Effectiveness of UV-C irradiation on controlling growth of *L. monocytogenes* on fresh cut broccoli

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ABSTRACT

Increasing numbers of foodborne disease outbreaks related to fresh produce are reported every year from around the world. This increase is partly attributed to increased consumption of fresh produce such as whole and fresh cut fruits and vegetables. Fresh produce can be contaminated at any time from field to table, providing channels for transmitting foodborne pathogens to humans. Studies have reported that human pathogens such as *Listeria monocytogenes,* which can grow and survive under refrigerated conditions, cannot be adequately removed by washing with commonly used chemical disinfectants. Consumers prefer products that have not been treated chemically, especially fresh products that are consumed without further processing before consumption. Physical treatments such as UV-C irradiation have shown promising effects in improving storage life, nutritional quality, and microbial safety of fresh produce. UV-C irradiation is beneficial due to its direct germicidal effect, and could possibly induce defence responses in fresh produce which may further improve quality and safety. However, direct germicidal effects would be limited by the uneven surfaces of fresh produce, with risk of survival of pathogens in areas shaded from direct UV exposure. It is also not clear whether induced defence related changes would be sufficient to offer any significant protection against human pathogens. Therefore, this study focused on evaluating the efficacy of postharvest UV-C irradiation to control growth of *L. monocytogenes* inoculated onto fresh cut broccoli at different times after UV-C treatment, and possible mechanisms of induced resistance.

UV-C irradiation supplied at a total dose of 5.2 kJ m^{-2} significantly reduced growth of *L. monocytogenes* inoculated onto fresh cut broccoli 6 h and 24 h after treatment, whereas a 2.6 kJ m^{-2} treatment was suppressive only 24 h after treatment. Neither dose of UV-C adversely affected the quality of fresh cut broccoli compared to untreated broccoli. The *in vitro* study of extracts of UV-C treated broccoli extracted 24 h after UV-C treatment showed that certain extracts were indeed suppressive against *L. monocytogenes.* Aqueous extract of UV-C treated broccoli extracted 24 h after treatment suppressed growth of *L. monocytogenes* compared to extracts of untreated broccoli. Phytochemical analysis of broccoli extracts by LC-HRMS revealed that UV-C irradiation significantly increased production of particular

phytochemical compounds. Compound identifications included raphanusamic acid, salicylic acid-β-glucoside, *p*-coumaryl quinic acid, all of which have been previously reported as being involved in plant defence. Therefore, UV-C irradiation appears to induce defence responses which may be effective systemically, and therefore not critically dependent on achieving an effective sanitising dose of UV-C across the entire broccoli surface. This may alleviate concerns about relying on uniform illumination for anticipated benefits.

Overall, these results suggest that treatment of broccoli with UV-C irradiation should lead to immediate microbial mortality, and a longer-lasting induction of defence systems in the tissues, which would suppress growth of pathogens such as *L. monocytogenes* if the tissue became contaminated during processing. Therefore this treatment can be recommended as a hurdle technology to improve microbial safety of packaged fresh produce.

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CFU	Colony forming units
CSLM	Confocal scanning laser microscopy
DMSO	Dimethyl sulfoxide
GC-MS	Gas chromatography - mass spectrometry
GLSs	Glucosinolates
H	Hours
ITC	Isothiocyanate
JA	Jasmonic acid
kJ	Kilo joules
LC-HRMS	Liquid chromatography - high resolution mass spectrometry
OD	Optical density
PAL	Phenylalanine ammonia-lyase
PCA	Principal component analysis
RT	Retention time
RTE	Ready - to – eat
SA	Salicylic acid
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TSB	Tryptic soy broth
UV	Ultraviolet

1 INTRODUCTION

1.1 Background

Outbreaks of foodborne diseases have been reported from many countries and are becoming a serious issue in the horticulture industry. Due to complex and busy life styles, many people prefer ready-to-eat foods (Appendini & Hotchkiss, 2002) including whole or fresh cut fruits and vegetables. The health benefits of eating fresh fruit and vegetables have led to an increase in the consumption of these products throughout the western world. Global food marketing now allows year-round availability of seasonal fresh fruit and vegetables and long food production chains may allow slow growth of pathogens during delivery to markets. This increase in consumption of fresh produce has been correlated with a rise in the number of foodborne disease outbreaks related to fresh produce (Olaimat & Holley, 2012). Foodborne diseases such as salmonellosis, and listeriosis are deadly diseases caused by foodborne bacteria. Therefore, proper preharvest and postharvest treatments are needed to prevent contamination as well as subsequent growth of human pathogens in fresh produce. Fresh fruit and vegetables are highly susceptible to contamination and deterioration by microorganisms after harvest. The most common method of eliminating microorganisms in fresh produce after harvest is washing with disinfectants and application of chemical preservatives. However, consumers are increasingly concerned about the detrimental effects of chemical preservatives on human health as well as on the environment. New approaches are needed to extend storage life and improve microbial safety of fresh produce without chemical additives (Soliva-Fortuny & Martín-Belloso, 2003).

