative stress change during the simultaneous UV-A and benzoic acid treatment

To evaluate the intracellular redox status of *Escherichia coli* O157:H7 during the treatment, intracellular glutathione (GSH) concentration was measured. The experiment was conducted using the Thiol Detection Assay Kit from Cayman Chemical (Ann Arbor, MI, USA) (Arbor, 2016). Briefly, a treatment sample containing 1×10^9 Log/mL *E. coli* and 0 or 15 mM BA was incubated under UV-A

exposure for 5 or 30 minute. A same control sample was incubated in dark for the same amount of time. A sample containing sterilized DI water with pH adjusted to 3.0 with hydrochloric acid was used as a control. Bacteria incubated with 0.3% hydrogen peroxide in dark was also used as a positive control. 1 mL of the incubated sample was washed once with sterilized DI water and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was re-suspended in 1 mL ice cold lysis buffer, which contained 100 mM Tris-HCL and 1 mM EDTA and were sterilized by filtration through a 0.2 μm syringe filter. 500 μL solution was then transferred to a 1.5 mL centrifuge tube containing approximate 0.5 mL silica beads. After vortexing at high speed for 10 minute, all the tubes were centrifuged for 10 minute at 16,000 × g at 4 °C. 50 μL supernatant was then collected and diluted in 200 μL working buffer (diluted 1:50 from the stock Thiol Assay Buffer (Cat700341)). 50 μL of diluted sample solution was transferred into an opaque 96 well plate separately and mixed with 50 μL detector (diluted 1:100 from the stock Thiol Fluorometric Detector (Cat700342)). After incubation in dark for 5 minute, fluorescence intensity was measured at an excitation wavelength of 385 nm and an emission wavelength of 515 nm on a SpectraMax M5e microplate reader (Molecular Devices, CA, USA).

The change of intracellular ROS level was also measured using CellROX® green reagent (C10444, Molecular Probes, Carlsbad, CA, USA). Treatments with BA and UV-A were first applied to bacterial cells as described above for 5 and 30 minutes. 0.3% hydrogen peroxide as well as samples with pH adjusted to 3 or 6 were used as controls. After the treatments, CellROX® green reagent was added to each of the samples to reach a final concentration of 5 μM (Molecular Probes, 2012; Xiong,

Siegel, & Ross, 2014). After incubating in dark at 37 °C for 30 minute, all samples were washed 3 times with 1× PBS and resuspended in 500 µL 1 × PBS. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm on the SpectraMax M5e microplate reader (Molecular Devices, CA, USA).

Dimethyl sulfoxide (DMSO) was used a ROS inhibitor (quenches hydroxyl radical) during the simultaneous UV-A and Benzoic Acid treatment (Mannan et al., 2010). 5% DMSO solution was made from the stock and sterilized by filtering through a 0.2 µm syringe filter. It was then added into treatment solution to reach final concentration of BA at 0 or 15 mM. 10^7 log CFU/mL *E.coli* culture was incubated in the solution under UV exposure for 30 minute. A control group of samples with the same condition were incubated in dark for the same amount of time. A serial dilution was then made in buffered peptone water for each sample. 100 µL solution at its proper dilution was plated on TSA and counted after overnight incubation at 37 °C

2.1.8 Change of intracellular pH during the simultaneous UV-A and BA treatment

To evaluate the intracellular pH (pH_i) change of *Escherichia coli* O157:H7 during the treatment, pHrodo™ Green AM Intracellular pH Indicator from Molecular Probes (CatP35373, Carlsbad, CA, USA) was used. Briefly, a treatment sample containing 1× 10^9 Log/mL *E. coli* and 0 or 15 mM BA was incubated under UV-A exposure for 30 minute. A same control sample was incubated in dark for the same amount of time. A sample containing sterilized DI water with pH being adjusted to 3.0 with hydrochloric acid was also used as a control sample. 1 mL of the incubated sample was then washed once with sterilized DI water and was further washed once

with 1 mL sterilized HEPES buffer (containing 20 mM HEPES). After centrifugation for 2 minute at 10,000 × g at room temperature, the pellet was re-suspended in 1 mL HEPES solution containing 1:1000 pHrodo Green AM Indicator. The culture was then incubated at 37 °C for 30 minute and washed again with sterilized HEPES buffer. After being diluted 10 times in sterilized HEPES buffer, the fluorescence intensity of each sample was measured at an excitation wavelength of 509 nm and an emission wavelength of 533 nm on a microplate reader as described above. The incubated samples were also examined under fluorescence microscope with excitation laser generator and emission filter set at the same excitation and emission wavelength as used in the microplate reader. A standard curve for pH_i was also plotted by using Intracellular pH Calibration Buffer Kit (CatP35379, Carlsbad, CA, USA) and following the same procedure in the same bacteria culture without the UV-A or BA treatment.

2.1.9 Investigation of the antimicrobial effect with the combined BA and UV-A treatment in a simulated continuous flow processing environment

To simulate the continuous flow processing environment, a commercial ultraviolet water purifier (MP16A, Atlantic Ultraviolet, Hauppauge, NY, USA) was used as the reactor. An 8W UV-A lamp (F8T5BLB, Prolume, Monroe, CT, USA) with 30.48 cm length was installed inside an inner quartz tube with peak emission at 365 nm as its major wavelength. Sample solution was pumped through 42 mm annular gap between the outer surface of the quartz tube and the inner surface of the cylinder. The flow rate was maintained at approximately 632 mL/min with a peristaltic pump (GP1000, Fisherbrand, Pittsburgh, PA, USA). The whole system

layout and dimensions can be found on Fig. 1. Before the experiment started, the whole system was first sanitized with chlorine at 100 ppm for one hour and then rinsed with sterilized DI water for one more hour with the UV lamp off. After the sanitation, 4 mL of overnight incubated *E. coli* culture was added to 4 L of sterilized treatment solution with 10 and 15 mM BA to reach an approximately 6 Log CFU/mL bacterial concentration. The UV lamp was activated after all the inoculated solution has been pumped into the system at the flow rate described above. Samples were collected from the reservoir in every 7 minute during the whole 35 minute treatment. An addition sample took at time zero was also saved and incubated in dark for 35 minute as a control. A serial dilution was then made in buffered peptone water for each sample. 100 µL solution at its proper dilution was plated on TSA. All agar plates were incubated overnight at 37 °C before counting. Experiments for each treatment were performed in duplicate.

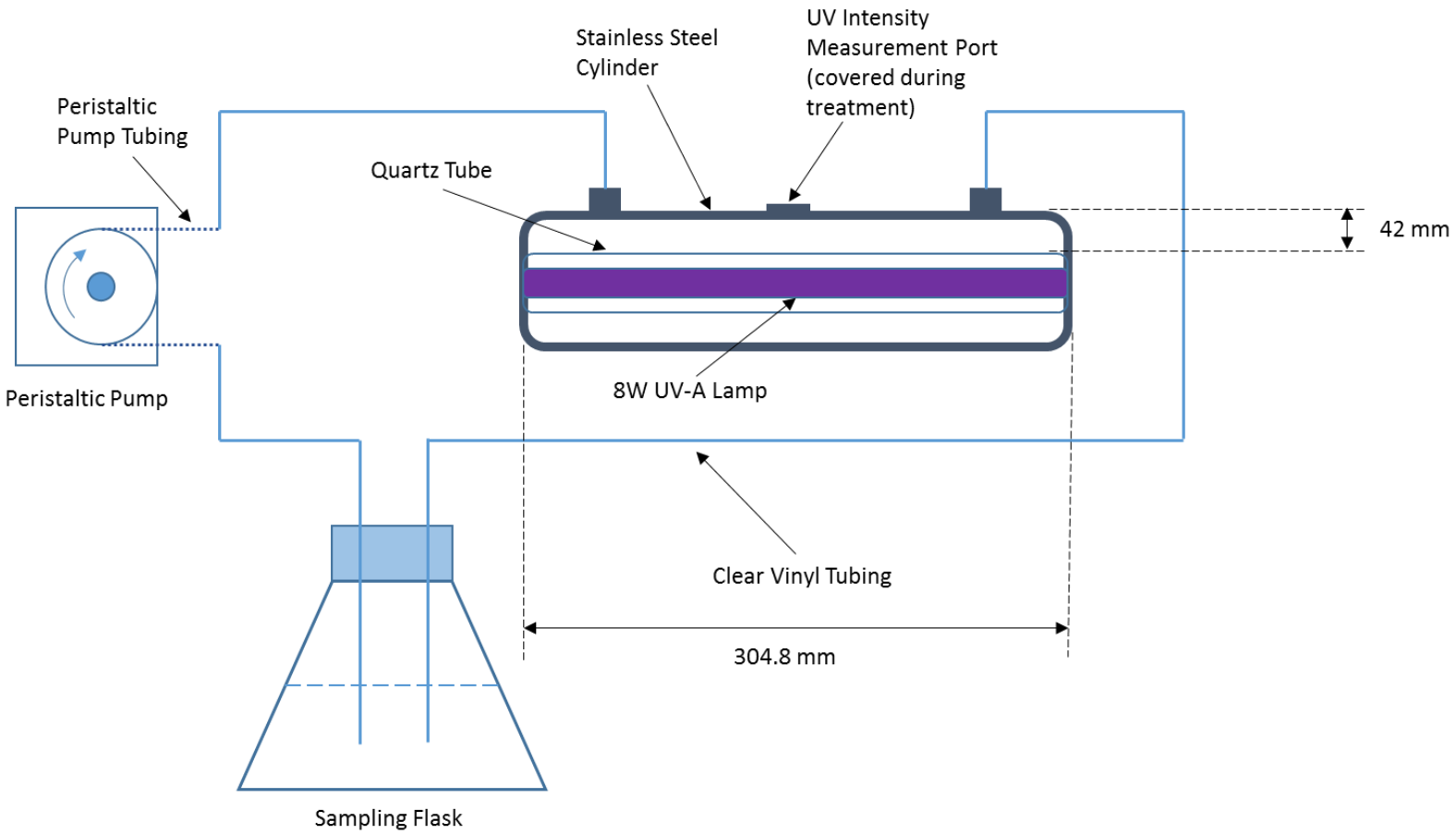


Figure1: Continuous flow UV-A treatment system layout

2.1.10 Statistical test

Unless specified above, all experiments were performed in triplicate. The significance between different treatments were calculated via unpaired Student's T test assuming equal variance by using Microsoft Excel 2013 (Seattle, WA, USA). The parameters in the Weibull model and the log-linear model were obtained via GRG nonlinear method using the Solver option in the same software. The antimicrobial activity data obtained from the study were also statistically analyzed by the Analysis of Variance (JMP Version 12.2, SAS Institute, NC, USA) and the means were separated according to Tukey's HSD test at a significance level of 0.05.

2.2 Results and discussions

2.2.1 Antibacterial activity of the simultaneous UV-A and BA treatment and the effect of EDTA and extracellular pH

Table 1 shows that while sample treated with UV-A light alone and 15 mM BA alone in dark for 30 minute had no significant antimicrobial activity ($p > 0.05$), a combined UV-A light and 15 mM BA treatment reduced the bacteria population by ~6 log (CFU/mL) in 30 minute. Interestingly, when the concentration of BA in the treatment was lowered to 10 or 8 mM, the antimicrobial effect of the combined treatment decreased significantly ($p < 0.05$) and only ~1 log (CFU/mL) reduction was obtained. Thus, the combined treatment of BA and UV-A light significantly improved the rate of microbial inactivation and the effect of combined treatment was dependent on the concentration of BA.

Indeed, given enough treatment time, both UV-A and BA treatments could be used alone to inhibit the growth of bacteria. As reviewed previously, UV-A irradiation has long been confirmed for its ability to generate free radicals and singlet oxygen through photosensitization and Type I- and II- mediated mechanism (Pattison & Davies, 2006). Similarly, BA, a commonly used weak organic acid preservative, is also known for its inactivation effect against many types of microorganisms due to intracellular acidification (Hazan, Levine, & Abeliovich, 2004). However, neither of the two treatments presented significant antimicrobial activity during the thirty minute treatment. The synergy of the combined treatment is apparent since the extent of bacteria inactivation in sample received the combined BA and UV-A treatment was significantly higher than the sum of antimicrobial activity between the two individual treatments ($p < 0.05$).

Results on Table1 also indicates that the antimicrobial effect of 15 mM BA and UV-A treatment completely disappeared ($p < 0.05$) when the solution pH was adjusted to 6.2 with PBS, proving that the antimicrobial activity of the combined UV-A and BA treatment was pH dependent, which corresponded to the characteristic of BA treatment where little antimicrobial activity was observed at neutral pH (Davidson et al., 2005). This can be attributed to the dissociation of BA at pH higher than its pK_a at 4.19 (Hollingsworth et al., 2002), which quickly becomes charged ions that cannot diffuse across the membrane freely (Hazan et al., 2004). Since only 1.44% of the BA will remain in its undissociated form at pH 6 (Rahn & Conn, 1944), and only undissociated BA can be taken up by cells (Macris, 1975), we may further conclude that instead of the extracellular BA, the internalization of BA has major impact on the antimicrobial effect of the combined treatment.

Ethylenediaminetetraacetic acid (EDTA) has been shown to be able to interact with cell membrane and thus increase membrane permeability (De Smet, Kingma, & Witholt, 1978; R. E. W. Hancock, 1984; R. E. W. Hancock & Wong, 1984; Nikaido, H; Varra, 1985). It is used in this experiment to test if it could help to increase the antimicrobial activity of the simultaneous UV-A and BA treatment. While 10 mM BA and 1 mM EDTA did not have a significant effect on the inactivation of the bacteria, the combined treatment caused more than 5 log (CFU/mL) reduction when exposed to UV-A light. Since it has been proved that EDTA can induce release of LPS on Gram negative bacteria outer membrane and increase the permeability of hydrophobic compound, the addition of EDTA may possibly increase BA uptake

during the combined treatment, which helps to lower the required BA concentration in the solution for the simultaneous UV-A and BA treatment.

To validate the role of EDTA in chelating metal ions, results in Table 1 shows that while the simultaneous UV-A, 8 mM BA, and 1 mM EDTA treatment had 4.46 ± 0.79 log (CFU/mL) reduction, the addition of 4 mM $CaCl_2$ and 4 mM $MgCl_2$ lowered the antimicrobial effect to 3.62 ± 0.73 log (CFU/mL). Although we observed that in each of the replicates, the addition of metal salts lowered the extent of inactivation, ($p < 0.05$ in paired t-test), unpaired t-test did not show significance ($p = 0.33$). This may be due to a significant variation in the quantitative results between the replicates. Nevertheless, the qualitative trend within the replicates was consistent in that addition of metal ions lowered the extent of inactivation by the combined EDTA+BA+UV-A treatment but did not fully eliminate it. These results validate the role of EDTA as a metal chelator.

Figure 2A and 2B shows the kinetic of microbial inactivation by the simultaneous UV-A and 15 mM BA treatment and the combine 10 mM BA, 1 mM EDTA and UV-A treatment. Since it had the better fit statistics ($R^2 > 0.99/ 0.99$) than log-linear model, the data was fitted into a nonlinear kinetic equation using the Weibull model (Couvert, O., Gaillard, S., Savy, N., Mafart, P., and Leguérinel, 2005), which has been used to describe the inactivation of many microorganisms through non-thermal processing techniques (Bialka, Demirci, & Puri, 2008; H. Chen, 2007; Rodrigo, Barbosa-Canovas, Martinez, & Rodrigo, 2003). The logarithm of the survivors at a given time *t* was calculated from the constants in the Weibull model

including p as a dimensionless shape parameter and δ as a parameter describing the time required to achieve the first 1-log reduction of the surviving population.

The mathematical formula used to describe the inactivation kinetics for the two treatments can be written as (Couvert, O., Gaillard, S., Savy, N., Mafart, P., and Leguérinel, 2005):

$$\log N = logN_0 - \left(\frac{t}{\delta}\right)^p$$

The specific values for each parameter used in the two equations and their R^2 values can be found in Table 2.

The above data has also been fitted into a log-linear model described by Ball and Olson (Ball & Olson, 1957) (result not shown on graph), where the logarithm of the survivors at a given time t was calculated from the constants in the log-linear model with parameter D as the decimal reduction value.

The mathematical formula used to describe the inactivation kinetics for the simultaneous 15 mM BA and UV-A treatment and simultaneous 10 mM BA, 1 mM EDTA and UV-A treatment can be written as (Ball & Olson, 1957) with D value at 6.27/ 6.49 (minute) and R^2 value at 0.94/ 0.95, respectively:

$$\log N = logN_0 - \frac{t}{D}$$

Since the Weibull regression model had higher coefficient of determination value than the log-linear model, it was adopted in this experiment to give a better statistical account for a growth-time distribution (Van Boekel, 2002). However, the model does provide information on the mechanism of inactivation. The p value could be used to help determine the susceptibility of bacteria towards the stress(es) being

applied (Van Boekel, 2002). Since in both cases, the *p* values were all larger than 1, suggesting that the remaining cells became increasingly susceptible to the treatment. The treatments will induce cumulative stress(es) on bacteria, such as membrane damage, production of ROS and decrease of intracellular pH.

Combined Treatments	UV-A	Dark
Water	0.04 ± 0.08^{c}	$< 0.01^{c}$
8 mM BA	0.89 ± 0.36^{c}	0.02 ± 0.1^{c}
10 mM BA	0.86 ± 0.13^{c}	0.09 ± 0.12^{c}
15 mM BA	5.80 ± 0.28^{a}	0.77 ± 0.10^{c}
15 mM BA (pH6.2)	0.05 ± 0.07^{c}	$< 0.01^{c}$
1 mM EDTA	0.24 ± 0.08^{c}	0.06 ± 0.13^{c}
8 mM BA+1 mM EDTA	4.46 ± 0.79^{b}	0.05 ± 0.02^{c}
10 mM BA + 1 mM EDTA	6.00 ± 0.07^{a}	0.33 ± 0.01^{c}
8 mM BA+1 mM EDTA+Metal Ions	3.62 ± 0.73^{b}	0.06 ± 0.02^{c}

Table 1. The Antimicrobial activity of the combined UV-A and BA + EDTA treatments against *E. coli* O157:H7. Results are presented as reduction in bacteria population. (Unit: Log (CFU/mL)). Results sharing the same small letters are significant at $p < 0.05$ according to Tukey's HSD test

Treatment	p	δ	Log N_0	Coefficient of Determination (R^2)
UVA + 15 mM BA	2.5605	12.9124	7.0779	0.9998
UVA + 10 mM BA + 1 mM EDTA	1.8411	11.07	7.07	0.9957

Table 2. Parameters for inactivation kinetics model of the combined BA/ EDTA + BA and UV-A treatment

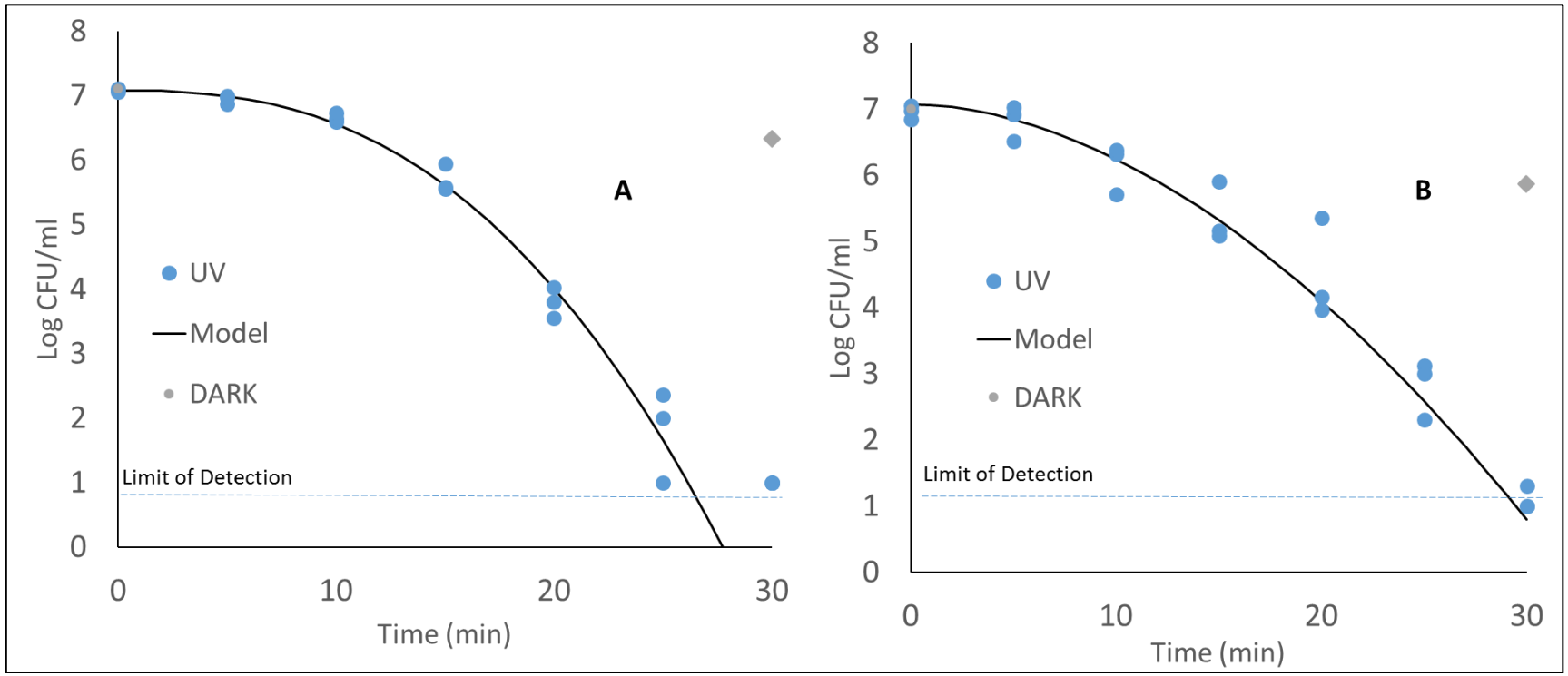


Figure 2: Inactivation kinetics for a simultaneous (A) 15 mM benzoic acid + UV-A light treatment/ (B) 10 mM benzoic acid + 1 mM EDTA+ UV-A light treatment

2.2.2 Antimicrobial activity of the sequential treatment between UV-A and benzoic acid

As shown on Figure 2, besides the simultaneous treatment, the antimicrobial effect of the sequential treatment between UV-A and BA was also evaluated. Bacteria first exposed to UV-A for 30 minute followed by 30 minute of incubation in 15 mM BA in dark (Fig. 3A) showed a reduction of 3.98 ± 0.66 log (CFU/mL), while treatment with the reverse sequence (BA first, then UV-A exposure) (Fig. 3B) reduced the bacterial population by 5.35 ± 0.94 log (CFU/mL). There was no significant difference between the BA-UV sequential treatment and the simultaneous treatment ($p = 0.54$), but the UV-BA sequential treatment had lower antimicrobial activity than the simultaneous treatment ($p = 0.02$). The explanation behind these findings may be that the bacteria exposed to stresses from one treatment make them become more susceptible to the subsequent treatment. BA and UV-A treatments may induce different stresses separately, and the sequence between the stresses may have different impact on how bacteria handling those stresses. For example, exposing the bacteria first to BA is likely to induce pH change inside bacteria cytoplasm, which may induce protein denaturation thus affect the activity of the enzymes that are crucial for bacterial metabolic activity, including enzymes such as superoxide dismutase and catalase that are responsible for maintaining intracellular oxidative stress (Smith & Raven, 1979). With the breakdown of the bacterial defense system against excessive oxidative stress, a lethal dose of ROS could soon be generated during the following UV-A treatment that leads to cell death. A simultaneous treatment between BA and UV-A, on the other hand, is likely to induce a combination of different stresses including cell membrane damage, cell acidification and ROS

generation at the same time, which could exceed the maximum stress threshold that bacterial cells could uphold, leading to their death eventually.

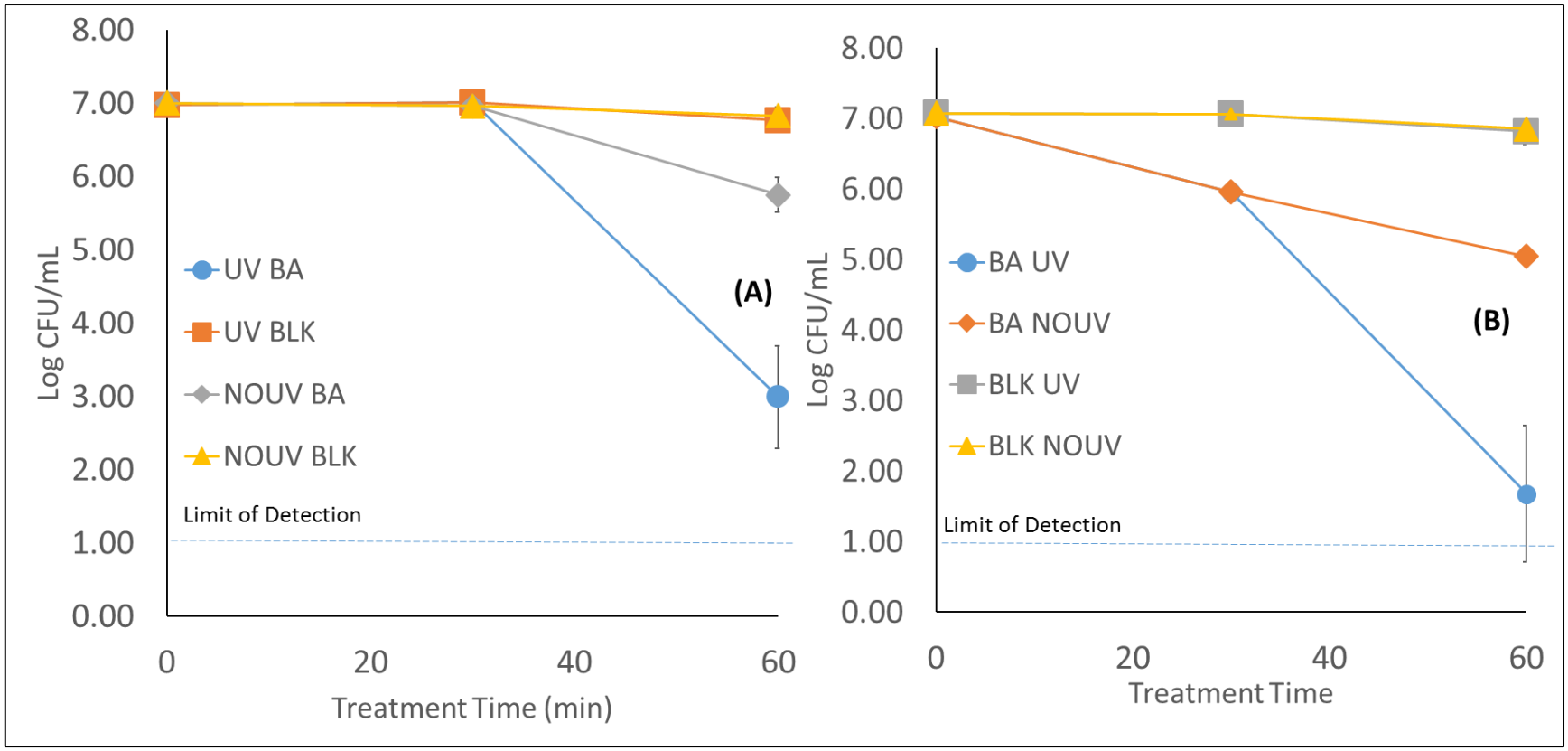


Figure 3: The antimicrobial activity from a sequential (A) BA-UV and (B) UV-BA treatment. Error bars represent the standard deviation of three observations

2.2.3 Change of membrane integrity during the simultaneous UV-A and BA treatment (Propidium iodide assay)

Figure 4 shows the results of propidium iodide (PI) uptake for bacteria treated with various treatments. The fluorescence signal from PI increases when it penetrates cells with damaged cytoplasmic membrane and intercalates to DNA with no sequence preference (Berney, Hammes, Bosshard, Weilenmann, & Egli, 2007) (Stiefel, Schmidt-Emrich, Maniura-Weber, & Ren, 2015). Therefore, it was used as an indicator for damaged cell membrane after various treatments. Results in Fig 4 suggested that the bacteria exposed to a combined EDTA and UV-A treatment presented higher fluorescence intensity than either EDTA or UV-A treatment alone ($p < 0.01$), and were therefore set as the reference to calculate the relative fluorescence value (as in Relative Fluorescence Unit (RFU)) for other treatments. Sample treated with UV-A alone for 30 minute had a signal intensity of 0.23 ± 0.06 RFU, which was higher ($p = 0.04$) than control sample in water receiving no UV-A exposure (0.10 ± 0.02 RFU). The fluorescence intensity for sample treated with 1 mM EDTA in dark was 0.28 ± 0.03 RFU, which was also higher ($p < 0.01$) than the control incubated in in dark. The results above indicated that both EDTA and UV-A treatment could cause cell membrane damage by themselves, while a much higher extent of membrane damage can be induced by applying the two treatments together. However, it can be noticed from results in Table 1 that neither UV-A nor EDTA treatment could achieve significant bacterial inactivation when used alone, indicating that the membrane damage caused by the two treatments are only sub-lethal and additional stresses from BA were required for an efficient inactivation.

It is apparent that not only EDTA but also UV-A could interfere with membrane integrity. It has been shown in previous studies that UV-A irradiation may induce lipid peroxidation and cause leakage in liposomal membrane (Bose & Chatterjee, 1995). The production of ROS by UV-A mediated photooxidation may lead to peroxidation of membrane phospholipids, which can affect cellular ion pumps and therefore increase membrane permeability (Bose & Chatterjee, 1995). The outer membrane of Gram negative bacteria acts as a barrier that protects cells against chemicals in extracellular environment. Its structure is supported by the crosslink between lipopolysaccharide molecules and divalent cations including Mg^{2+} and Ca^{2+} (Alakomi, Saarela, & Helander, 2003). EDTA can potentially chelate those stabilizing divalent cations, leading to the release of LPS and increasing membrane permeability (Alakomi et al., 2003).

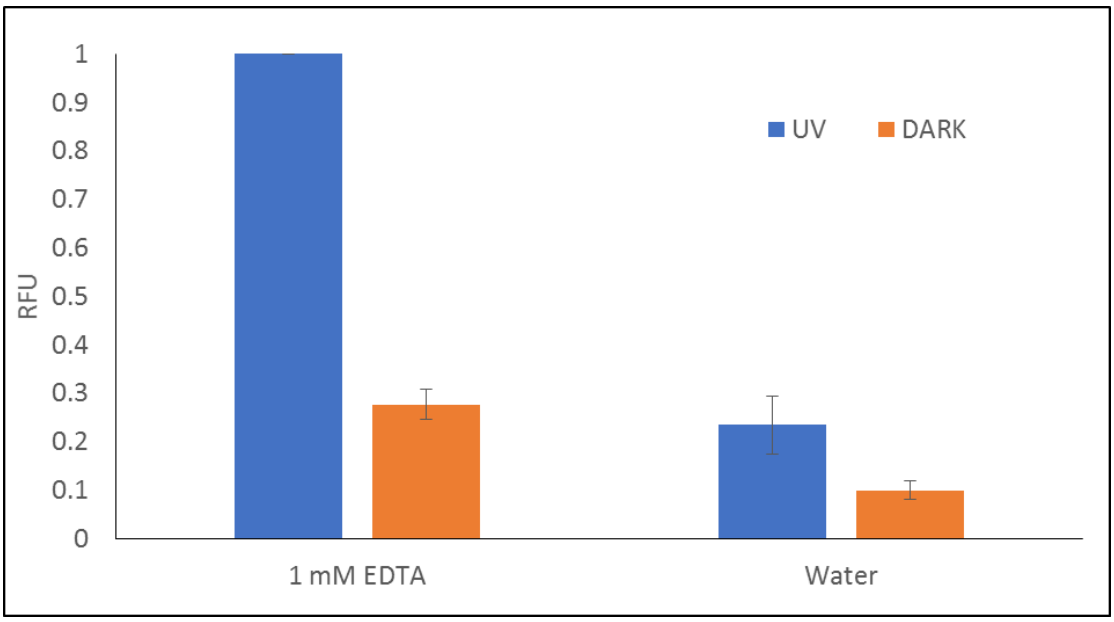


Figure 4: Detection of membrane damage induced by a combined 1 mM EDTA and UV-A treatment (propidium iodide assay). Error bars represent the standard deviation of three observations.

2.2.4 SEM

SEM was used in this study to directly visualize the morphological changes in *E. coli* treated with UV-A and BA. In Figure 4, it could be noticed that the control bacterium (Fig. 5A1-5A3) had a unique rod shape of *E. coli* with a smooth cell surface, while bacterium in samples receiving simultaneous UV-A and BA treatment (Fig. 5B1-5B3) was shown to have surface that was far more uneven and appeared shriveled, further confirming the damage to the cell wall and membrane from the combined treatment.