Physical postharvest technologies such as heat treatment, pressure processing, and ultraviolet (UV) irradiation have confirmed their effectiveness in preserving quality of fresh produce and their potential use as substitutes for chemical additives (Soliva-Fortuny & Martín-Belloso, 2003; Gonzalez-Aguilar et al., 2010). However, these treatments have been focused mostly on sanitization and improving physicochemical properties of fresh produce after harvest. The increasing number of foodborne disease outbreaks all over the world raises the question of whether these treatments are effective in controlling human pathogens attached to fresh produce. Refrigeration

is the most common method of preserving quality of fresh produce, and many foodborne pathogens may be suppressed or inhibited during low temperature storage. Unfortunately, psychrotrophic pathogens such as *Listeria monocytogenes* can tolerate low temperature storage and multiply up to harmful levels by the time consumers eat the produce. Therefore, it is important to find measures to control growth of such high risk psychrotrophic pathogens on fresh products during storage.

This chapter reviews the literature pertaining to foodborne disease outbreaks associated with fresh produce and technologies to control growth and survival of foodborne pathogens attached to fresh produce surfaces after harvest. Background on the types of foodborne diseases linked to fresh produce with particular attention to *L. monocytogenes* is provided followed by sections explaining the factors affecting contamination of fresh produce and methods to prevent *L. monocytogenes* contamination of fresh produce. Different types of postharvest treatments used to control human pathogens are critically evaluated. This is followed by a review of studies conducted on UV-C irradiation as a postharvest treatment for fresh produce and characteristics of UV irradiation in order to critically evaluate the possibility of using it as a postharvest treatment to control growth and survival of *L. monocytogenes* inoculated onto fresh cut broccoli after treatment. The regulations governing the application of UV irradiation in the food industry and the special characteristics of broccoli as a model fresh produce are discussed. The impact of UV-C irradiation on physiological processes of broccoli such as changes in quality, antioxidant activity, gene expression are explained. Finally aims and objectives of this research and outline of the thesis are stated.

1.2 Fresh produce and human pathogens

Fresh products such as fruit and vegetables are often eaten fresh without further processing, generating a risk of diseases due to pathogenic microorganisms. Primary washing by dipping in water or washing under running tap water may not adequately reduce the load of pathogenic microorganisms in fresh produce. In addition, fresh produce may be stored at relatively high temperatures (4 - 20 °C) in retail stores which favour the growth of pathogenic microorganisms such as *L. monocytogenes*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. Therefore it is important to employ handling and postharvest practices that will minimize the risk of human pathogen contamination of fresh produce.

1.2.1 Severity of foodborne illnesses related to fresh produce

Global consumption of fresh produce including fruit and vegetables has increased yearly by about 4.5 % on average between 1990 to 2004 (Olaimat & Holley, 2012). Advanced technological interventions in agronomy, processing, postharvest, storage, distribution, and marketing in recent years have contributed to availability and continuous supply of all types of fresh fruit and vegetables to consumers (Beuchat, 2002). However, these same technologies may generate a greater risk of foodborne illnesses due to pathogenic microorganisms that can contaminate fresh produce at any stage of production, transportation, or storage (Beuchat, 1995, 2002). Millions of cases of foodborne illness associated with fresh produce occur in the world every year and many more cases are undiagnosed and unreported (CDC, 2014). For example, 14.8% of the foodborne disease outbreaks in the US were due to consumption of fresh produce such as salads, vegetables, and fruit (CSPI, 2009). The most recent and dangerous outbreak occurred in 2011 in the United States (US) associated with consumption of cantaloupe contaminated with *L. monocytogenes*, leading to 146 reported illnesses, 30 deaths (20.5%) and one miscarriage (CDC, 2011b). The largest outbreak, related to *E. coli O157:H7*, and linked to consumption of raw radish sprouts, occurred in Japan in 1996, killing 12 people and resulting in 12,000 illnesses. The same pathogen caused an outbreak in the US and Canada in 2006, which was linked to consumption of contaminated spinach causing 199 illnesses with 3 deaths and 16% of the infected persons developed acute renal failure (CDC, 2006). In 2010 another *E. coli* O157:H7 outbreak associated with shredded lettuce was reported from five states in the US; with 26 confirmed cases. Another strain of *E. coli* which is a shiga toxin-producing *E. coli* (STEC) O104:H4 was linked to an infection that occurred in Europe, in 2011. Nearly 4,000 people in 16 countries were affected and the outbreak was linked to consumption of contaminated vegetable sprouts (EFSA, 2011). A large *Salmonella* outbreak was reported in 2008 over 43 states of the US and Canada with 1442 reported illnesses due to consumption of hot peppers (CDC, 2008). In 2011 also another *Salmonella* outbreak occurred in the US leading to 140 reported illnesses due to consumption of contaminated alfalfa sprouts (CDC, 2011a).

Green salad, lettuce, seed sprouts, tomatoes, and cantaloupe are the most common types of fresh produce associated with foodborne disease outbreaks (Olaimat &

Holley, 2012). Table 1.1 shows the outbreaks related to fresh produce from 2005 to 2011.

1.2.2 Common foodborne illnesses caused by human pathogens

Consumption of foods that are contaminated by pathogens such as bacteria, viruses, or parasites causes foodborne illnesses in humans. But most outbreaks from fresh produce are linked to bacterial origins (Beuchat, 1995). According to the Centre for Disease Control and Prevention (CDC) in the United States, around 48 million people get sick, 128,000 are hospitalized, and 3000 people die due to foodborne illnesses in the US each year. This may cause considerable impacts on the economy of a country demanding implementation of high cost preventive and surveillance systems and loss of work force due to illnesses. Ribera and others demonstrated that the cost of preventing such foodborne diseases is far less than the costs incurred by producers due to outbreaks caused by contaminated fresh produce (Ribera et al., 2012).