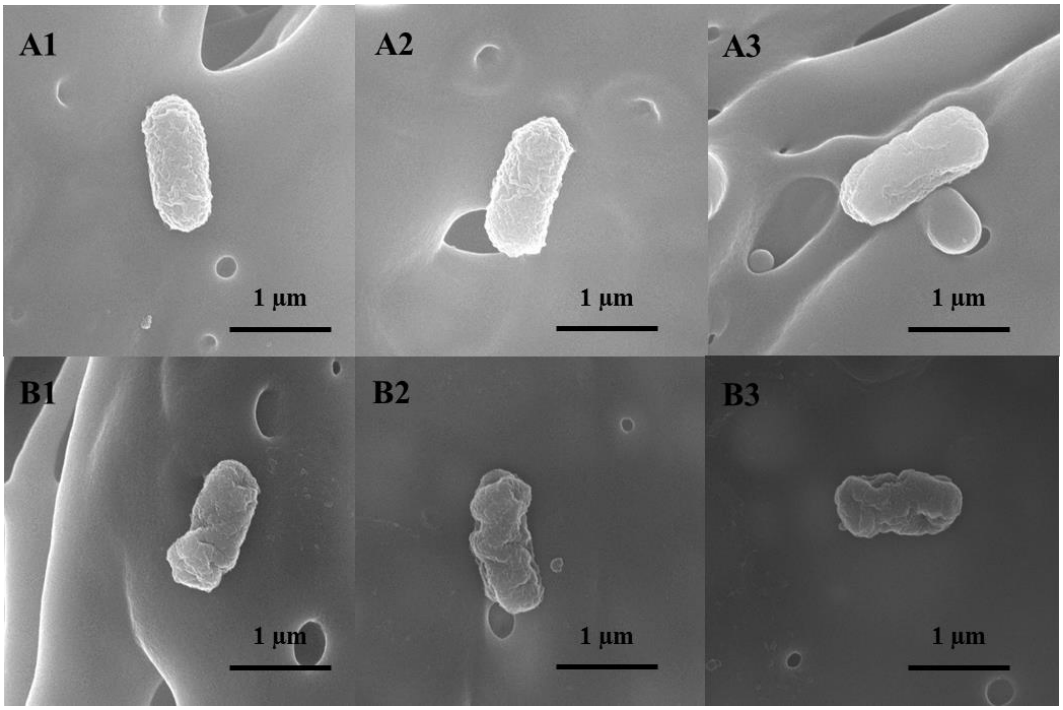


Figure 5: SEM images on *E. coli* O157:H7 after a simultaneous 15 mM BA and UV-A treatment

2.2.5 Generation of the oxidative stress during the simultaneous UV-A and BA treatment

Fig. 6A shows the relative concentration of free thiols in bacteria exposed to various treatments. The intracellular thiol content was measured as an indicator of redox state of *E. coli* using Thiol Fluorometric Detector, which reacts with free thiol groups (such as glutathione) in lysed bacterial solution and emits fluorescence signal. A lower fluorescence signal reflects a lower concentration of free thiols, indicating a high intracellular oxidative stress. The fluorescence intensity for the control sample incubated in water in dark was set as the reference value to calculate the relative fluorescence value for other treatments in the experiment. Results in Fig. 6A suggested that bacteria treated with UV-A and 15 mM BA together for 30 minute was 0.14 ± 0.02 RFU, while the value for sample treated with UV-A treatment at pH3 for the same amount of time was 0.73 ± 0.16 RFU. The thiol concentration in bacteria treated with UV-A and BA treatment simultaneously was therefore significantly lower ($p < 0.01$) than the treatment with UV-A alone, indicating that treatment with BA contributes to the increase of intracellular oxidative stress. However, in neither the 30 minute treatment nor the 5 minute sub-lethal treatment tested could we observe any significant difference between samples treated with UV-A and samples incubated in dark for the same amount of time ($p > 0.07$), making it difficult to determine the role of UV-A during the simultaneous BA and UV-A treatment in affecting the intracellular oxidative stress. Bacterial cells will release free thiol compounds into the cytoplasm as a response to the oxidative stress caused by intracellular activity like metabolic activities or extracellular stress such as UV-A exposure (Cabiscol et al., 2000). However, when bacteria are treated with BA, the internalized organic acid

molecules will cause change in intracellular pH and therefore affect the efficacy for bacteria to adjust its intracellular oxidative stress, leading to a large decrease in free thiol concentration, regardless of whether the bacterial cells received the UV-A treatment. It has been shown that acid stress can upregulate the transcription of a number of oxidation damage response genes, including katG, trxC, and regulatory RNA oxyS, indicating an increase in oxidative stress (King, Lucchini, Hinton, & Gobius, 2010) experienced by bacteria. The CellROX assay, which could directly monitor the generation of ROS, was therefore employed to further tease out the mechanism.

The CellROX Green reagent is a cell permeable fluorogenic probe used to measure the intracellular ROS levels. It has been shown in endothelial cells that the CellROX Green reagent can specifically detect menadione-induced ROS including hydroxyl radicals (Molecular Probes, 2012) (Kim, Shin, Sohn, & Lee, 2014). The probeexhibits strong fluorescence signal under oxidation state while being non-fluorescent at its reduced state (Molecular Probes, 2012). In Fig. 6B, the fluorescence intensity for the 30min combined UV-A and 15 mM BA treatment was significantly higher than the fluorescence signal for treatment with 15 mM BA alone ($p < 0.01$) or treatment with UV-A alone ($p < 0.01$), which was set as the reference value for the experiment. Although lower than the signal from 30-minute treatment with 0.3% H_2O_2 at the same pH (pH3) (3.57 ± 0.27 RFU, data not shown on the graph), the simultaneous UV-A and BA treatment was still able to induce a higher amount of ROS production than treatment with either UV-A or BA alone, suggesting that the signals detected from the simultaneous treatment could be a result from both

internalized BA and UV-A radiation. The fact that the simultaneous BA and UV-A treatment generated more ROS inside the treated bacteria than the treatment with pH 3 water could be contributed to (a) the internalized BA diffused inside cell cytoplasm and forms acid anions, which will accumulate inside the cells, generating high turgor pressure and leading to production of ROS (b) ROS was produced through the photooxidation of internalized BA with UV-A. After the pH of the 15 mM BA solution was adjusted to 6.27 with PBS, the fluorescence signal for samples received 30 minute of combined BA and UV-A treatment was only 0.21 ± 0.01 RFU, which was significantly lower than the simultaneous treatment at its original pH (pH3) ($p < 0.01$). It proves that extracellular BA was not responsible for the increased concentration of ROS. During the 5-minute sub-lethal treatment, the fluorescence intensity for the simultaneous UV-A and BA treatment (0.90 ± 0.04 RFU) was still higher than 15 mM BA treatment in dark for the same amount of time (0.56 ± 0.02 RFU) ($p < 0.01$), which validates that ROS production precedes microbial inactivation.

Dimethyl sulfoxide (DMSO) is a specific and effective free radical scavenger for hydroxyl radicals (Reilly, Schiller, & Bulkley, 1991). It can penetrate cell membrane and act directly at the intracellular sites of free radical production (Jacob & Herschler, 1986). Therefore, it was used in this experiment to validate the role played by hydroxyl radical in the antimicrobial effect of the simultaneous UV-A and BA treatment. Fig. 6C indicates that sample treated with simultaneous UV-A and BA treatment for 30 minute had 5.97 ± 0.06 log (CFU/mL) reduction, while an addition of 5% DMSO to the simultaneous treatment induced a significantly lower bacterial

reduction by only 4.74 ± 0.44 log (CFU/mL) during the same amount of time. The significant difference ($p = 0.02$) between these two treatments indicated that hydroxyl radicals were generated during the simultaneous UV-A and BA treatment and were one of the dominant ROS that contributed to the intracellular oxidative stress increase. Hydroxyl radicals are generally formed through reduction of hydrogen peroxide, which is generated during the one-electron reduction of molecular oxygen (Krumova & Cosa, 2016). This process could take place during both the metabolic reaction of bacterial cells and the exposure to UV-A light (Pattison & Davies, 2006). With their antioxidant enzyme system being deactivated at low intracellular pH, the accumulation of hydroxyl radicals inside bacterial cells may induce severe oxidative damage. This result also proves that the ROS induced intracellular oxidative stress change is a critical antimicrobial factor of the simultaneous treatment and generation of hydroxyl radical seems to be one of the causes behind the observed antimicrobial effect. It is possible that other ROS, such as singlet oxygen, superoxide anion and peroxyl radicals may have also been produced during the process and inducing intracellular oxidative stress change together with hydroxyl radicals.

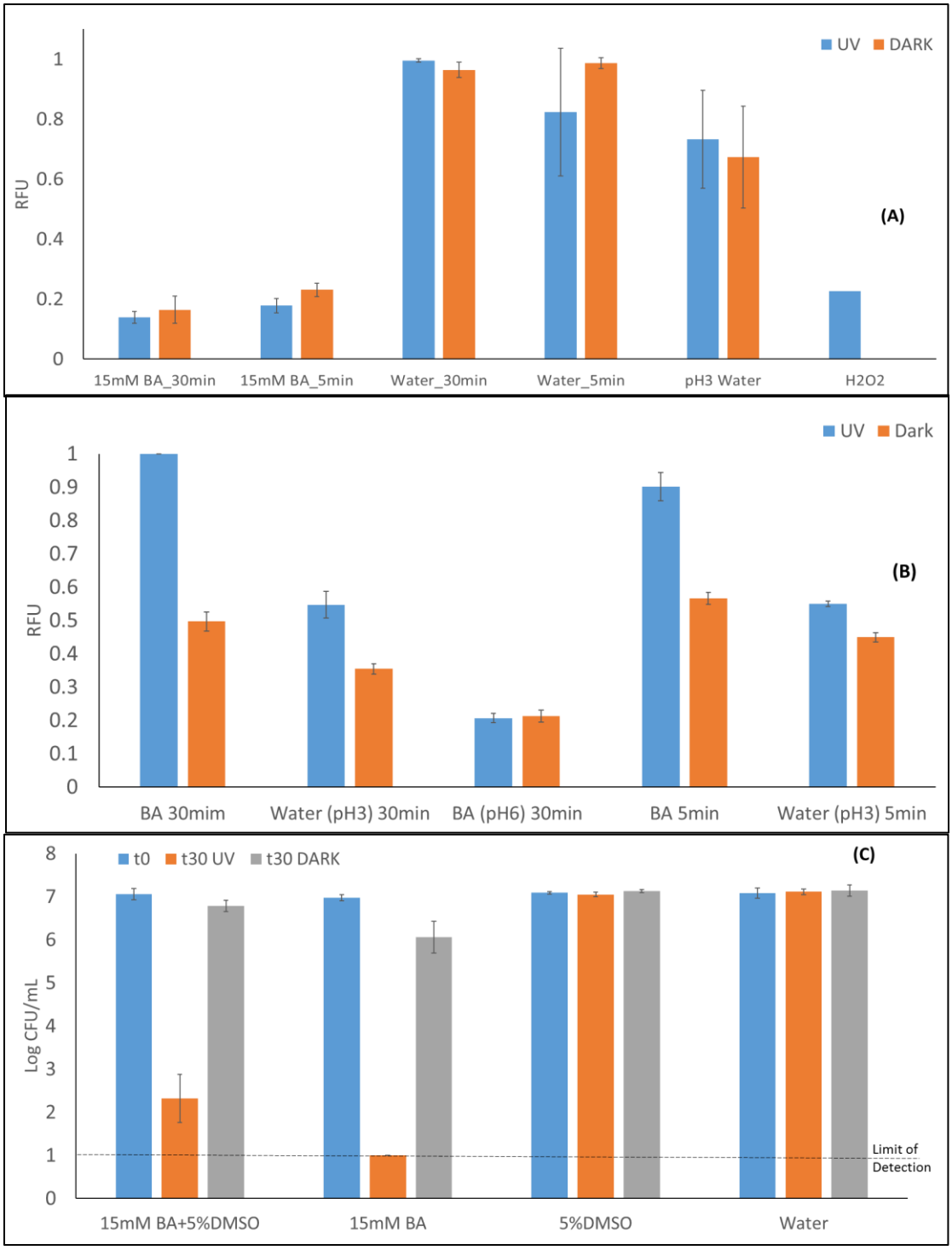


Figure 6: Detection of intracellular oxidative stress induced by a simultaneous 15 mM BA and UV-A treatment using (A) Thiol Oxidation Assay (B) CellROX Assay, and (C) Effect of a ROS inhibiter on antimicrobial activity of a simultaneous 15 mM BA + UV-A treatment. Error bars represent the standard deviation of three observations.

2.2.6 Change of intracellular pH during the simultaneous UV-A and BA treatment

The pHrodo™ Green AM Intracellular pH Indicator is a fluorogenic probe that can permeate cell membranes and is retained inside cells once cleaved by nonspecific esterase. The probe is weakly fluorescent at neutral pH, but the intensity increases with a decrease in pH (LifeTechnologies, 2013). Fig. 7A shows that as the intracellular pH decreased, the fluorescence signal of the probe increased in a linear manner ($R^2 = 0.94$). Therefore, by measuring the fluorescence intensity of different samples, we can calculate the actual intracellular pH value by using the standard curve being plotted on Fig. 7A. As shown on Fig. 7B, bacteria treated with 15 mM BA alone had lower pH_i value than bacteria treated with water in dark ($P < 0.01$), which corresponds to the effect of benzoic acid to penetrate cell membrane and dissociate inside cytoplasm to cause decrease of pH_i. However, it is worth noticing that there was no significant difference between the bacteria treated with UV-A in water (either at pH 3 or neutral pH) with bacteria incubated in dark at corresponding pH values ($P > 0.05$). Interestingly, the intracellular pH value for bacteria treated with UV-A and 15 mM BA simultaneously was 3.77 ± 0.25 unit, which was lower than pH_i of bacteria treated with 15 mM BA in dark (4.70 ± 0.13 unit) or UV-A alone at the same extracellular pH (pH3) for the same amount of time (4.59 ± 0.24 unit) ($p = 0.02/ 0.03$). Since we have demonstrated earlier that UV-A can increase the membrane damage, this observation supports the hypothesis that exposure to UV-A increased the uptake of BA thus lowering the cytoplasmic pH by one order of magnitude. Fluorescence micrographs for the bacteria in samples treated with the simultaneous UV-A and BA treatment was shown on Fig. 7C, which validated that

the fluorescence signal detected in the experiment originated from bacterial cytoplasm.

The decrease of pH_i was mainly contributed by the dissociation of internalized BA, where the undissociated BA molecules were placed in an environment with near neutral pH and were therefore forced to dissociate into charged ions, including protons and BA anions, both of which would be trapped inside cell plasma (Lambert & Stratford, 1999). The increase of proton concentration inside the plasma induced the decrease of pH_i and may cause disruption to cellular metabolic activities such as glycolysis (Krebs et al., 1983) and inhibition of active transport (Freese et al., 1973). As a cellular response towards the disruption on pH_i, it will start to remove the excessive protons through an efflux pump, which requires the consumption of energy as in ATPs (Warth, 1988). With the membrane being interrupted by UV-A, the continuous influx of BA may eventually lead to ATP depletion and cell death.

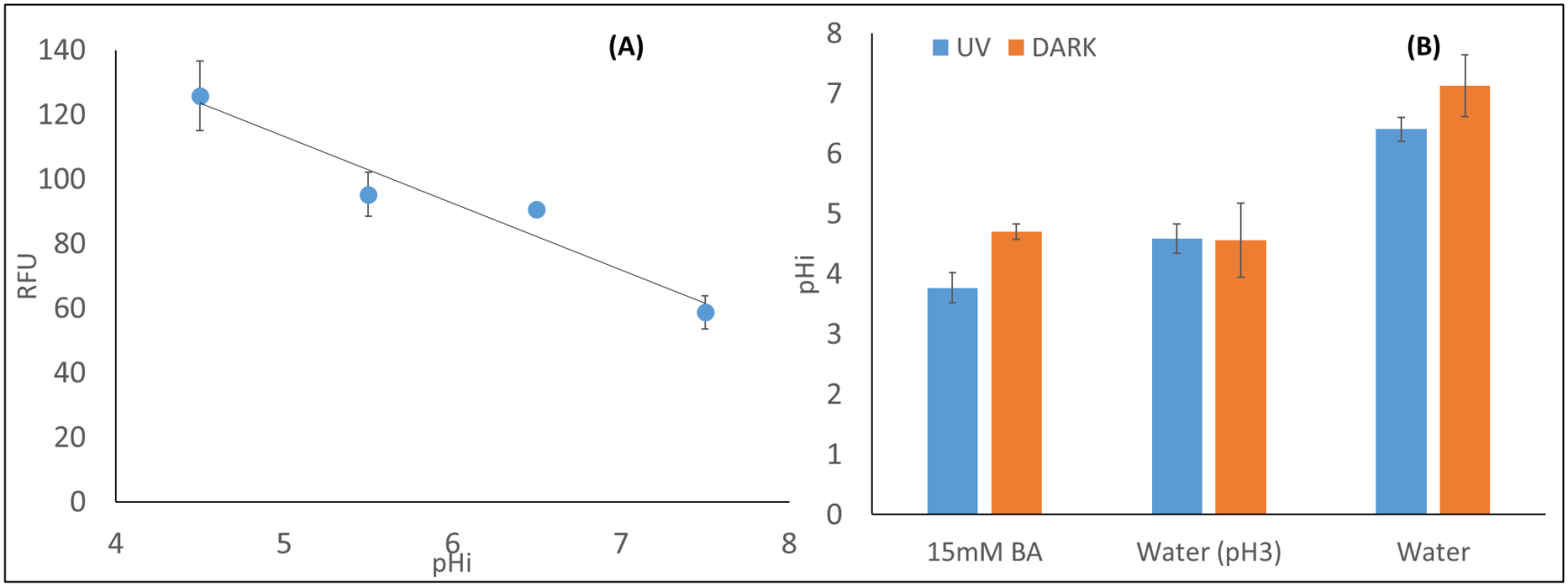


Figure 7: Detection of intracellular pH induced by a simultaneous 15 mM BA and UV-A treatment (A) standard curve (B) Intracellular pH of *E. coli* after the treatment. Error bars represent the standard deviation of three observations.

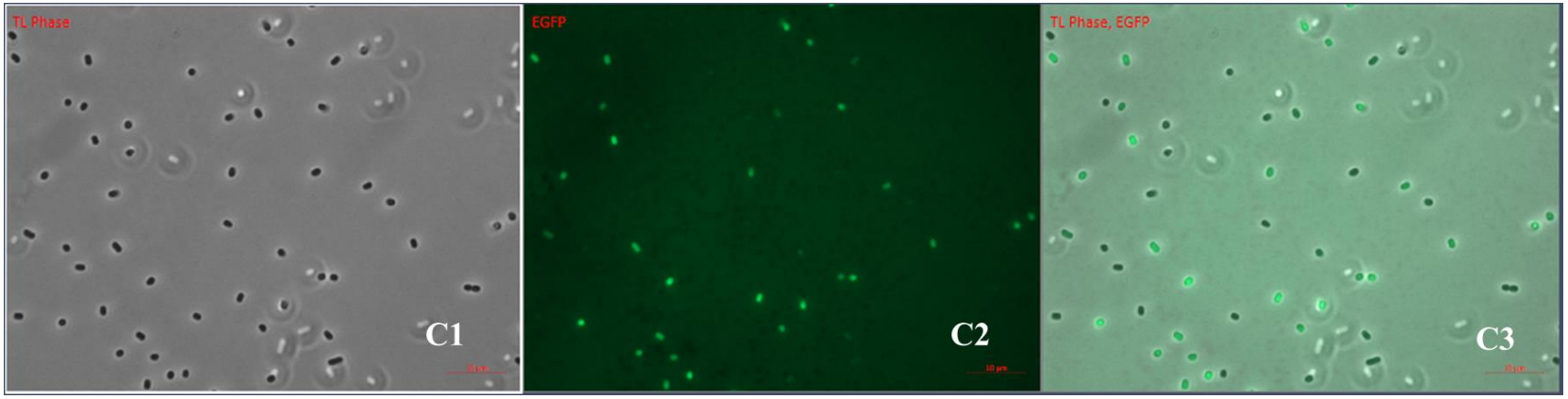


Figure 7C: Fluorescence microscopic images for pHrodo Assay during a simultaneous 15 mM BA and UV-A treatment (C1) TL Phase (C2) EGFP (C3) Combined TL Phase & EGFP

2.2.7 Application of the simultaneous UV-A and BA treatment in a simulated continuous flow processing environment

UV treatments are typically performed in a continuous, flow-through systems (Keyser, Muller, Cilliers, Nel, & Gouws, 2008; Koutchma, Parisi, & Unluturk, 2006). Therefore, we investigated whether the proposed treatment was able to retain its effectiveness in a scaled-up, continuous system. Figure 8 indicates that 10mM BA combined with simultaneous UV-A treatment received around 0.6 log (CFU/mL) reduction after approximate nine passes through the reactor (35 minute). While under the same settings, 15mM BA could effectively reduce bacteria population by 4 log (CFU/mL) together with UV-A light during the same amount of treatment time. As can be seen from the result, similar to the batch system, bacterial population could be effectively reduced after the simultaneous treatment between UV-A light and BA at the simulated continuous flow environment and the increase of total treatment volume (from 2 mL to 3.5 L) did not impair the antimicrobial effect of our proposed treatment. However, the treatment time was significantly higher than what is typically desirable for sanitation operations, which is around 15 seconds or less (Duffy et al., 2000; Koutchma, 2008; Koutchma et al., 2006). We attribute this mainly to the un-optimized nature of the equipment we used. An optimum UV system for non-water applications would typically be designed to accommodate more than one UV lamp (CiderSure) (Hanes et al., 2002) and to generate (a) a thin film of liquid within UV reactors that would counter the increased absorbance of the solution due to presence of various solutes (BA), or (b) a turbulent flow within a UV reactor to achieve an equal distribution of UV exposure for the entire solution (Koutchma et al., 2006). However, the UV systems that are currently prevalent in the market are designed for

sanitation of water that typically does not contain any UV-absorbing additives (such as BA) and are designed for UV-C lamps. Since we made use of one of these available systems, it had significant design constraints such as: (a) use of a single UV-A lamp, (b) relatively large flow thickness (42 mm) that prevented effective penetration of UV-A light in UV absorbing solution (absorbance for 15 mM BA at 365 nm was at 0.06). Despite these constraints, we were able to achieve promising results for the scaled-up treatment. Nevertheless, this study highlights the need for specifically designing UV-A/B/C equipment for food industry, where the solutions typically have high absorbance values.

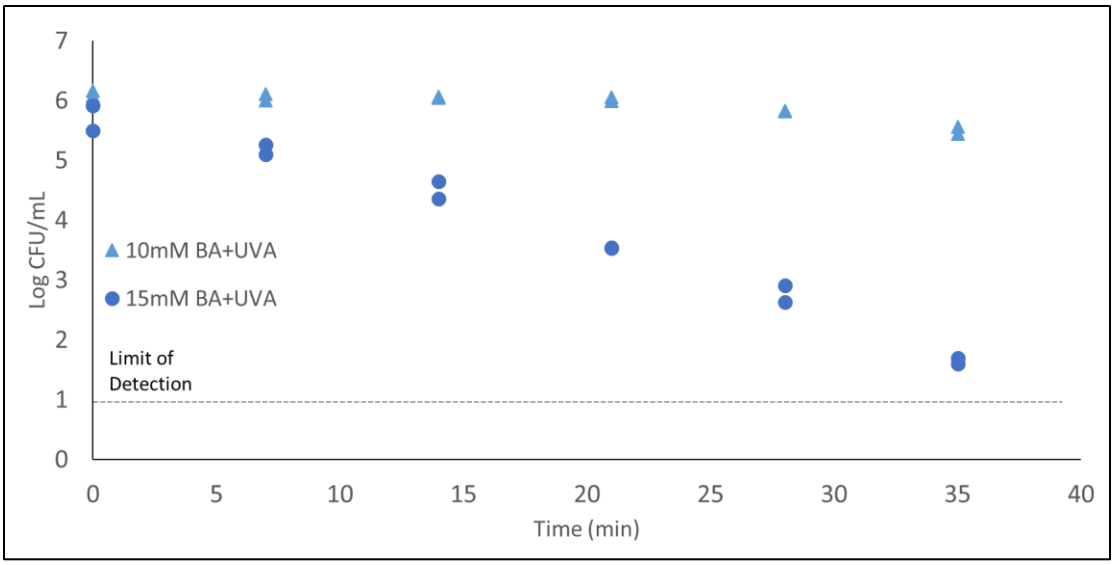


Figure 8: The antimicrobial activity of a simultaneous 15 mM BA and UV-A treatment within a continuous flow system

3 Chapter 3: The antimicrobial effect of propyl paraben treatment combined with UV-A light on *Escherichia coli* O157:H7

3.1 Methods and materials

3.1.1 Investigation of antimicrobial activity of simultaneous treatment between UV-A light and parabens against *E. coli* O157:H7

Methyl, Ethyl or Propyl Paraben (MP, EP and PP) (Acros Organics, NJ, USA) was prepared in 10% ethyl alcohol solutions that was sterilized by passing through a 0.2 μm syringe filters. Overnight incubated *E. coli* culture was diluted in sterilized DI water to reach a concentration of approximately 1×10^8 CFU/mL. It was further diluted with sterilized paraben solutions to achieve final concentrations at 0, 2, 3, and 5.5 mM for PP and 5.5 mM for MP and EP with approximately 1×10^7 CFU/mL for bacteria (only one replicate was conducted for treatments with 5.5 mM MP, EP, and PP). 2 mL of the solutions prepared as described above was transferred into a well of a 6-well flat bottom polystyrene plate and exposed to UV-A light for up to 30 minute. Controls for this experiment consist of incubating bacteria and PP in dark and exposing bacteria to UV-A light in the absence of PP for the same amount of time. Samples were obtained periodically during the UV exposure, serially diluted in 0.2% buffered peptone water and 100 μL solution of each dilution was plated on Eosin methylene blue agar (EMB). Only colonies displaying a distinctive metallic green sheen color were identified as *E. coli* colonies to be counted. All agar plates were incubated overnight at 37 °C before counting.

3.1.2 Investigation of the cell membrane integrity during PP and UV-A treatments

Propidium iodide was used as an indicator of the integrity of cell membrane. A treatment sample containing 1×10^9 Log/mL *E. coli* and 0 or 3 mM PP dissolved in 10% ethanol was exposed to UV-A for 30 minute. A control sample was incubated in dark for the same amount of time. 1 mL of the incubated sample was then washed once with sterilized DI water and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was then re-suspended in 5 μM PI solution and incubated at room temperature for an additional 15 minute. The incubated solution was further washed with 1 × PBS and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was resuspended in 500 μL of 1 × PBS. 100 μL of the solution was transferred into an opaque 96 well plate separately. The fluorescence intensity was measured at an excitation wavelength of 535 nm and an emission wavelength of 617 nm on a SpectraMax M5e microplate reader (Molecular Devices, CA, USA).

3.1.3 Prevention of cross contamination on spinach leaves in simulated wash water with the simultaneous treatment between UV-A light and propyl paraben

5 pieces of organic baby spinach (Nature's Promise Organic, products purchased from local supermarket) leaves with no visual cracks or injures on their surface were collected and rinsed in 20 mL sterilized DI water separately, air dried in sterilized petri dishes (Fisher Scientific, Waltham, MA, USA) until further needed. Overnight incubated *E. coli* culture was washed once with sterilized DI water at 7830 rounds per minute (rpm) for 10 minutes and then diluted ten times to reach a concentration of approximately 1×10^8 CFU/mL. Two leaves were placed in 15 mL diluted bacteria cultures separately and incubated for 30 minutes at room temperature

before they were removed from the solution and air dried in sterilized petri dishes for another 30 minute to have bacteria attached to the surface of leaves. One of the inoculated leaves was used to determine the initial bacterial load before treatment. 3 non-inoculated leaves were mixed with the other inoculated leaf and 30 mL of either 0 or 3 mM PP solution in a sterilized petri dish and constantly stirred on a portable magnetic stirrer (Cole-Parmer, Vernon Hills, IL, USA) inside the UV crosslinker described previously. After 30 minute of UV-A exposure, each leaf (inoculated and non-inoculated) was weighed separately after being taken out of the solution. Each leaves was added to a sterilized stomach bag separately (WHIRL-PAK, Nasco, Fort Atkinson, WI, USA) together with nine times as much of sterilized 0.2% peptone buffer. Samples were obtained after stomaching at normal speed for 5 minutes with a Seward stomacher (Seward, Davie, FL, USA), serially diluted in 0.2% buffered peptone water and 100 µL solution of each dilution was plated on Trypticase™ soy agar (TSA). All agar plates were incubated overnight at 37 °C before counting.

3.1.4 Evaluation of color change on spinach leave after combined PP and UV-A treatment

After being treated in the simulated wash water using the simultaneous 3 mM PP and UV-A treatment as described above, the changes on the visual color of the spinach leaves before and after treatments were evaluated using with a HunterLab colourimeter model EZ-45/0 CX2405 (Hunter Associates Laboratory, Reston, VA, USA) calibrated with standard white and black tiles. Data was recorded as in Hunter *L* (lightness), *a* and *b* values. The maximum *L* (100) represented a perfect reflect diffuser, while the minimum of it (0) stands for black. The *a* value measure redness and greenness, with positive value towards red and negative value towards green. The

b value measure yellowness and blueness, with positive value towards yellow and negative value towards blue (Nisha, Singhal, & Pandit, 2004). When used together, the uniform Hunter *L, a, b* color scale can give a good indication of sample color based on the numeric values. Three leaf samples before and after treatments were individually placed above the light source and covered with the black cover from the instrument, which would instantly give the Hunter *L, a* and *b* values for each sample.

3.1.5 Statistical test

Unless specified above, all experiments were performed in triplicate. The significance between different treatments were calculated via unpaired Student's T test assuming equal variance by using Microsoft Excel 2013 (Seattle, WA, USA). The parameters in the log-linear model were obtained via GRG nonlinear method using the Solver option in the same software.

3.2 Results and discussions

3.2.1 Antimicrobial activity of the simultaneous UV-A and parabens treatment against *Escherichia coli* O157:H7

As indicated in figure 9A, while no significant antimicrobial activity was observed from either 5.5 mM MP or 5.5 mM EP after 30 minute UV-A exposure, while 5.5 mM PP was able to achieve more than 6 Log CFU/mL reduction when incubated in dark for the same amount of time (data not shown). The combined UV-A and PP treatment was equally effective when the concentration of PP was reduced to 3 mM, which was only 60% of the maximum level allowed by the FDA. The results also validate that paraben with longer alkyl chain length has higher antimicrobial activity, making PP the best chemical to be studied in the combined treatment. Samples treated with UV-A light alone or 3 mM PP alone in dark for 30 minute had significantly less antimicrobial effect ($p < 0.01$) than the simultaneous treatment, proving that the combined treatment was synergistic. It can also be noticed that when the concentration of PP in the treatment was lowered to 2.2 mM, the antimicrobial effect of the combined treatment although significant ($p < 0.01$) was lowered to only ~1 log (CFU/mL) reduction. Thus, the simultaneous UV-A light and PP treatment can significantly improve the microbial inactivation rate and the effect of combined treatment was dependent on the concentration of PP.

Figure 9B shows the kinetic of microbial inactivation by the simultaneous UV-A and 3 mM PP treatment. The data was fitted into a log-linear model described by Ball and Olson (Ball & Olson, 1957), which is based on the assumption of a linear correlation between bacterial population and treatment dose. The logarithm of the

survivors at a given time *t* calculated from the constants in the log linear model with parameter *D* as the decimal reduction value.

The mathematical formula used to describe the inactivation kinetics can be written as (Ball & Olson, 1957) with *D* value at 5.37 (minute) and R^2 value of 0.9981:

$$\log N = logN_0 - \frac{t}{D}$$

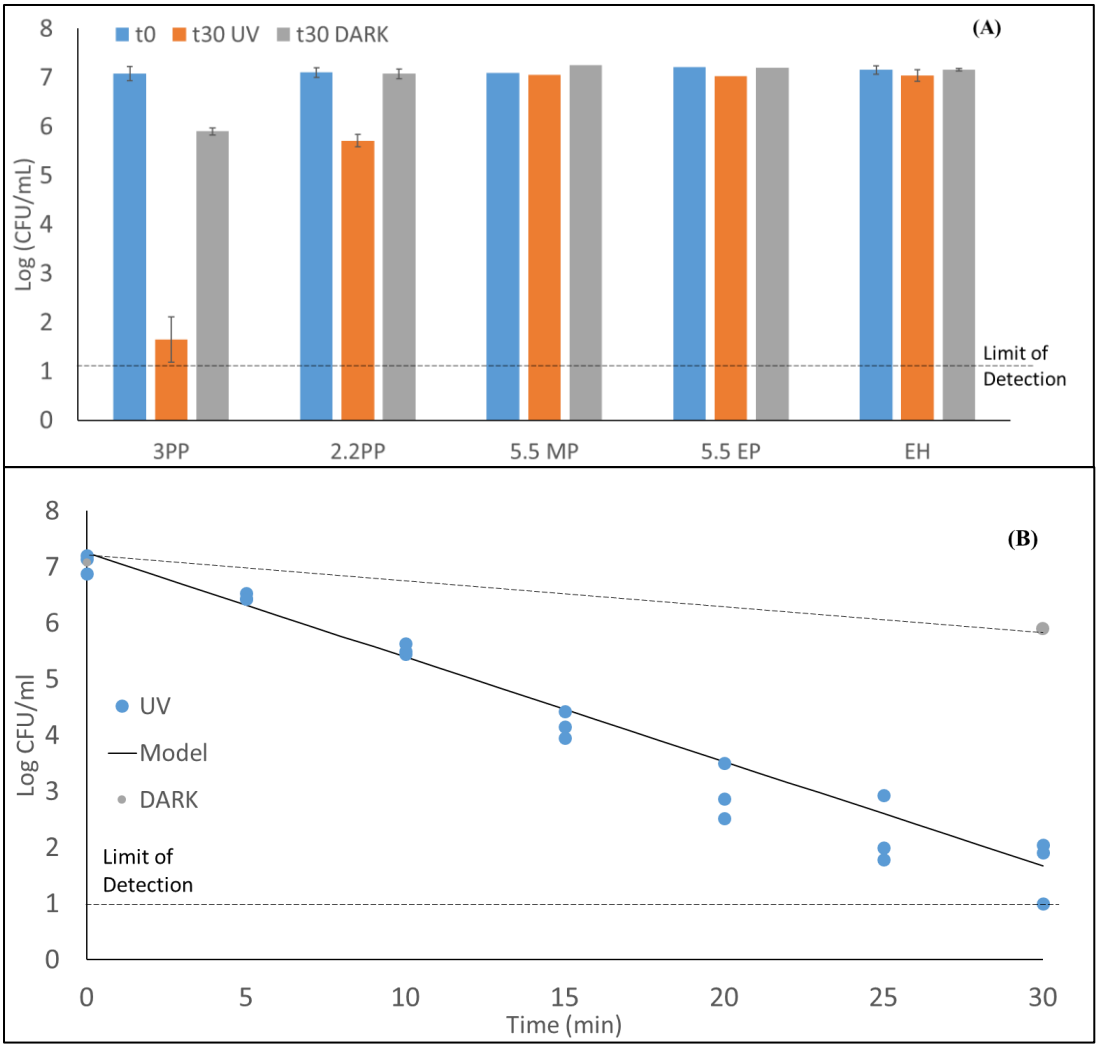


Figure 9: (A) Antimicrobial activity and (B) Inactivation kinetics of a simultaneous treatment between parabens and UV-A; EH represents for 10% ethanol solution. Error bars represent the standard deviation of three observations.