There are about 250 identified foodborne diseases but three organisms were responsible for most disease outbreaks related to fresh produce in the US (Table 1.1).

1.2.2.1 Salmonellosis

Salmonellosis is caused by bacteria belonging to genus *Salmonella*; a rod shaped, Gram negative facultative anaerobic bacterium (Cray & Fedorka-Cray, 2002). *Salmonella* serotype *typhymurium* and *enteritidis* are the common foodborne pathogens (Swartz, 2002). Other than food poisoning, *Salmonella* may cause typhoid and paratyphoid fever. Any food, including vegetables, may be easily contaminated as this bacterium is commonly found in human and animal faeces. Common symptoms include diarrhoea, fever, and abdominal cramps, which start within 12 to 72 h after infection and may last for 4 to 7 days. However infected persons may fully recover without treatment. *Salmonella* infection can be transmitted from intestine to the blood system and to other organs of the body causing serious problems (Cray & Fedorka-Cray, 2002). About 40,000 cases of salmonellosis are reported in the US, and 400 people die each year from acute salmonellosis (CDC, 2014). Raw or undercooked eggs, poultry or meat, unpasteurized milk, and fresh produce pose the greatest risk to human health from *Salmonella*. Most of these high risk products are of animal origin and may cross contaminate other products as their natural

Table 1.1 Outbreaks linked to fresh produce from 2005 to 2011

Country	Year	Pathogens	Produce	Cases (deaths)
Canada	2005	*Salmonella*	Mung bean sprouts	592
USA	2005	S*almonella*	Tomatoes	459
Australia	2006	*Salmonella*	Alfalfa sprouts	125
USA Canada	2006	*Salmonella*	Fruit salad	41
USA	2006	*Salmonella*	Tomatoes	183
Australia	2006	*Salmonella*	Cantaloupe	115
Europe	2007	*Salmonella*	Alfalfa sprouts	45
Europe	2007	*Salmonella*	Baby spinach	354
North America/ Europe	2007	*Salmonella*	Basil	51
USA Canada	2008	*Salmonella*	Peppers	1442 (2)
UK	2008	*Salmonella*	Basil	32
USA	2008	*Salmonella*	Cantaloupe	51
USA Canada	2008	*Salmonella*	Peanut butter	714 (9)
USA	2009	*Salmonella*	Alfalfa sprouts	235
USA	2010	*Salmonella*	Alfalfa sprouts	44
USA	2011	*Salmonella*	Alfalfa & mixed sprouts	140
USA	2011	*Salmonella*	Cantaloupe	20
USA	2011	*Salmonella*	Papaya	106
USA	2006	*E. coli* O157:H7	Spinach	199 (3)
USA	2006	*E. coli* O157:H7	Lettuce	81
USA	2006	*E. coli* O157:H7	Spinach	22
USA Canada	2008	*E. coli* O157:H7	Lettuce	134
USA	2010	*E. coli* O145	Lettuce	26
Europe	2011	*E. coli* O104:H4	Vegetable sprouts	3911 (47)
USA	2011	*E. coli* O157:H7	Strawberries	15 (1)
USA	2011	*E. coli* O157:H7	Lettuce	60
USA	2010	*L. monocytogenes*	Fresh cut produce	10 (5)
USA	2011	*L. monocytogenes*	Cantaloupe	146 (31)

Source: Modified information from Olaimat et al. (2012)

contamination is high. To avoid *Salmonella* infection, food should be refrigerated at all times and cooked thoroughly for at least 10 minutes before eating, but fresh products are at a risk of transmitting the pathogen because they are often eaten fresh. Good hygiene is also important including washing hands before food preparation, after using toilets, and after contact with animal faeces (WHO, 2013).

1.2.2.2 Escherichia coli

E. coli is a rod shaped, Gram negative facultative anaerobic bacterium commonly found in the intestine of humans and animals. Most *E. coli* are harmless and constitute part of normal gut flora, benefiting the host in many ways. Different serotypes of pathogenic *E. coli* have been categorized into different groups depending on their virulence mechanisms: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), enterohemorrhagic (EHEC), uropathogenic *E. coli* (UPEC), and verotoxin-producing *E. coli* (Fratamico et al., 2002). ETEC are the most common cause of travellers' diarrhoea and about 79,420 cases of ETEC are estimated in the US each year (CDC, 2014). This pathogen can be easily transmitted through food and water contaminated with human or animal faeces. Enterohemorrhagic *E. coli* (EHEC) causes illness by producing a toxin called Shiga toxin. The most common shiga toxin-producing *E. coli* (STEC) is *E. coli* O157:H7. Symptoms of STEC are severe stomach cramps, diarrhoea (mostly bloody), vomiting, and low fever. About 5 to 10% of people diagnosed with STEC infection may develop haemolytic uremic syndrome (HUS) which is a serious life threatening complication (Fratamico et al., 2002). Foods such as unpasteurized milk, soft cheese made with raw milk, contaminated water, contact with cattle, and unpasteurized apple cider were identified as causes for previous outbreaks in the US. There are about 265,000 STEC cases reported in the US of which *E. coli* O157:H7 accounts for 36% (CDC, 2014).