3.2.2 Damage of membrane integrity during the simultaneous UV-A and PP treatment (Propidium iodide assay)

Figure 10 shows the results of propidium iodide (PI) uptake for bacteria receiving the simultaneous UV-A and 3 mM PP treatment. The fluorescence signal from PI increases when it penetrates cells with damaged cytoplasmic membrane and intercalates to DNA with no sequence preference (Berney et al., 2007) (Stiefel et al., 2015). Therefore, it was used as an indicator for damaged cell membrane after various treatments. Results on Fig 4 suggested that the bacteria exposed to a combined PP and UV-A treatment presented higher fluorescence intensity than either PP or UV-A treatment alone ($p < 0.01$), and were therefore set as the reference to calculate the RFU for other treatments. Sample treated with 3 mM PP alone for 30 minute had a signal intensity of 0.43 ± 0.04 RFU, which was higher ($p < 0.01$) than control sample in 10% ethanol treated with or without UV-A light (0.11 ± 0.01/ 0.06 ± 0.02 RFU). The results above indicated that both 3 mM PP and UV-A treatment could induce significant cell membrane damage by themselves, while a much higher extent of membrane damage can be induced by applying the two treatments together, which validate the capacity PP and UV-A to induce a significant damage to bacterial cell membrane and corresponding to the high antimicrobial activity observed from samples receiving the combined treatment. The fluorescence intensity in bacteria treated with simultaneous 3 mM PP treatment is higher than the combined 1 mM EDTA treatment ($p < 0.01$), indicating membrane damage induced by treatment with PP is more significant than treatment with EDTA at the given levels, which is likely to be one of the main mechanism behind the antimicrobial activity of the combined treatment with PP.

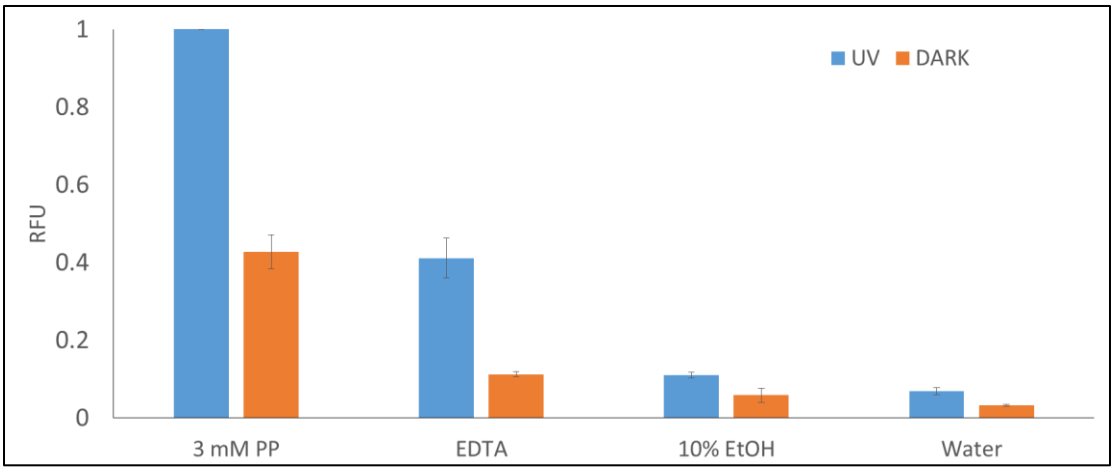


Figure 10: Detection of membrane damage induced by a combined 3 mM PP treatment (propidium iodide assay). Error bars represent the standard deviation of three observations.

3.2.3 Prevention of cross contamination in wash water by the simultaneous UV-A and PP treatment

Being one of the most important leafy vegetables consumed all over the world, the food safety issue related to spinach has always been a major concern for the fresh produce industry (Nisha et al., 2004). Since baby spinach leaves can be consumed as fresh, pureed or processed, the importance to reduce the risk of cross contamination during washing process is becoming increasingly important. As indicated by figure 11, the presence of 3 mM PP combining with UV-A treatment in wash water significantly lowered the contamination level on un-inoculated spinach leaves by ~3 log (CFU/g) after 30 minute of simulated washing treatment ($p < 0.01$), while the simulated washing process with water in dark could only reduce the bacterial load on un-inoculated leaves by only ~1 log (CFU/g). Initial bacteria load at 5 log (CFU/g) is considered as high inoculum level (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008b), and does not reflect real life situation that usually starts at a contamination level below 3 log (CFU/g) (Gombas et al., 2017). Therefore, the simultaneous UV-A and PP treatment can effectively prevent the cross-contamination between leaves during the washing procedure.

Previous studies have shown that lettuce leaf (Wachtel & Charkowski, 2002; Zhang, Ma, Phelan, & Doyle, 2009), fresh cut escarole (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008a) are susceptible to cross-contamination of *E. coli* O157:H7 when contamination source was presented in the wash water. The results above indicated that sanitation method(s) on wash water had significant impact on controlling the level of cross-contamination happened during the washing process. Currently, the most widely used method to treat wash water in the fresh produce

industry is the administration of chlorine due to the its highly efficient antimicrobial activity and cost efficiency (Tomás-Callejas, López-Velasco, Artés, & Artés-Hernández, 2012). However, although being an efficient oxidizer, hypochlorous acid (HClO), as the main chemical species when chlorine dissolved in water, is known for its instability at low pH environments and may lose its antimicrobial effect rapidly (Gombas et al., 2017). Organic load in the wash water may also affect the antimicrobial activity of HClO. The accumulation of organic materials released by the leafy vegetable commodity in wash water, including dissolved and suspended solids, is likely to combine with and depletes the effective chlorine level. Therefore, being a GRAS material with relatively low toxicity (Davidson et al., 2005), the use of PP together with UV-A light treatment can become a substitute for the currently used wash waster sanitation methods.

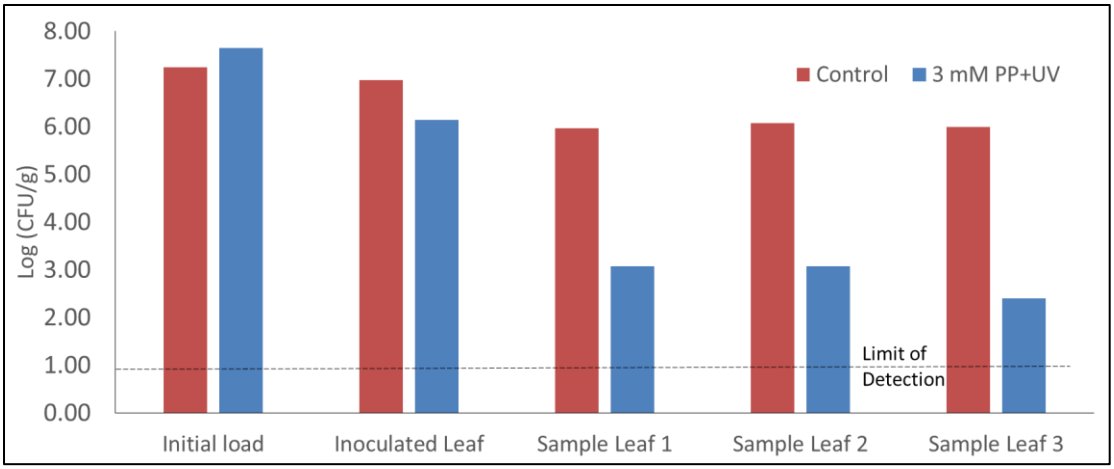


Figure 11: Effect of a combined 3 mM PP and UV-A treatment on preventing *E. coli* cross-contamination during simulated washing process of spinach

3.2.4 Evaluation of color change on spinach leave after combined PP and UV-A treatment

Visually, both samples before and after the treatment exhibited a medium to light green color. Hunter colorimeter data presented on Table 2 indicated that no visual color change was observed during the 30-minute simultaneous treatment between 3 mM PP and UV-A light. There was no significant difference between *L*, *a* and *b* values in spinach leaf samples before and after the combined treatment ($P > 0.05$), suggesting little change in light to dark (*L* value), red to green (*a* value), and yellow to blue (*b* value) color between all samples. Therefore, it can be concluded that the 30-minute PP and UV-A treatment will not induce visual discoloration. Since color poses a significant impact during sensory quality evaluation via visual recognition and surface and subsurface properties assessment on the visual appearance of food products, the acceptance of fresh produce is largely dependent on this property (Nisha et al., 2004) (Klockow & Keener, 2009). Being a treatment with little negative effect from discoloration, the simultaneous UV-A light and PP treatment has been proved to have large potential to be applied during fresh produce processing.

	L	**a**	**b**
Before Treatment	42.08 ± 2.54	-8.92 ± 0.56	20.39 ± 1.88
After Treatment	41.86 ± 2.35	-8.43 ± 0.42	20.27 ± 1.80

Table 3 Hunter *L, a, b* color analysis on spinach received a simultaneous 3 mM PP and UV-A treatment during simulated washing process

4 Chapter 4: Conclusions and suggestions for future studies

The results of this study demonstrated that a combination of UV-A and BA or PP treatment could lead to a significant antimicrobial effect against *E. coli* O157:H7 at an appropriate dose level. The simultaneous treatment could induce more than 5 log (CFU/mL) reduction under bench top settings, which has met the performance criterion defined by FDA for fruit and vegetable juice pasteurization towards the target pathogen of concern (U.S. Food and Drug Administration, 2000b). This synergistic antimicrobial effect could only be observed when the BA or PP and UV-A treatments are applied simultaneously to the target microorganisms. The antimicrobial effect of the combined treatment between BA and UV-A was mainly contributed by the decrease of intracellular pH, the increase of the intracellular oxidative stress, and the membrane damage. For the simultaneous UV-A and PP treatment, membrane damage solely is likely to be one of its main mechanisms of inactivation. EDTA and UV-A have been validated for their role as enhancers for the antibacterial treatment based on their ability to damage membrane integrity thus increasing the permeability of extracellular antimicrobial compound such as benzoic acid. The antimicrobial activity of the simultaneous UV-A and BA treatment was also validated in a continuous flow system, while the combined UV-A and PP treatment has been shown to have inhibitory effect against cross-contamination during the washing process for fresh produce. Both findings indicate significant potential of these two treatments to be adopted by food and fresh produce industry.

Although we demonstrated a novel approach for using the simultaneous BA and UV-A treatment in the sanitation process in a continuous flow system for produce

and food industry and discussed some of the likely mechanisms behind the enhanced inactivation, we still don't have enough evidence to identify the exact mechanism for the generation of ROS from the combined treatment or have a detailed understanding of how do bacterial cells respond to acidic stresses, neither can we confirm if membrane damage is the dominant inactivation mechanism for the combined PP and UV-A treatment or how severe is the damage to cells. Therefore, it may be worthwhile to test the potential ROS production in a simulated intracellular environment with the combined BA and UV-A treatment, as well as to examine the morphology of bacteria via SEM and the leakage of intracellular protein after the simultaneous treatment with PP and UV-A light the future. It may also be interesting to investigate the antimicrobial effect of the two simultaneous treatments on Gram positive pathogen such as *Listeria monocytogen* and to design an optimized continuous flow system to reach the maximum antimicrobial activity of the simultaneous BA and UV-A treatment in commercial food product such as apple cider.

Appendices

Detection of in vitro ROS production in UV irradiated BA

In order to test if BA could generate ROS with UV exposure in vitro, BA solution was exposed to UV light at different wavelengths to investigate the production of hydrogen peroxide and singlet oxygen during this process.

Materials and Methods

The amount of singlet oxygen generated by BA during UV exposure was measured with furfuryl alcohol (FFA). 15 ml of 10 mM benzoic acid solution with an additional 40 µM FFA was exposed to UV-A, B and C light for up to 60 minutes in a UV crosslinker as described previously, respectively. The sample was hold in a crystallizing dish while being stirred continuously with a magnetic stirrer during the treatment. In addition, the solution with same composition was also used as control and was incubated in dark for the same amount of time. 1 ml sample was obtained periodically during the UV exposure for analysis. A set of FFA standard solutions were also prepared at concentrations from 2.5 µM to 40 µM. The FFA concentration in the solutions were measured through high-performance liquid chromatography (HPLC) with a modified method from a previous study (Nayak, Muniz, Sales, & Tikekar, 2016). The column used was a 250*4.6 mm SELECTOSIL C18 column with the column oven temperature set at 33 °C. 84% 0.1N H_3PO_4 - 16% acetonitrile solution was used as the mobile phase with the isocratic flow rate set as 0.6 ml/min. The UV-Vis detector was set at the wavelength of 215 nm. The absorption peak for FFA appeared at around 9.4 minute. The singlet oxygen generation rate was

calculated by measuring the degradation of FFA in samples based on the reaction constant between FFA and singlet oxygen, which is 8.3×10^7 M^{-1} s^{-1} according to literature value (Latch, Stender, Packer, Arnold, & McNeill, 2003).

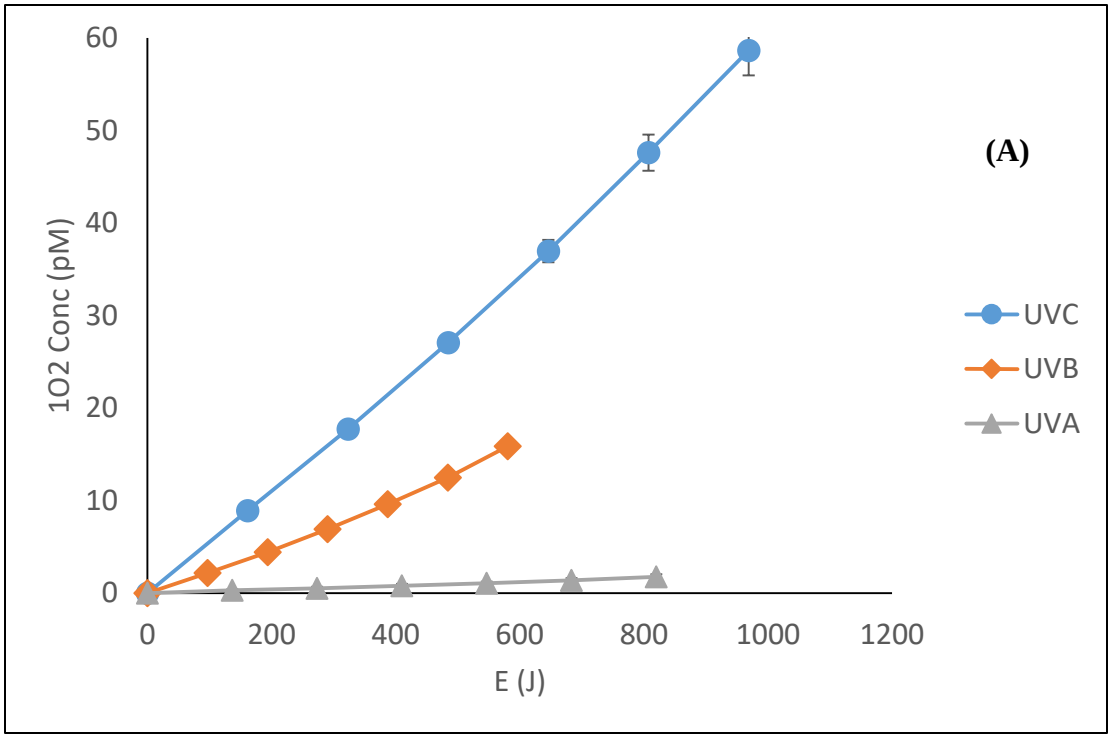
The amount of hydrogen peroxide generated by BA during UV exposure was measured with ferrous oxidation−xylenol orange (FOX) assay (Wang, Durand, Elias, & Tikekar, 2016). The FOX assay reagent was prepared with 250 mM sulfuric acid, 2.5 mM ferrous sulfate, 1 M sorbitol and 1 mM xylenol orange. 15 ml of 10 mM benzoic acid solution was exposed to UV-A, B and C light for up to 50 minutes in a UV crosslinker as described previously, respectively. The sample was hold in a crystallizing dish while being continuously stirred with a magnetic stirrer during the treatment. In addition, the solution with same concentration of BA was used as control and was incubated in dark for the same amount of time. 350 µl sample was obtained periodically during the UV exposure and mixed with 50 µl FOX assay reagent. A set of standard solutions were also prepared with hydrogen peroxide at concentrations from 0 µM to 2.20 µM. After incubating at room temperature for 30 minutes, the absorbance of the incubated samples and standard solutions were transferred into a clear bottom 96-well plate and read at 560 nm with a microplate reader as described previously.

Results and Discussion

Furfuryl alcohol (FFA) was a well-defined kinetic probe for singlet oxygen, as being reviewed in previous literatures (Latch et al., 2003; Wilkinson, Helman, & Ross, 1995). It is used in this experiment to quantify the concentration of steady-state singlet oxygen generated from BA solution exposed to UV lights at different

wavelength. As shown in Figure 12A, 1.78 ± 0.25 pM, 15.87 ± 0.47 pM and 58.66 ± 2.70 pM singlet oxygen was detected in benzoic acid solutions received UV-A, B and C treatments, respectively. No singlet oxygen production was detected in the control sample incubated in dark. The singlet oxygen generation rate of UV-C was higher than UV-A and UV-B ($P < 0.01$).

The ferrous oxidation−xylenol orange (FOX) assay was used to quantitively measure the production of hydrogen peroxide in BA solution treated with UV lights at different wavelength. Previous study (Gay, Collins, & Gebicki, 1999) has proved that the excess Fe^{2+} ions in the FOX assay solution will be oxidized by hydrogen peroxide in samples at low pH. The generated Fe^{3+} ions will form the Fe–XO complex with the dye xylenol orange (XO), which can be measured in the visible absorbance range. The sensitivity of the assay is enhanced by sorbitol, which acts as a radical chain carrier. As shown in Figure 12B, 0.06 ± 0.01 μM, 1.69 ± 0.10 μM and 1.35 ± 0.06 μM hydrogen peroxide was detected in benzoic acid solutions received UV-A, B and C treatments, respectively. No significant hydrogen peroxide production was detected in the control sample incubated in dark. The singlet oxygen generation rate of UV-B and UV-C were both higher than UV-A ($P < 0.01$/ $P < 0.01$).



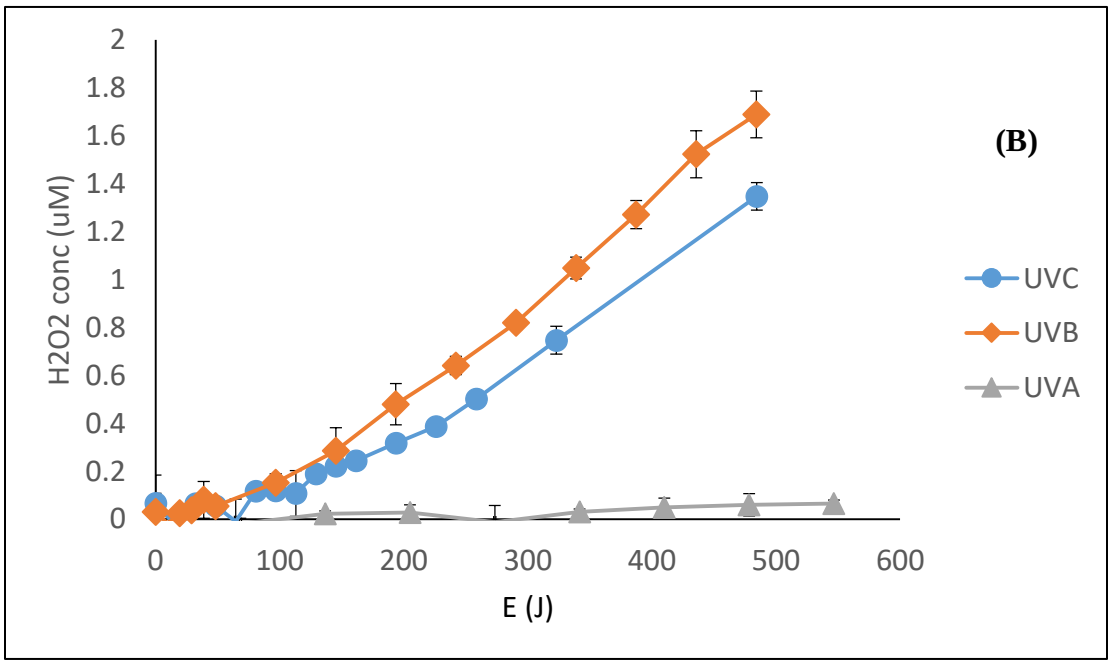


Figure 12A/ 12B Detection of *in vitro* singlet oxygen/ hydrogen peroxide production in BA during UV light treatments

Investigation of membrane fluidity change during the simultaneous UV-A and Benzoic acid treatment

Materials and Methods

The fluorescence polarization of the membrane inserted probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was measured as an indicator for bacterial cytoplasmic membrane fluidity change (Mykytczuk, Trevors, Leduc, & Ferroni, 2007). The probe was first dissolved in tetrahydrofuran to make a stock solution with a concentration at 4 mM. A working solution was then prepared by diluting the stock solution in sterilized PBS to reach a final concentration of 4 μM. Treatment sample containing 1×10^9 Log/mL *E. coli* and 0, 10 or 15 mM BA and/ or 1 mM EDTA was incubated under UV-A exposure for 5 or 30 minute. A same control sample was incubated in dark for the same amount of time. A sample containing sterilized DI water with pH adjusted to 3.0 with hydrochloric acid was used as a control. 1 mL of the incubated sample was washed twice with sterilized PBS and centrifuged for 2 minute at 10,000 × g at room temperature. The pellet was re-suspended in the working solution containing 4 μM DPH. After vortexing at high speed for 30 seconds, the mixture was incubated in dark for 30 minutes. The fluorescence polarization values were determined at an excitation wavelength of 358 nm and an emission wavelength of 428 nm by using the SpectraMax M5e microplate reader. The excitation polarized light source was set to produce polarized beam in the vertical position. The emission polarizers collected and measured the emission intensity of light emitted both parallel (I_{VH}) and perpendicular (I_{VV}) to the plane of excitation. The polarization value was calculated by the following formula:

$$P = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + G \times I_{VH}}$$

Where G is the instrument dependent grating factor, which was assumed to be 1 for the instrument in use. To make results easier to read, all fluorescence polarization values obtained from this experiment were expressed in *mP*, which equals to 1000 times *P*.

Results and Discussions

To complement the PI assay and to further understand the physiological status of the cellular cytoplasmic membrane, bacterial membrane fluidity was measured as in fluorescence polarization values presented in Fig. 12C. The DPH probe can penetrate into the cytoplasmic membrane and position itself parallel to the fatty acid side chains, where its rotational movement is restricted by the lipid order and acyl chain interactions on the membrane (Adler & Tritton, 1988; Mykytczuk et al., 2007). A lower chain interaction presented inside the membrane will lead to a lower polarization value by promoting probe rotation, leading to florescence emission in all directions; with higher restriction from membrane structure interaction, however, the probe motion will be reduced, resulting in a high polarization ratio (Mykytczuk et al., 2007). Therefore, the membrane fluidity and the polarization ratio detected through the probe is inversely proportional. From the results in Fig. 12C, there is no significant difference ($P > 0.05$) in polarization values between bacteria received different treatments, indicating that treatments used in the experiments (BA, EDTA. UVA and their combinations) did not disrupt cell membrane integrity through the alteration on bacterial cytoplasmic membrane fluidity.

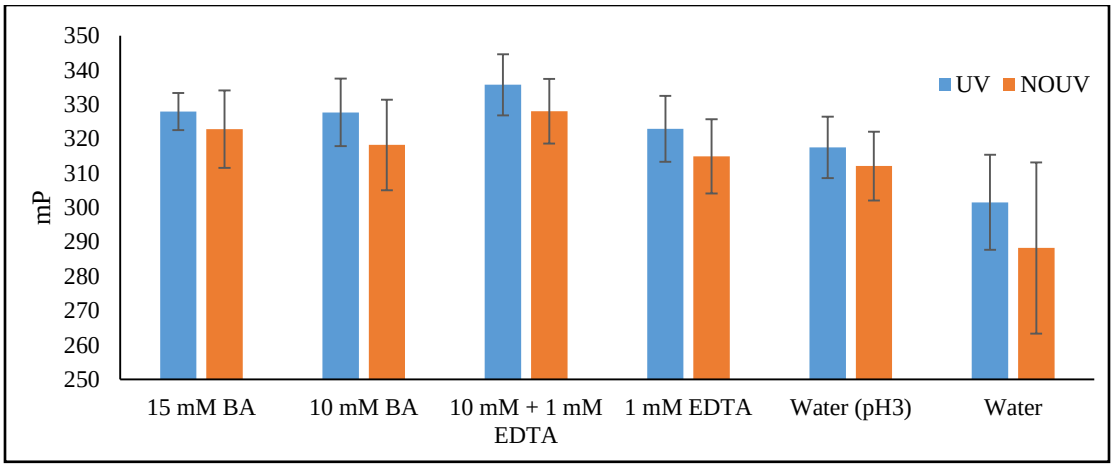


Figure 12C Detection of membrane fluidity change during combined BA + EDTA + UV-A treatments

Bibliography

Aalto, T. R., Firman, M. C., & Rigler, N. E. (1953). p-Hydroxybenzoic Acid Esters as Preservatives*. *Journal of the American Pharmaceutical Association (Scientific Ed.)*, *42*(8), 449–457. https://doi.org/10.1002/jps.3030420802

Abdullah, M. I., Young, J. C., & Gamed, D. E. (1994). Supercritical Fluid Extraction of Carboxylic and Fatty Acids from. *J Agric Food Chem*, 1. 1An T.T. and Thu T.N.H. (2006), Hóa phân tích,.

Ackers, M.-L., Mahon, B. E., Leahy, E., Goode, B., Damrow, T., Hayes, P. S., … Slutsker, L. (1998). An outbreak of Escherichia coli O157:H7 infections associated with leaf lettuce consumption. *The Journal of Infectious Disease*, *177*(6), 1588–1593. https://doi.org/10.1109/iembs.1995.579676

Ackers, M. L., Mahon, B. E., Leahy, E., Goode, B., Damrow, T., Hayes, P. S., … Slutsker, L. (1998). An outbreak of Escherichia coli O157:H7 infections associated with leaf lettuce consumption. *The Journal of Infectious Diseases*, *177*(6), 1588–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9607837

Adler, M., & Tritton, T. R. (1988). Fluorescence depolarization measurements on oriented membranes. *Biophysical Journal*, *53*(6), 989–1005. https://doi.org/10.1016/S0006-3495(88)83179-5

Alakomi, H. L., Saarela, M., & Helander, I. M. (2003). Effect of EDTA on Salmonella enterica serovar Typhimurium involves a component not assignable to lipopolysaccharide release. *Microbiology*, *149*(8), 2015–2021. https://doi.org/10.1099/mic.0.26312-0

Allende, A., Selma, M. V., López-Gálvez, F., Villaescusa, R., & Gil, M. I. (2008a).

Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. *Postharvest Biology and Technology*, *49*(1), 155–163. https://doi.org/10.1016/j.postharvbio.2007.12.010

Allende, A., Selma, M. V, López-Gálvez, F., Villaescusa, R., & Gil, M. I. (2008b). Impact of wash water quality on sensory and microbial quality, including Escherichia coli cross-contamination, of fresh-cut escarole. *Journal of Food Protection*, *71*(12), 2514–2518. https://doi.org/10.4315/0362-028X-71.12.2514

Apel, K., & Hirt, H. (2004). REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. *Annual Review of Plant Biology*, *55*(1), 373–399. https://doi.org/10.1146/annurev.arplant.55.031903.141701

Arbor, A. (2016). *Thiol Detection Assay Kit*.

Bachmann, R. (1975). STERILIZATION BY INTENSE ULTRAVIOLET-RADIATION. *Brown Boveri Review*, *62*(5), 206–209.

Baldwin, E. A., Nisperos, M. O., Chen, X., & Hagenmaier, R. D. (1996). Improving storage life of cut apple and potato with edible coating. *Postharvest Biology and Technology*, *9*(2), 151–163. https://doi.org/10.1016/S0925-5214(96)00044-0

Ball, C. O., & Olson, F. C. W. (1957). *Sterilization in Food Technology Theory, Practice and Calculation*. New York: McGraw-Hill Book.

Bargiota, E., Rico-Munoz, E., & Davidson, P. M. (1987). Lethal effect of methyl and propyl parabens as related to Staphylococcus aureus lipid composition. *International Journal of Food Microbiology*, *4*(3), 257–266. https://doi.org/10.1016/0168-1605(87)90042-0

Beacham, I. R., Kahana, R., Levy, L., & Yagil, E. (1973). Mutants of Escherichia coli K-12 “cryptic,” or deficient in 5’-nucleotidase (uridine diphosphate-sugar hydrolase) and 3’-nucleotidase (cyclic phosphodiesterase) activity. *Journal of Bacteriology*, *116*(2), 957–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4355492

Bell, B. P., Goldoft, M., Griffin, P. M., Davis, M. A., Gordon, D. C., Tarr, P. I., … Wells, J. G. (1994). A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. *JAMA*, *272*(17), 1349–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7933395

Benjamin, M. M., & Datta, A. R. (1995). Acid tolerance of enterohemorrhagic Escherichia coli. *Applied and Environmental Microbiology*, *61*(4), 1669–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7747983

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H. U., & Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied and Environmental Microbiology*, *73*(10), 3283–3290. https://doi.org/10.1128/AEM.02750-06

Beuchat, L. (1997). Produce Handling and Processing Practices. *Emerging Infectious Diseases*, *3*(4), 459–465. https://doi.org/10.3201/eid0304.970407

Bialka, K. L., Demirci, A., & Puri, V. M. (2008). Modeling the inactivation of Escherichia coli O157:H7 and Salmonella enterica on raspberries and strawberries resulting from exposure to ozone or pulsed UV-light. *Journal of Food Engineering*, *85*(3), 444–449.

https://doi.org/10.1016/j.jfoodeng.2007.08.007

Bintsis, T., Litopoulou-Tzanetaki, E., & Robinson, R. K. (2000). Existing and potential applications of ultraviolet light in the food industry - a critical review. *Journal of the Science of Food and Agriculture*, *80*(6), 637–645. https://doi.org/10.1002/(SICI)1097-0010(20000501)80:6<637::AID-JSFA603>3.0.CO;2-1

Bose, B., & Chatterjee, S. N. (1995). Correlation between UVA-induced changes in microviscosity, permeability and malondialdehyde formation in liposomal membrane. *Journal of Photochemistry and Photobiology, B: Biology*, *28*(2), 149–153. https://doi.org/10.1016/1011-1344(94)07102-T

Bosund, I. (1963). The Action of Benzoic and Salicylic Acids on the Metabolism of Microorganisms. *Advances in Food Research*, *11*, 331–353. https://doi.org/10.1016/S0065-2628(08)60068-3

Brey, R. N., Beck, J. C., & Rosen, B. P. (1978). Cation/proton antiport systems in Escherichiacoli. *Biochemical and Biophysical Research Communications*, *83*(4), 1588–1594. https://doi.org/10.1016/0006-291X(78)91403-1

Brown, L., Wolf, J. M., Prados-Rosales, R., & Casadevall, A. (2015). Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nature Reviews Microbiology*, *13*(10), 620–630. https://doi.org/10.1038/nrmicro3480

Buchanan, R. ., & Klawitter, L. A. (1992). The effect of incubation temperature, initial pH, and sodium chloride on the growth kinetics of Escherichia coli O157:H7. *Food Microbiology*, *9*(3), 185–196. https://doi.org/10.1016/0740-

0020(92)80046-7

Buchanan, R. L., & Bagi, L. K. (1994). Expansion of response surface models for the growth of Escherichia coli O157:H7 to include sodium nitrite as a variable. *International Journal of Food Microbiology*, *23*(3–4), 317–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7873334

Buchanan, R. L., & Doyle, M. P. (1997). Foodborne disease significance of Escherichia coli O157:H7 and other enterohemorrhagic E. coli. *Food Technology*, *51*(10), 69–76.

Burt, S. a, & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against Escherichia coli O157:H7. *Letters in Applied Microbiology*, *36*(3), 162–7. https://doi.org/10.1046/j.1472-765X.2003.01285.x

Cabiscol, E., Tamarit, J., & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*, *3*(1), 3–8. https://doi.org/10.2436/im.v3i1.9235

Cadenas, E. (1989). Biochemistry of Oxygen Toxicity. *Annual Review of Biochemistry*, *58*(1), 79–110. https://doi.org/10.1146/annurev.bi.58.070189.000455

Cadet, J., Douki, T., Ravanat, J.-L., & Di Mascio, P. (2009). Sensitized formation of oxidatively generated damage to cellular DNA by UVA radiation. *Photochemical & Photobiological Sciences : Official Journal of the European Photochemistry Association and the European Society for Photobiology*, *8*(7), 903–11. https://doi.org/10.1039/b905343n

Carrondo, M. A. (2003). NEW EMBO MEMBER'S REVIEW: Ferritins, iron uptake

and storage from the bacterioferritin viewpoint. *The EMBO Journal*, *22*(9), 1959–1968. https://doi.org/10.1093/emboj/cdg215

Ceylan, E., Fung, D. Y. C., & Sabah, J. R. (2004). Antimicrobial Activity and Synergistic Effect of Cinnamon with Sodium Benzoate or Potassium Sorbate in Controlling Escherichia coli O157:H7 in Apple Juice. *Journal of Food Science*, *69*(4), FMS102--FMS106. https://doi.org/10.1111/j.1365-2621.2004.tb06348.x

Char, C. D., Mitilinaki, E., Guerrero, S. N., & Alzamora, S. M. (2010). Use of High-Intensity Ultrasound and UV-C Light to Inactivate Some Microorganisms in Fruit Juices. *Food and Bioprocess Technology*, *3*(6), 797–803. https://doi.org/10.1007/s11947-009-0307-7

Chen, C. S., Shaw, P. E., & Parish, M. E. (1993). Orange and tangerine juices. *Fruit Juice Processing Technology*, 110–165.

Chen, H. (2007). Use of linear, Weibull, and log-logistic functions to model pressure inactivation of seven foodborne pathogens in milk. *Food Microbiology*, *24*(3), 197–204. https://doi.org/10.1016/j.fm.2006.06.004

Chen, M.-C., Yeh, G. H.-C., & Chiang, B.-H. (1996). ANTIMICROBIAL AND PHYSICOCHEMICAL PROPERTIES OF METHYLCELLULOSE AND CHITOSAN FILMS CONTAINING A PRESERVATIVE. *Journal of Food Processing and Preservation*, *20*(5), 379–390. https://doi.org/10.1111/j.1745-4549.1996.tb00754.x

Chipley, J. R. (2005). Sodium benzoate and benzoic acid. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in Food* (3rd ed., p. 11). Taylor Francis Group.