1.2.2.3 Listeriosis

Listeriosis is caused by *L. monocytogenes*; a Gram positive, facultative, anaerobic, rod shaped psychrotrophic bacterium (Harris, 2002). It mostly affects pregnant women, neonates, and immunocompromised people and can cause meningitis in newborns (Little et al., 2007). Symptoms of a normal infection may include fever, muscle aches, and sometimes diarrhoea. There are about 1600 illnesses and 260

deaths occurring in the US annually due to listeriosis (CDC, 2014). *L. monocytogenes* can be found freely in the environment: air, soil, and water. Therefore, fresh produce is highly susceptible to contamination by this pathogen. Even though pasteurization or proper cooking can destroy *L. monocytogenes* in food, fresh produce is at a risk of causing foodborne illnesses as they may be eaten fresh without cooking.

1.2.3 Significance of *L. monocytogenes*

L. monocytogenes is a psychrotrophic bacterium that causes foodborne disease called listeriosis in humans with high hospitalization and fatality rates (Little et al., 2007; Little et al., 2009). This bacterium is one of the most virulent foodborne pathogens and causes a higher percentage of deaths compared to other foodborne pathogens (table 1.1). *L. monocytogenes* is widely distributed in the environment and adapted to diverse environmental conditions. For example, it is capable of growing at refrigeration temperatures (2 - 4 °C), in acidic foods and high salt foods (Gandhi & Chikindas, 2007; Little et al., 2007; Luber et al., 2011). This adaptability and ubiquitous nature of the bacterium and high fatality rate of the disease challenge food industry to find mechanisms to improve microbial safety of fresh produce.

1.2.3.1 Low temperature and low pH adaptation

Under the low temperature (7 °C) conditions, *L. monocytogenes* is capable of maintaining the ideal fluidity of the cell membrane required for enzyme activity and solute transport by changing the composition of the cell membrane fatty acids (Beales, 2004). *L. monocytogenes* is also capable of producing cold shock proteins (CSPs) when temperature drops and cold acclimation proteins (CAPs) during survival at the low temperatures (Bayles et al., 1996). Acidic nature of low pH food and gastrointestinal fluid may also challenge the growth and survival of *L. monocytogenes*. To tolerate such acid stress, *L. monocytogenes* utilizes the glutamate decarboxylase system which consists of three genes: gadA, gadB, gadC. One of the proteins (GroEL) that was induced in cold shock was induced under acid stress also in addition to ATP synthase and various other transcriptional regulators (Gandhi & Chikindas, 2007). Because of these adaptive mechanisms *L. monocytogenes* is capable of growing at the low temperatures which are ideal for storing fresh produce.

This makes *L. monocytogenes* an important issue that fresh produce industry must deal with to ensure microbial safety of fresh produce.

1.2.3.2 Biofilm formation and quorum sensing

L. monocytogenes can exist as individual cells or as communities called biofilms, in which individuals are attached to a surface as well as to each other. Biofilms of *L. monocytogenes* can exist as either monoculture or as mixed cultures, coexisting with other types of bacteria (Bremer et al., 2001). In a biofilm, bacteria are enclosed in a matrix of exopolysaccharides (Donlan, 2002) which provides them protection from disinfectants and sanitizers that are commonly used for washing fresh produce and cleaning food processing surfaces (Olmez & Temur, 2010). Biofilms have been isolated from a wide range of surfaces including food processing facilities, medical devices, and even on fresh produce surfaces (Bremer et al., 2001; Harvey et al., 2007; Bonsaglia et al., 2014). For example, Bonsaglia and others have observed biofilm formation behaviour of *L. monocytogenes* and reported that this pathogen is capable of forming biofilms even at the low temperatures (4 °C) on different types of surfaces used in food processing facilities such as stainless steel, glass and polystyrene (Bonsaglia et al., 2014). In a similar study biofilm formation of *L. monocytogenes* on lettuce leaf surface was observed by scanning electron microscopy (SEM): a range of sanitizers were tested and none were effective in removing biofilms 48 h after inoculating the pathogen onto the leaf surface (Olmez & Temur, 2010). Biofilm formation by microorganisms including human pathogens is a complex process affected by many factors including surface properties such as hydrophobicity, cell surface properties such as surface charge, and environmental factors such as temperature and pH (Donlan, 2002). Biofilm formation is induced through production of chemical signalling molecules termed as autoinducers which are involved in cell to cell communication; a process known as quorum sensing (QS) (Gandhi & Chikindas, 2007; Riedel et al., 2009). Concentration of these signalling molecules increases with increasing cell density, and bacteria in a community sense accumulation of a minimum threshold stimulatory concentration of the signalling molecules and alter gene expression and function as multicellular organisms by synchronizing their behaviours (Waters & Bassler, 2005). Gram positive bacteria including *B. cereus* and *Staphylococci* produce oligopeptides while Gram negative bacteria produce acylated homoserine lactones (HSLs) as autoinducers (Riedel et al.,

2009). Genome sequence analysis of *L. monocytogene*s has revealed the presence of a QS system which resembles that found in *S. aureus* and *Staphylococci*, referred to as the agr system. Agr QS system is composed of a four-gene operon encoding four proteins namely agrA, agrB, agrC, and agrD which are responsible for virulence of *L. monocytogenes* including biofilm formation (Autret et al., 2003). Riedel et al. (2009) confirmed the incapability of an agrD mutant *L. monocytogenes* to form biofilms in brain-heart infusion medium (BHI). Identification of phytochemicals which are anti-quorum sensing or anti pathogenic, and that may influence the cell to cell signalling mechanism of pathogenic bacteria may be useful as a tool to control growth and/or virulence of foodborne pathogens.

1.3 Factors affecting pathogen contamination of fresh produce

1.3.1 Sources of contamination of fresh produce

Fresh produce can be contaminated by pathogens while it is in the field, green house or orchard, or before or after harvest. The major sources of contamination can be environmental, animal, or human origin (Beuchat, 2002). Fresh produce may be contaminated by soil, irrigation water, dust, manure, insects, wild or domestic animals, or people who handle the produce (Olaimat & Holley, 2012). Therefore, proper cultural practices in the field and handling practices after harvest need to be designed to minimize pathogen contamination (Warriner et al., 2009; Olaimat & Holley, 2012). Cultivated land can be contaminated with human pathogens when animal manure is used as fertilizer. Particularly, manure of ruminants and sewage have been shown to be contaminated with *Salmonella, E. coli O157:H7,* and *C. jejuni*, and these pathogens can survive in the soil for 45 to 100 days (Nicholson et al., 2005). Most of these pathogens are natural gastrointestinal microflora of poultry, pigs, and cattle (Warriner et al., 2009). Most *E. coli* strains are harmless and live in human intestinal tract. *L. monocytogenes* by contrast is not a normal resident of the intestinal tract but is commonly found in nature: in soil, dust, and decaying vegetation. Waste water used for irrigating fresh produce also imposes a risk of human pathogen contamination. Not only the water quality but the irrigation method plays a role here because flooding and spray irrigation methods allow the contaminated water to directly contact the surfaces of edible plant parts (Warriner et al., 2009). Therefore WHO/FAO has recommended a maximum limit of fecal

coliforms (1000 cfu /100 mL) in irrigation water used for fresh produce (Olaimat & Holley, 2012).

Harvesting and processing steps are also of concern because most of the human and mechanical contacts with fresh produce happen during these processes (Brackett, 1999). Workers who are sick or carriers of pathogens may be a source of contamination of fresh produce during harvesting and processing (Warriner et al., 2009). Processing equipment can also harbour pathogens if not cleaned properly. Cutting, peeling, and slicing allow nutrients to leak from tissues, providing pathogens a source of nutrients and promoting their growth after contamination (Harris et al., 2003).

Fresh produce may be cross contaminated during processing if a few contaminated samples are present in a batch. Selecting appropriate and efficient packaging that minimizes growth of microorganisms followed by appropriate storage temperatures is important to improve the microbial safety of fresh produce (Little et al., 2007). Many fresh products are better stored under 5 °C to maintain quality and reduce the growth of spoilage and pathogenic microorganisms (Rediers et al., 2009). Temperature abuse (undesirable increase in storage temperature) during transportation and retail storage may increase the growth of foodborne pathogens (Little et al., 2009; Luber et al., 2011). Even though low temperature storage can reduce or even stop the growth of many pathogens, *L. monocytogenes* is a psychrotrophic bacterium which can grow even under refrigerated conditions (Brackett, 1999) compelling the food industry to find better solutions for controlling the growth and survival of this harmful foodborne pathogen. Postharvest treatments that reduce growth and survival of such psychrotrophic bacteria must be applied to improve microbial safety of fresh produce. There are many studies explaining the use of different postharvest treatments in reducing initial microbial pathogen counts on fresh produce, but no research has been done on evaluating the effect of postharvest treatments on controlling human pathogens inoculated onto surface of fresh produce after treatment. Some postharvest treatments not only kill pathogens on surface of fresh produce, but also induce phytochemical production from plant metabolism. Production of such compounds which may be antimicrobial may effective in suppressing growth of pathogens inoculated onto fresh produce surface after treatment.

1.3.2 Surface-pathogen interaction

Different types of foodborne pathogens attach in varying degrees to fresh produce surfaces (Takeuchi et al., 2000). Hydrophobicity of the surface of fresh produce due to presence of a waxy cuticle layer couples with hydrophobicity of bacteria cell surface may encourage the bacteria to adhere (Mendonca, 2005). It is generally recognized that the bacterial attachment process occurs in three steps (Ukuku et al., 2005): reversible adsorption, primary adhesion, and colonization. During the first step, bacteria attach to the surface by weak van der Waal's interactions at a distance of 50 nm and at the second stage, bacteria attach more closely by electrostatic forces at a distance of 10-20 nm from the surface. The third step is colonization where bacteria form biofilms. By the time the distance between bacteria and the surface is less than 1.0 nm, bacteria bind to the surface by strong hydrogen bonding, cation bridging, and receptor-ligand interactions improving resistance to sanitizers and disinfectants (Ukuku et al., 2005). In contrast, negatively charged surfaces of both fresh produce and the bacteria may create electrostatic repulsion upon contact. Bacterial appendages such as fimbriae, extracellular polysaccharide or outer membrane proteins can bridge the gap created by electrostatic repulsion (Mendonca, 2005). For example, fimbriae and bacterial exopolysaccharides produced by *E. coli* O157:H7 can function as plant surface adhesins whereas *Salmonella* strongly binds to produce surfaces by producing cellulose and curli (Ukuku et al., 2005). Another factor affecting pathogen attachment is the surface roughness or topography. A rougher cell surface may provide a larger contact area for the pathogen to attach to and protection by shading the pathogen from sanitizing treatments (Wang et al., 2009). The higher surface roughness of peach compared to pear reduced the efficacy of UV-C in reducing *E. coli* O157:H7 populations on the peach surface, whereas a greater than three log cycle reduction was observed on intact pear surface (Roopesh et al., 2013).