Chung, D., Chikindas, M. L., & Yam, K. L. (2001). Inhibition of Saccharomyces cerevisiae by slow release of propyl paraben from a polymer coating. *Journal of Food Protection*, *64*(9), 1420–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11563521

Comes, J. E., & Beelman, R. B. (2002). Addition of Fumaric Acid and Sodium Benzoate as an Alternative Method To Achieve a 5-log Reduction of Escherichia coli O157:H7 Populations in Apple Cider. *Journal of Food Protection*, *65*(3), 476–483. https://doi.org/10.4315/0362-028X-65.3.476

Couvert, O., Gaillard, S., Savy, N., Mafart, P., and Leguérinel, I. (2005). Survival curves of heated bacterial spores: effect of environmental factors on Weibull parameters. *International Journal of Food Microbiology*, *101*(1), 73–81. https://doi.org/10.1016/j.ijfoodmicro.2004.10.048

Davidson, P. M., Sofos, J. N., & Branen, A. L. (2005). *Antimicrobials in Food* (3rd ed.). New York: Taylor & Francis.

Davidson, P. M., Taylor, T. M., & Schmidt, S. E. (2013). Chemical Preservatives and Natural Antimicrobial Compounds. In *Food Microbiology* (pp. 765–801). American Society of Microbiology. https://doi.org/10.1128/9781555818463.ch30

De Smet, M. J., Kingma, J., & Witholt, B. (1978). The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of escherichia coli. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *506*(1), 64–80. https://doi.org/10.1016/0005-2736(78)90435-2

Deuel, H. J., Alfin-Slatee, R., Weil, C. S., & Smyth, H. E. (1954). SORBIC ACID AS

A FUNGISTATIC AGENT FOR FOODS.. *Journal of Food Science*, *19*(1–6), 1–12. https://doi.org/10.1111/j.1365-2621.1954.tb17417.x

DiRienzo, J. M., Nakamura, K., & Inouye, M. (1978). The Outer Membrane Proteins of Gram-Negative Bacteria: Biosynthesis, Assembly, and Functions. *Annual Review of Biochemistry*, *47*(1), 481–532. https://doi.org/10.1146/annurev.bi.47.070178.002405

Dizdaroglu, M., Rao, G., Halliwell, B., & Gajewski, E. (1991). Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. *Archives of Biochemistry and Biophysics*, *285*(2), 317–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1654771

Dobiás, J., Chudackova, K., Voldrich, M., & Marek, M. (2000). Properties of polyethylene films with incorporated benzoic anhydride and/or ethyl and propyl esters of 4-hydroxybenzoic acid and their suitability for food packaging. *Food Additives and Contaminants*, *17*(12), 1047–53. https://doi.org/10.1080/02652030010014394

Dock, L. L., Floros, J. D., & Linton, R. H. (2000). Heat Inactivation of Escherichia coli O157:H7 in Apple Cider Containing Malic Acid, Sodium Benzoate, and Potassium Sorbate. *Journal of Food Protection*, *63*(8), 1026–1031. https://doi.org/10.4315/0362-028X-63.8.1026

Doyle, M. P., & Schoeni, J. L. (1984). Survival and growth characteristics of Escherichia coli associated with hemorrhagic colitis. *Applied and Environmental Microbiology*, *48*(4), 855–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6391379

Duffy, S., Churey, J., Worobo, R. W., & Schaffner, D. W. (2000). Analysis and modeling of the variability associated with UV inactivation of Escherichia coli in apple cider. *J Food Prot*, *63*(11), 1587–1590.

Dukan, S., Farewell, A., Ballesteros, M., Taddei, F., Radman, M., & Nystrom, T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proceedings of the National Academy of Sciences*, *97*(11), 5746–5749. https://doi.org/10.1073/pnas.100422497

Eklund, T. (1980). Inhibition of Growth and Uptake Processes in Bacteria by Some Chemical Food Preservatives. *Journal of Applied Bacteriology*, *48*(3), 423–432. https://doi.org/10.1111/j.1365-2672.1980.tb01031.x

El-Shenawy, M. A., & Marth, E. H. (1988). Sodium Benzoate Inhibits Growth of or Inactivates Listeria monocytogenes. *Journal of Food Protection*, *51*(7), 525–530. https://doi.org/10.4315/0362-028X-51.7.525

Escalona, V. H., Aguayo, E., Martínez-Hernández, G. B., & Artés, F. (2010). UV-C doses to reduce pathogen and spoilage bacterial growth in vitro and in baby spinach. *Postharvest Biology and Technology*, *56*(3), 223–231. https://doi.org/10.1016/j.postharvbio.2010.01.008

Faith, N. G., Shere, J. A., Brosch, R., Arnold, K. W., Ansay, S. E., Lee, M. S., … Kaspar, C. W. (1996). Prevalence and clonal nature of Escherichia coli O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology*, *62*(5), 1519–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8633851

Farr, S. B., D'Ari, R., & Touati, D. (1986). Oxygen-dependent mutagenesis in Escherichia coli lacking superoxide dismutase. *Proceedings of the National*

Academy of Sciences, *83*(21), 8268–8272. https://doi.org/10.1073/pnas.83.21.8268

Farr, S. B., & Kogoma, T. (1991). Oxidative stress responses in Escherichia coli and Salmonella typhimurium. *Microbiological Reviews*, *55*(4), 561–85. Retrieved from http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=1779927

Farr, S. B., Touati, D., & Kogoma, T. (1988). Effects of oxygen stress on membrane functions in Escherichia coli: role of HPI catalase. *Journal of Bacteriology*, *170*(4), 1837–1842. https://doi.org/10.1128/jb.170.4.1837-1842.1988

Flint, D. H., Tuminello, J. F., & Emptage, M. H. (1993). The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *The Journal of Biological Chemistry*, *268*(30), 22369–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8226748

Fluhr, J. W., & Gloor, M. (1997). The antimicrobial effect of narrow-band UVB (313 nm) and UVA1 (345-440 nm) radiation in vitro. *Photodermatology, Photoimmunology & Photomedicine*, *13*(5–6), 197–201. https://doi.org/10.1111/j.1600-0781.1997.tb00230.x

Foster, J. W. (2004). Escherichia coli acid resistance: tales of an amateur acidophile. *Nature Reviews Microbiology*, *2*(11), 898–907. https://doi.org/10.1038/nrmicro1021

Franz, C. M. A. P., Specht, I., Cho, G. S., Graef, V., & Stahl, M. R. (2009). UV-C-inactivation of microorganisms in naturally cloudy apple juice using novel inactivation equipment based on Dean vortex technology. *Food Control*, *20*(12),

1103–1107. https://doi.org/10.1016/j.foodcont.2009.02.010

Freese, E., Sheu, C. W., & Galliers, E. (1973). Function of lipophilic acids as antimicrobial food additives. *Nature*, *241*(5388), 321–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4633553

Fukahori, M., Akatsu, S., Sato, H., & Yotsuyanagi, T. (1996). Relationship between uptake of p-hydroxybenzoic acid esters by Escherichia coli and antibacterial activity. *Chemical & Pharmaceutical Bulletin*, *44*(8), 1567–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8795274

Furr, J. R., & Russell, A. D. (1972). Some factors influencing the activity of esters of p-hydroxybenzoic acid against Serratia marcescens. *Microbios*, *5*(19), 189–98. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4594255

Gale, E. F. (1943). FACTORS INFLUENCING THE ENZYMIC ACTIVITIES OF BACTERIA. *Bacteriological Reviews*, *7*(3), 139–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/440869

Gay, C., Collins, J., & Gebicki, J. M. (1999). Hydroperoxide Assay with the Ferric–Xylenol Orange Complex. *Analytical Biochemistry*, *273*(2), 149–155. https://doi.org/10.1006/abio.1999.4208

Gelting, R. J., Baloch, M. A., Zarate-Bermudez, M. A., & Selman, C. (2011). Irrigation water issues potentially related to the 2006 multistate E. coli O157:H7 outbreak associated with spinach. *Agricultural Water Management*, *98*(9), 1395–1402. https://doi.org/10.1016/j.agwat.2011.04.004

Gombas, D., Luo, Y., Brennan, J., Shergill, G., Petran, R., Walsh, R., … Deng, K. (2017). Guidelines To Validate Control of Cross-Contamination during Washing

of Fresh-Cut Leafy Vegetables. *Journal of Food Protection*, *80*(2), 312–330. https://doi.org/10.4315/0362-028X.JFP-16-258

Graham, G. S., Treick, R. W., & Brunner, D. P. (1979). Effect of Ca2+ and Mg2+ upon the reassociation byEscherichia coli of material released by ethylenediaminetetraacetate. *Current Microbiology*, *2*(6), 339–343. https://doi.org/10.1007/BF02602872

Greenberg, J. T., & Demple, B. (1988). Overproduction of peroxide-scavenging enzymes in Escherichia coli suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in oxyR-mutants. *The EMBO Journal*, *7*(8), 2611–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/457135

Greer, A. (2006). Christopher Foote's Discovery of the Role of Singlet Oxygen [1 O 2 (1 Δ g)] in Photosensitized Oxidation Reactions. *Accounts of Chemical Research*, *39*(11), 797–804. https://doi.org/10.1021/ar050191g

Griffin, P. M., & Tauxe, R. V. (1991a). The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*, *13*(1), 60–98.

Griffin, P. M., & Tauxe, R. V. (1991b). The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*, *13*(1), 60–98. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1765120

Guerrero-Beltran, J. A., & Barbosa-Canovas, G. V. (2004). Advantages and Limitations on Processing Foods by UV Light. *Food Science and Technology International*, *10*(3), 137–147. https://doi.org/10.1177/1082013204044359

Hancock, R. E. (1984). Alterations in outer membrane permeability. *Annual Review of Microbiology*, *38*, 237–64. https://doi.org/10.1146/annurev.mi.38.100184.001321

Hancock, R. E. W. (1984). Alterations in structure of the cell envelope. *Ann. Rev. Microbiol.*, *38*, 237–264. https://doi.org/10.1146/annurev.mi.38.100184.001321

Hancock, R. E. W., & Wong, P. G. W. (1984). Compounds Which Increase the Permeability of the Pseudomonas-Aeruginosa Outer-Membrane. *Antimicrobial Agents and Chemotherapy*, *26*(1), 48–52. https://doi.org/10.1128/AAC.26.1.48

Hanes, D. E., Worobo, R. W., Orlandi, P. A., Burr, D. H., Miliotis, M. D., Robl, M. G., … Jackson, G. J. (2002). Inactivation of Cryptosporidium parvum Oocysts in Fresh Apple Cider by UV Irradiation. *Applied and Environmental Microbiology*, *68*(8), 4168–4172. https://doi.org/10.1128/AEM.68.8.4168-4172.2002

Hazan, R., Levine, A., & Abeliovich, H. (2004). Benzoic Acid, a Weak Organic Acid Food Preservative, Exerts Specific Effects on Intracellular Membrane Trafficking Pathways in Saccharomyces cerevisiae Benzoic Acid , a Weak Organic Acid Food Preservative , Exerts Specific Effects on Intracellular Membr. *Applied and Environmental Microbiology*, *70*(8), 4449–4457. https://doi.org/10.1128/AEM.70.8.4449

Hazelbauer, G. L. (1975). Role of the receptor for bacteriophage lambda in the functioning of the maltose chemoreceptor of Escherichia coli. *Journal of Bacteriology*, *124*(1), 119–26. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1100597

Hollingsworth, C. A., Seybold, P. G., & Hadad, C. M. (2002). Substituent effects on

the electronic structure and pKa of benzoic acid. *International Journal of Quantum Chemistry*, *90*(4–5), 1396–1403. https://doi.org/10.1002/qua.10362

Humpf, H.-U., & Schreier, P. (1991). Bound aroma compounds from the fruit and the leaves of blackberry (Rubus laciniata L.). *Journal of Agricultural and Food Chemistry*, *39*(10), 1830–1832. https://doi.org/10.1021/jf00010a028

Hutchinson, F. (1985). Chemical changes induced in DNA by ionizing radiation. *Progress in Nucleic Acid Research and Molecular Biology*, *32*, 115–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3003798

Imlay, J. a. (2003). Pathways of oxidative damage. *Annual Review of Microbiology*, *57*, 395–418. https://doi.org/10.1146/annurev.micro.57.030502.090938

Jacob, S. W., & Herschler, R. (1986). Pharmacology of DMSO. *Cryobiology*, *23*(1), 14–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3007027

Keene, W. E., McAnulty, J. M., Hoesly, F. C., Williams, L. P., Hedberg, K., Oxman, G. L., … Fleming, D. W. (1994). A swimming-associated outbreak of hemorrhagic colitis caused by Escherichia coli O157:H7 and Shigella sonnei. *The New England Journal of Medicine*, *331*(9), 579–84. https://doi.org/10.1056/NEJM199409013310904

Keyser, M., Muller, I. A., Cilliers, F. P., Nel, W., & Gouws, P. A. (2008). Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. *Innovative Food Science and Emerging Technologies*, *9*(3), 348–354. https://doi.org/10.1016/j.ifset.2007.09.002

Kim, Y. J., Shin, Y. K., Sohn, D. S., & Lee, C. S. (2014). Menadione induces the formation of reactive oxygen species and depletion of GSH-mediated apoptosis

and inhibits the FAK-mediated cell invasion. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *387*(9), 799–809. https://doi.org/10.1007/s00210-014-0997-x

King, T., Lucchini, S., Hinton, J. C. D., & Gobius, K. (2010). Transcriptomic analysis of Escherichia coli O157:H7 and K-12 cultures exposed to inorganic and organic acids in stationary phase reveals acidulant- and strain-specific acid tolerance responses. *Applied and Environmental Microbiology*, *76*(19), 6514–6528. https://doi.org/10.1128/AEM.02392-09

Klockow, P. A., & Keener, K. M. (2009). Safety and quality assessment of packaged spinach treated with a novel ozone-generation system. *LWT - Food Science and Technology*, *42*(6), 1047–1053. https://doi.org/10.1016/j.lwt.2009.02.011

Koutchma, T. (2008). UV Light for Processing Foods. *Ozone: Science & Engineering*, *30*(1), 93–98. https://doi.org/10.1080/01919510701816346

Koutchma, T. (2009). Advances in Ultraviolet Light Technology for Non-thermal Processing of Liquid Foods. *Food and Bioprocess Technology*, *2*(2), 138–155. https://doi.org/10.1007/s11947-008-0178-3

Koutchma, T. (2014). *Preservation and Shelf Life Extension : UV Applications for Fluid Foods*. Elsevier Science. Retrieved from http://public.eblib.com/choice/publicfullrecord.aspx?p=1657930

Koutchma, T., Parisi, B., & Patazca, E. (2007). Validation of UV coiled tube reactor for fresh juices. *Journal of Environmental Engineering and Science*, *6*(3), 319–328. https://doi.org/10.1139/s06-058

Koutchma, T., Parisi, B., & Unluturk, S. (2006). Evaluation of Uv Dose in Flow-Through Reactors for Fresh Apple Juice and Cider. *Chemical Engineering*

Communications, *193*(6), 715–728. https://doi.org/10.1080/00986440500266842

Krebs, H. A., Wiggins, D., Stubbs, M., Sols, A., & Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *The Biochemical Journal*, *214*(3), 657–63. https://doi.org/10.1111/j.1469-8137.1979.tb07471.x

Krishnamurthy, K., Demirci, A., & Irudayaraj, J. M. (2007). Inactivation of Staphylococcus aureus in milk using flow-through pulsed UV-light treatment system. *Journal of Food Science*, *72*(7). https://doi.org/10.1111/j.1750-3841.2007.00438.x

Krumova, K., & Cosa, G. (2016). *Singlet Oxygen : Applications in Biosciences and Nanosciences*. (S. Nonell & C. Flors, Eds.) (1st ed., Vol. 1). Cambridge: Royal Society of Chemistry. https://doi.org/10.1039/9781782622208

Kudva, I. T., Hatfield, P. G., & Hovde, C. J. (1996). Escherichia coli O157:H7 in microbial flora of sheep. *Journal of Clinical Microbiology*, *34*(2), 431–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8789031

Lambert, R. J., & Stratford, M. (1999). Weak-acid preservatives: Modelling microbial inhibition and response. *Journal of Applied Microbiology*, *86*(1), 157–164. https://doi.org/10.1046/j.1365-2672.1999.00646.x

Latch, D. E., Stender, B. L., Packer, J. L., Arnold, W. A., & McNeill, K. (2003). Photochemical Fate of Pharmaceuticals in the Environment: Cimetidine and Ranitidine. *Environmental Science & Technology*, *37*(15), 3342–3350. https://doi.org/10.1021/es0340782

Leistner, L. (1992). Food preservation by combined methods. *Food Research International*, *25*(2), 151–158.

Leive, L. (1974). THE BARRIER FUNCTION OF THE GRAM-NEGATIVE ENVELOPE. *Annals of the New York Academy of Sciences*, *235*(1 Mode of Actio), 109–129. https://doi.org/10.1111/j.1749-6632.1974.tb43261.x

LifeTechnologies. (2013). *Intracellular pH (pHi) Detection*. Carlsbad. Retrieved from https://tools.thermofisher.com/content/sfs/manuals/mp35372.pdf

Liu, X., Yousef, A. E., & Chism, G. W. (1996). Combination of Organic Acids and Pulsed Electric Field. *Journal of Food Safety*, *16*(1997), 287–299.