Trichomes, cut edges, crevices, stomata and damaged tissues of leaf surface were the preferred places by pathogens for attachment and SEM and confocal scanning laser microscopic (CSLM) images illustrated that human pathogens including *Salmonella* spp, *E. coli,* and *L. monocytogenes* tended to aggregate close to those sites on cabbage and lettuce, and formed biofilms within 24 h in most cases (Seo & Frank, 1999; Takeuchi et al., 2000; Ells & Lisbeth, 2006; Elhariry, 2011). It may be interesting to study mechanisms of human pathogen attachment to surfaces as

mechanisms may be similar to or different from plant pathogens which have evolved for plant pathogenicity. Very recently some researchers have studied the genes involved in *L. monocytogenes* attachment to fresh produce surfaces (Bae et al., 2013; Salazar et al., 2013; Martinez-Vaz et al., 2014). Bae et al. (2013) identified 32 genes encoding surface proteins and lipases of *L. monocytogenes* and five of them were significantly up-regulated during attachment to lettuce surfaces. In further studies they have screened a gene encoding a protein with a putative cellulose binding domain that may be responsible for attachment to lettuce, baby spinach, and cantaloupe surfaces and named it *Listeria* cellulose binding protein (LCP) (Bae et al., 2013). Salazar et al. (2013) identified a putative family transcription factor Lmo0753 [cyclic AMP receptor proteins (Crp)/fumarate reductase regulator (Fnr)] which is highly specific to human outbreak-associated genetic lineages of *L. monocytogenes*. The Lmo0753 contains two functional domains analogous with the gene encoding positive regulatory factor A (PrfA). *L. monocytogenes* cells deficient in the Lmo0753 gene showed significantly less attachment to romaine lettuce and cantaloupe rind compared to the wild type suggesting a key role of the Lmo0753 gene in surface attachment to fresh produce (Salazar et al., 2013). These results imply that *L. monocytogenes* may utilize virulence factors evolved for human pathogenicity, to attach to fresh produce; a likely the reason for its survival in a wide range of environments and recent emergence in foodborne outbreaks linked to fresh produce (Salazar et al., 2013). It is evident that most foodborne human pathogens linked to fresh produce have evolved many mechanisms to attach to surfaces and survive through basic washing and other postharvest treatments. Therefore, it is important to control growth of contaminated pathogens on fresh produce through postharvest treatments where even low temperature storage may not be adequate. One possibility of reducing the problem of contamination by psychrotrophic pathogens like *L. monocytogenes* is a hurdle technology (see 1.5.2). For example, use of different hurdles for pathogen growth at different stages of processing: use of UV-C irradiation to treat fresh cut broccoli after hot water washing and then pack in modified atmosphere packages to improve postharvest quality and safety of fresh produce.

1.4 Controlling *Listeria monocytogenes* on fresh produce

Psychrotrophic pathogens like *L. monocytogenes* are of great concern because of their ability to grow under refrigerated conditions. The ubiquitous nature of *L. monocytogenes* in the environment provides plenty of opportunities for the pathogens to attach to fresh produce (Beuchat, 2002). The ability to form biofilms and adapt to different environmental conditions make them resistant to sanitizers and disinfectants and hence difficult to remove from surfaces (Olmez & Temur, 2010). Food contaminated by *L. monocytogenes* may cause outbreaks of listeriosis which affects immunocompromised people and leads to high mortality rates. Therefore, the food industry needs to be vigilant about *L. monocytogenes*. (Luber et al., 2011). Increased consumption of fresh and minimally processed foods that are eaten without further processing and development of new technologies that extend storage life of food have implications on microbial safety of food including fresh produce (Ukuku et al., 2005; Gandhi & Chikindas, 2007). In addition, some of the pathogens including *L. monocytogenes* and *E. coli* O157:H7 may become internalized in the tissues or aggregate in crevices or on damaged surfaces, reducing the effectiveness of postharvest sanitization treatments (Mendonca, 2005). It is therefore important to employ possible interventions that are capable of eliminating or controlling the growth of pathogenic bacteria to improve microbial safety of fresh produce (Luber et al., 2011). In 2008, WHO/FAO published a list of recommendations covering a range of aspects involved in production of fresh produce to ensure microbial safety of fresh produce (Olaimat & Holley, 2012) including the following:

1. Minimizing pathogen contamination by wildlife in crops
2. Topographical and climate risk assessment of crops before field planting
3. Proper management of irrigation and other types of water supply used in growing and manufacturing of fresh produce
4. Prevention of faecal contamination of surface water, underground water, and cultivated areas
5. Application of good agricultural practices and good manufacturing practices; proper sanitation of humans and equipment associated with cultivation, harvesting, processing, and distribution

6. Appropriate training and education of farm workers and consumers
7. Implementation of good manufacturing practices and hygiene practices with standard operating procedures
8. Proper sanitation of food processing areas, equipment and contact surfaces
9. Food packaging design and materials
10. Packaging material or gases should not compromise safety and suitability of food for consumption

In the case of *L. monocytogenes,* different countries have developed their own protocols and threshold contamination levels. For example, the European Union has enforced levels less than 100 cfu/g during shelf life of ready-to-eat (RTE) foods that do not support the growth of *L. monocytogenes* and complete absence from infant food and foods intended for special medical purposes throughout their shelf life (Little et al., 2007). The US on the other hand, has a zero tolerance policy. However, the competence of these regulations in controlling *L. monocytogenes* contamination of food is uncertain because there are still a lot of outbreaks and cases reported annually even from these countries. Therefore, there is a compelling need for innovative technologies for preventing contamination and controlling growth of pathogens during storage of food.

1.5 Different postharvest techniques studied to control human pathogens on fresh produce

Many studies have demonstrated that quality and safety of fresh produce can be achieved by applying good postharvest treatments such as cold storage, chemical treatments (washing with disinfectants, adding food additives), physical treatments (heat, irradiation, pressure, UV-C, coating), packaging technologies (modified atmosphere packaging, active packaging), and use of antagonistic microorganisms (Lactic acid bacteria, bacteriocins, and bacteriophages) among many other postharvest treatments. Temperature control is the most widely used method to protect produce quality and safety (Francis et al., 2012; Olaimat & Holley, 2012). However *L. monocytogenes* is a psychrotrophic bacterium that can grow even at refrigerated temperatures. Temperatures less than 4 °C reduce the growth rate but do

not kill *L. monocytogenes* and growth may occur at or above 8 °C in packed vegetables (Francis et al., 2012). It is now clear that temperature abuse during storage or transportation can detrimentally affect the microbial safety of fresh produce. Therefore, it is important to identify the possible points in the supply chain where contamination can occur through Hazard Analysis and Critical Control Point (HACCP) programs in order to recommend appropriate postharvest technology for controlling pathogens (Francis et al., 2012).

1.5.1 Hormesis

Hormesis is a phenomenon that occurs as a response in organisms as a result of exposure to different kinds of potentially toxic agents (Stebbing, 1982). The word "hormesis" is derived from the Greek word "hormaein" which means "to excite" (Lingegowdaru, 2007). Luckey (1980) defined it as 'stimulation of beneficial responses by low doses of any potentially harmful agent'. A range of harmful agents such as heavy metals, ionizing and non-ionizing radiation, insecticides, alcohol, oxygen poisoning, cyanide, and antibiotics has been studied for their potential use in stimulating stresses in live organisms at lower doses. Beneficial doses of these stressors are termed as hormetic doses (Stevens et al., 1997). Hormetic doses of a harmful physical or chemical agent that induce stress in fresh produce can be used successfully as postharvest treatments to stimulate beneficial responses (Charles & Arul, 2007).

1.5.2 Hurdle Technology

No single intervention can provide 100% assurance of a safe and high quality food product (Sommers & Xuetong, 2006). Therefore, a multiple barrier (hurdle) approach has been recommended which combines several types of preservation techniques. For example, UV-C irradiation combined with chemical or antimicrobial treatments may increase the effectiveness more than individual techniques. However, the appropriate combination of hurdles should be selected depending on the produce in order to optimize the overall impact on the desired level of pathogen reduction and final quality of the produce.

1.5.3 Postharvest treatments

1.5.3.1 Chemical treatment

Basically a sanitizing step, chemical washing or dipping is practised in the postharvest industry. Plant debris, pesticide residues and other external contaminations are removed by rinsing in tap water followed by a dip in chlorinated water to reduce the microbial population attached to the surface. In order to assure the original sensorial qualities of chlorine washed produce, they should be rinsed in clean water to eliminate residual chlorine from the surface. (Soliva-Fortuny & Martín-Belloso, 2003). Rewashing with non-chlorinated water may increase the risk of reintroducing pathogens to the product. Effectiveness of sodium hypochlorite (NaOCl)- a source of chlorine, on microorganisms depends on pH, temperature, concentration, organic matter, time of exposure, and load of initial microbes. However, negative consequences such as formation of chloroform and trihalomethanes, which are known to be carcinogenic or mutagenic substances may occur if the recommended pH range (6.5 to 7.5) is not maintained (Artés et al., 2009). To overcome these issues as well as to improve postharvest quality, a variety of alternative chemical treatments such as chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), peroxyacetic acid, citric acid, ascorbic acid, calcium lactate, electrolyzed water, and natural plant extracts have been studied (Rico et al., 2007; Artés et al., 2009; Oms-Oliu et al., 2010). For example, application of calcium based solutions (e.g. calcium lactate) increases firmness, delays senescence, reduces chlorophyll and protein losses in fresh produce while ensuring microbial quality (Martin-Diana et al., 2005). Organic acids such as citric acid, acetic acid and their derivatives act as antimicrobial, antibrowning, and antioxidative compounds which contribute to extending the postharvest storage life of fruit and vegetables (Rico et al., 2007; Oms-Oliu et al., 2010). Recent studies have shown that very low concentrations of 1-methylcyclopropene (1-MCP) have beneficial effects especially on climacteric fruits in which 1-MCP can block the ethylene binding sites and as a result, effectively delay ethylene induced fruit ripening and senescence (Soliva-Fortuny & Martín-Belloso, 2003). This also helps to retain natural disease resistance ability in immature fruit and vegetables (Mao et al., 2007).