Lück, E., & Jager, M. (1997). *Antimicrobial Food Additives* (2nd ed.). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-59202-7

Macris, B. (1975). Mechanism of benzoic acid uptake by Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, *30*(4), 503–506. Retrieved from papers3://publication/uuid/86A55C51-9B16-45E2-B708-CA9012BA01C3

Mannan, A., Liu, C., Arsenault, P. R., Towler, M. J., Vail, D. R., Lorence, A., & Weathers, P. J. (2010). DMSO triggers the generation of ROS leading to an increase in artemisinin and dihydroartemisinic acid in Artemisia annua shoot cultures. *Plant Cell Reports*, *29*(2), 143–152. https://doi.org/10.1007/s00299-009-0807-y

Marlatt, C., Ho, C. T., & Chien, M. (1992). Studies of aroma constituents bound as glycosides in tomato. *Journal of Agricultural and Food Chemistry*, *40*(2), 249–252. https://doi.org/10.1021/jf00014a016

Matak, K., & Churey, J. (2005). Efficacy of UV light for the reduction of Listeria monocytogenes in goat's milk. *Journal of Food Protection*, *68*(10), 2212–2216.

Retrieved from http://www.ingentaconnect.com/content/iafp/jfp/2005/00000068/00000010/art00032

McLaggan, D., Stephen, J., & Booth, I. R. (1998). Chapter 5 Regulation of cytoplasmic pH in bacteria. *Principles of Medical Biology*, *9*(C), 65–77. https://doi.org/10.1016/S1569-2582(97)80142-0

Meng, J., Zhao, S., Zhao, T., & Doyle, M. P. (1995). Molecular characterisation of Escherichia coli O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. *Journal of Medical Microbiology*, *42*(4), 258–63. https://doi.org/10.1099/00222615-42-4-258

Molecular Probes. (2012). CellROX ® Oxidative Stress Reagents, 1–6.

Muschel, L. H., & Gustafson, L. (1968). Antibiotic, detergent, and complement sensitivity of Salmonella typhi after ethylenediaminetetraacetic acid treatment. *Journal of Bacteriology*, *95*(6), 2010–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4970219

Mykytczuk, N. C. S., Trevors, J. T., Leduc, L. G., & Ferroni, G. D. (2007). Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. *Progress in Biophysics and Molecular Biology*, *95*(1–3), 60–82. https://doi.org/10.1016/j.pbiomolbio.2007.05.001

Nayak, S., Muniz, J., Sales, C. M., & Tikekar, R. V. (2016). Fructose as a novel photosensitizer: Characterization of reactive oxygen species and an application in degradation of diuron and chlorpyrifos. *Chemosphere*, *144*, 1690–7. https://doi.org/10.1016/j.chemosphere.2015.10.074

Niederhoffer, E. C., Naranjo, C. M., Bradley, K. L., & Fee, J. A. (1990). Control of Escherichia coli superoxide dismutase (sodA and sodB) genes by the ferric uptake regulation (fur) locus. *Journal of Bacteriology*, *172*(4), 1930–1938. https://doi.org/10.1128/jb.172.4.1930-1938.1990

Nikaido, H; Varra, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Mol Biol Rev*, *67*(1), 593–656. https://doi.org/10.1128/mmbr.67.4.593-656.2003

Nikaido, H., & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews*, *49*(1), 1–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2580220

Nisha, P., Singhal, R. S., & Pandit, A. B. (2004). A study on the degradation kinetics of visual green colour in spinach (Spinacea oleracea L.) and the effect of salt therein. *Journal of Food Engineering*, *64*(1), 135–142. https://doi.org/10.1016/j.jfoodeng.2003.09.021

Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne Pathogens in Milk and the Dairy Farm Environment: Food Safety and Public Health Implications. *Foodborne Pathogens and Disease*, *2*(2), 115–129. https://doi.org/10.1089/fpd.2005.2.115

Padan, E., Zilberstein, D., & Schuldiner, S. (1981). pH homesstasis in bacteria. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes*, *650*(2–3), 151–166. https://doi.org/10.1016/0304-4157(81)90004-6

Page, B. D., Conacher, H. B. S., Weber, D., & Lacroix, G. (1992). A survey of benzene in fruits and retail fruit juices, fruit drinks, and soft drinks. *Journal of*

AOAC International, *75*(2), 334–340.

Palva, E. T., & Makela, P. H. (1980). Lipopolysaccharide Heterogeneity in Salmonella typhimurium Analyzed by Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. *European Journal of Biochemistry*, *107*(1), 137–143. https://doi.org/10.1111/j.1432-1033.1980.tb04634.x

Pattison, D. I., & Davies, M. J. (2006). Actions of ultraviolet light on cellular structures. *Exs*, (96), 131–157. https://doi.org/10.1007/3-7643-7378-4_6

Peak, M. J., Peak, J. G., Moehring, M. P., & Webs, R. B. (1984). ULTRAVIOLET ACTION SPECTRA FOR DNA DIMER INDUCTION, LETHALITY, AND MUTAGENESIS IN Escherichia coli WITH EMPHASIS ON THE UVB REGION. *Photochemistry and Photobiology*, *40*(5), 613–620. https://doi.org/10.1111/j.1751-1097.1984.tb05349.x

Rahn, O., & Conn, J. E. (1944). Effect of Increase in Acidity on Antiseptic Efficiency. *Industrial & Engineering Chemistry*, *36*(2), 185–187. https://doi.org/10.1021/ie50410a020

Rai, P., Cole, T. D., Wemmer, D. E., & Linn, S. (2001). Localization of Fe2+ at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe2+ and H2O211Edited by M. F. Summers. *Journal of Molecular Biology*, *312*(5), 1089–1101. https://doi.org/10.1006/jmbi.2001.5010

Rajkowski, K. T., & Marmer, B. S. (1995). Growth of Escherichia coli O157:H7 at Fluctuating Incubation Temperatures. *Journal of Food Protection*, *58*(12), 1307–1313. https://doi.org/10.4315/0362-028X-58.12.1307

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., & Swerdlow, D. L. (2005).

Epidemiology of Escherichia coli O157:H7 Outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*, *11*(4), 603–609. https://doi.org/10.3201/eid1104.040739

Reilly, P. M., Schiller, H. J., & Bulkley, G. B. (1991). Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. *American Journal of Surgery*, *161*(4), 488–503. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2035771

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., … Cohen, M. L. (1983). Hemorrhagic Colitis Associated with a Rare Escherichia coli Serotype. *New England Journal of Medicine*, *308*(12), 681–685. https://doi.org/10.1056/NEJM198303243081203

Rodrigo, D., Barbosa-Canovas, G. V, Martinez, A., & Rodrigo, M. (2003). Weibull distribution function based on an empirical mathematical model for inactivation of Escherichia coli by pulsed electric fields. *Journal of Food Protection*, *66*(6), 1007–1012.

Ross, A. I. V, Griffiths, M. W., Mittal, G. S., & Deeth, H. C. (2003). Combining nonthermal technologies to control foodborne microorganisms. *International Journal of Food Microbiology*, *89*(2–3), 125–138. https://doi.org/10.1016/S0168-1605(03)00161-2

Rusul, G., & Marth, E. H. (1988). Food additives and plant components control growth and aflatoxin production by toxigenic aspergilli: A review. *Mycopathologia*, *101*(1), 13–23. https://doi.org/10.1007/BF00455664

Sado, I. (1973). Synergistic Toxicity of Official Permissible Preservative Food

Additives. *Nippon Eiseigaku Zasshi (Japanese Journal of Hygiene)*, *28*(5), 463–476. https://doi.org/10.1265/jjh.28.463

Salmond, C. V, Kroll, R. G., & Booth, I. R. (1984). The Effect of Food Preservatives on pH Homeostasis in Escherichia coIi. *Journal of General Microbiology*, *130*(May), 2845–2850. https://doi.org/10.1099/00221287-130-11-2845

Sanders, D., & Slayman, C. L. (1982). Control of intracellular pH. Predominant role of oxidative metabolism, not proton transport, in the eukaryotic microorganism Neurospora. *The Journal of General Physiology*, *80*(3), 377–402. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6292329

Schweizer, M., Hindennach, I., Garten, W., & Henning, U. (1978). Major Proteins of the Escherichia coli Outer Cell Envelope Membrane. Interaction of Protein II* with Lipopolysaccharide. *European Journal of Biochemistry*, *82*(1), 211–217. https://doi.org/10.1111/j.1432-1033.1978.tb12013.x

Shama, G. (1999). Ultraviolet light.

Sizer, C. E., & Balasubramaniam, V. M. (1999). New intervention processes for minimally processed juices. *Food Technology*, *53*(10), 64–67.

Smith, F. A., & Raven, J. A. (1979). Intracellular PH and its Regulation. *Annual Review of Plant Physiology*, *30*(1), 289–311. https://doi.org/10.1146/annurev.pp.30.060179.001445

Solomon, E. B., Pang, H.-J., & Matthews, K. R. (2003). Persistence of Escherichia coli O157:H7 on lettuce plants following spray irrigation with contaminated water. *Journal of Food Protection*, *66*(12), 2198–2202. https://doi.org/10.4315/0362-028X-66.12.2198

Solomon, E. B., Yaron, S., & Matthews, K. R. (2002). Transmission of Escherichia coli O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied and Environmental Microbiology*, *68*(1), 397–400. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11772650

Steele, M., & Odumeru, J. (2004). Irrigation Water as Source of Foodborne Pathogens on Fruit and Vegetables. *Journal of Food Protection*, *67*(12), 2839–2849. https://doi.org/10.4315/0362-028X-67.12.2839

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., & Ren, Q. (2015). Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiology*, *15*, 36. https://doi.org/10.1186/s12866-015-0376-x

Storz, G., Christman, M. F., Sies, H., & Ames, B. N. (1987). Spontaneous mutagenesis and oxidative damage to DNA in Salmonella typhimurium. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(24), 8917–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3321061

Storz, G., Tartaglia, L. a., Farr, S. B., & Ames, B. N. (1990). Bacterial defenses againsts oxidative stress. *Trends in Genetics*, *6*(11), 363–368.

Stratford, M., & Rose, A. H. (1986). Transport of Sulphur Dioxide by Saccharomyces cerevisiae. *Microbiology*, *132*(1), 1–6. https://doi.org/10.1099/00221287-132-1-1

Sutton, J. C., Yu, H., Grodzinski, B., & Johnstone, M. (2000). Relationships of

ultraviolet radiation dose and inactivation of pathogen propagules in water and hydroponic nutrient solutions. *Canadian Journal of Plant Pathology*, *22*(3), 300–309. https://doi.org/10.1080/07060660009500479

Tatsuguchi, K., Kuwamoto, S., Ogomori, M., Ide, T., & Watanabe, T. (1991). Membrane Disorders of Escherichia coli Cells and Liposomes Induced by p-Hydroxybenzoic Acid Esters. *Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi)*, *32*(3), 121–127_1. https://doi.org/10.3358/shokueishi.32.121

Thevelein, J. M. (1994). Signal transduction in yeast. *Yeast*, *10*(13), 1753–1790. https://doi.org/10.1002/yea.320101308

Thompson, D. P. (1994). Minimum inhibitory concentration of esters of p-hydroxybenzoic acid (paraben) combinations against toxigenic fungi. *Journal of Food Protection*, *57*(2), 133–135.

Tomás-Callejas, A., López-Velasco, G., Artés, F., & Artés-Hernández, F. (2012). Acidified sodium chlorite optimisation assessment to improve quality of fresh-cut tatsoi baby leaves. *Journal of the Science of Food and Agriculture*, *92*(4), 877–885. https://doi.org/10.1002/jsfa.4664

Tran, M. T. T., & Farid, M. (2004). Ultraviolet treatment of orange juice. *Innovative Food Science and Emerging Technologies*, *5*(4), 495–502. https://doi.org/10.1016/j.ifset.2004.08.002

U.S. Food and Drug Administration. 21 CFR part 179 (2000). Silver Spring: Federal Register.

U.S. Food and Drug Administration. Irradiation in the Production, Processing and Handling of Food., 21 CFR Part 179. 71056–71058 (2000). Retrieved from

https://www.fda.gov/OHRMS/DOCKETS/98fr/112900a.htm

U.S. Food and Drug Administration. Code of Federal Regulations Title21 (2016). Silver Spring. Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=184.1021

U.S. Food and Drug Administration. Code of Federal Regulations Title 21 (2016). Silver Spring. Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=112.44

U.S. Food and Drug Administration. Code of Federal Regulations Title 21 (2016).

U.S. Food and Drug Administration. Code of Federal Regulations Title 21 (2016). U.S. Food and Drug Administration.

Uraih, N., & Offonry, S. (1981). Inhibition of aflatoxin production in groundnut with benzoic acid derivatives and possible toxic effect of their aromatic residues. *Microbios*, *31*(124), 93–102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6799751

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews*, *56*(3), 395–411. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1406489

Van Boekel, M. A. J. S. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology*, *74*(1–2), 139–159. https://doi.org/10.1016/S0168-1605(01)00742-5

Vega-Mercado, H., Martín-Belloso, O., Qin, B.-L., Chang, F. J., Marcela Góngora-Nieto, M., Barbosa-Cánovas, G. V., & Swanson, B. G. (1997). Non-thermal food preservation: Pulsed electric fields. *Trends in Food Science & Technology*, *8*(5), 151–157. https://doi.org/10.1016/S0924-2244(97)01016-9

Vega-Mercado, H., Pothakamury, U. R., Chang, F.-J., Barbosa-Cánovas, G. V., & Swanson, B. G. (1996). Inactivation of Escherichia coli by combining pH, ionic strength and pulsed electric fields hurdles. *Food Research International*, *29*(2), 117–121. https://doi.org/10.1016/0963-9969(96)00015-4

Wachtel, M. R., & Charkowski, A. O. (2002). Cross-Contamination of Lettuce with Escherichia coli O157:H7. *Journal of Food Protection*, *65*(3), 465–470. https://doi.org/10.4315/0362-028X-65.3.465

Wachtel, M. R., Whitehand, L. C., & Mandrell, R. E. (2002). Association of Escherichia coli O157:H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. *Journal of Food Protection*, *65*(1), 18–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11808792

Wang, Q., Durand, E., Elias, R. J., & Tikekar, R. V. (2016). Generation of reactive oxidative species from thermal treatment of sugar solutions. *Food Chemistry*, *196*(1), 301–308. https://doi.org/10.1016/j.foodchem.2015.09.044

Warth, A. D. (1988). Effect of Benzoic Acid on Growth Yield of Yeasts Differing in Their Resistance to Preservatives Effect of Benzoic Acid on Growth Yield of Yeasts Differing in Their Resistance to Preservatives, *54*(8), 2091–2095.

Wilkinson, F., Helman, W. P., & Ross, A. B. (1995). Rate Constants for the Decay and Reactions of the Lowest Electronically Excited Singlet State of Molecular

Oxygen in Solution. An Expanded and Revised Compilation. *Journal of Physical and Chemical Reference Data*, *24*(2), 663–677. https://doi.org/10.1063/1.555965

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters*, *82–83*(C), 969–974. https://doi.org/10.1016/0378-4274(95)03532-X

Xiong, R., Siegel, D., & Ross, D. (2014). Quinone-induced protein handling changes: Implications for major protein handling systems in quinone-mediated toxicity. *Toxicology and Applied Pharmacology*, *280*(2), 285–295. https://doi.org/10.1016/j.taap.2014.08.014

Yamada, H., & Mizushima, S. (1980). Interaction between Major Outer Membrane Protein (O-8) and Lipopolysaccharide in Escherichia coli K12. *European Journal of Biochemistry*, *103*(1), 209–218. https://doi.org/10.1111/j.1432-1033.1980.tb04305.x

Ye, Z., Forney, L. J., Koutchma, T., Giorges, A. T., & Pierson, J. A. (2008). Optimum UV Disinfection between Concentric Cylinders. *Industrial & Engineering Chemistry Research*, *47*(10), 3444–3452. https://doi.org/10.1021/ie0703641

Yousef, A. E., El-Shenawy, M. A., & Marth, E. H. (1989). Inactivation and Injury of Listeria monocytogenes in a Minimal Medium as Affected by Benzoic Acid and Incubation Temperature. *Journal of Food Science*, *54*(3), 650–652. https://doi.org/10.1111/j.1365-2621.1989.tb04673.x

Zhang, G., Ma, L., Phelan, V. H., & Doyle, M. P. (2009). Efficacy of Antimicrobial

Agents in Lettuce Leaf Processing Water for Control of Escherichia coli O157:H7. *Journal of Food Protection*, *72*(7), 1392–1397. https://doi.org/10.4315/0362-028X-72.7.1392

Zhao, T., Doyle, M., & Besser, R. (1993a). Fate of enterohemorrhagic Escherichia coli O157: H7 in apple cider with and without preservatives. *Applied and Environmental*, *59*(8), 2526–2530. Retrieved from http://aem.asm.org/content/59/8/2526.short

Zhao, T., & Doyle, M. P. (1994). Fate of Enterohemorrhagic Escherichia coli O157: H7 in Commercial Mayonnaise. *Journal of Food Protection*, *57*(9), 780–783. https://doi.org/10.4315/0362-028X-57.9.780

Zhao, T., Doyle, M. P., & Besser, R. E. (1993b). Fate of enterohemorrhagic Escherichia coli O157:H7 in apple cider with and without preservatives. *Applied and Environmental Microbiology*, *59*(8), 2526–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8368839

Zhao, T., Doyle, M. P., Shere, J., & Garber, L. (1995). Prevalence of enterohemorrhagic Escherichia coli O157:H7 in a survey of dairy herds. *Applied and Environmental Microbiology*, *61*(4), 1290–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7747951