However, there is a huge public concern over health and environmental issues of chemicals used as preservatives and sanitizers in the food industry and non-chemical

alternative technologies have been developed in the recent past that are more environment and human friendly. Natural antimicrobial materials derived from plants and microorganisms are good substitutes for chemicals to be used as preservatives and additives in the food industry.

1.5.3.2 Heat treatment

Heat treatment can be considered as an alternative to chemical treatments and an effective method of postharvest quarantine and decay control by which many issues of physiological and pathological nature can be overcome. In addition, it is effective in inhibiting the ripening process (Vicente et al., 2006) and inducing resistance to chilling injury in some fruit and vegetables (Ma et al., 2014). Temperature and exposure time have an effect on produce quality and the tolerance between an effective treatment and damage due to overheating can be as low as 1 to 2°C (Lu et al., 2007). Heat treatments may lead to undesirable quality changes in fresh produce such as loss of flavor, appearance, firmness, vitamins and minerals, and modifications of the biopolymers unless proper temperature regimes are used (Rico et al., 2007). The high temperature - short time (HTST) concept has been used as a general approach to minimize these adverse effects of heat treatment assuming that the inactivation of microorganisms is directly associated with the high temperature whereas the time of exposure affects the development of undesirable quality changes (Ohlsson, 1980, 1994). Heat treatment can be applied as blanching (hot water dips and hot air flush), heat shock, infrared radiation and electric heating. Although blanching may result in nutrient loss due to thermal degradation and loss of texture and appearance, it is useful as a disinfection treatment as it uses comparatively higher temperatures (approx. 85-100°C). Heat shock is also a HTST method which involves a washing step at a comparatively low temperature (45-70°C) for less than 5 minutes which reduces quality deterioration (Rico et al., 2007). However, the main disadvantage of heat treatments in general is the inability to produce a uniform transfer of heat to the produce. Electric heating can be used effectively to overcome this shortfall as it heats up the entire volume equally (Ohlsson, 1994). However, removing products from heat immediately after treatment is important to avoid overheating, perhaps by immersing in cold water which may reintroduce microorganisms to the fresh produce.

1.5.3.3 Modified atmosphere packaging

As the term implies, the composition of the atmosphere surrounding a commodity can be altered by using an appropriate packaging technique. This can be achieved passively by sealing the package under normal atmospheric conditions or actively by flushing the packages with desired gas mixtures before closing. The gas composition inside the package is altered due to respiration of the produce and the gas permeability of the film used for packaging (Oms-Oliu et al., 2008). Low levels of oxygen and high levels of carbon dioxide inside the package reduce the respiration rate of produce, delaying senescence (Allende et al., 2006). The appropriate levels of O_2 and CO_2 concentrations vary with the produce and extremely low levels of O_2 can induce anaerobic respiration producing undesirable metabolites and other physiological disorders (Soliva-Fortuny et al., 2002) such as fermentation and off flavours and odours, reducing the quality of the produce (Rico et al., 2007). On the other hand, high CO_2 concentrations inside the package may be responsible for certain physiological disorders as it dissolves in the cell fluid inhibiting the activities of some enzymes of the Krebs' cycle including succinate dehydrogenase which result in an accumulation of succinic acid, which is toxic to the tissues (Soliva-Fortuny & Martín-Belloso, 2003).

1.5.3.4 Irradiation

Irradiation of food and other agricultural commodities with ionizing radiation is a useful sanitary and phytosanitary treatment used to prevent the growth of spoilage or pathogenic microorganisms by damaging the DNA and RNA of the cells, and also to delay ripening, senescence, and control certain fruit flies (Lacroix & Vigneault, 2007). DNA and RNA of microorganisms absorb the radiation energy, leading to prevention of cell multiplication and consequent death of cells (Monk et al., 1995). Ionizing radiation at appropriate doses may enhance the synthesis of phenolic compounds, flavonoid and vitamin content in fruits and vegetables (Lacroix & Vigneault, 2007).

Irradiation technology employs ionizing radiation which represents a portion of the electromagnetic spectrum having shorter wave lengths and higher associated energy levels than the ultraviolet spectral region (Sommers & Xuetong, 2006). For example, X-rays and gamma-rays are the most common ionizing radiation used in the horticulture industry. Use of low dose irradiation for food commodities has been

approved by many regulatory agencies around the world including United State Food and Drug Administration (USFDA) for the purpose of controlling pathogens such as bacteria, parasites, and protozoans (Rico et al., 2007). Maximum level of irradiation allowed for fruits and vegetables by the USFDA is 1.0 kGy (U.S. FDA, 2010) and such dosages conserve sensory qualities without altering the freshness of the produce. However, higher doses of radiation needed to inactivate pathogens such as bacteria can lead to tissue damage and impair the quality of the produce (Rico et al., 2007) and may cause severe public concerns. Therefore, it is important to measure the distribution of absorbed energy to determine the maximum and minimum doses absorbed by the food in order to confirm that the approved food preservation and quality control procedures have been followed (Lacroix & Vigneault, 2007).

1.5.3.5 Pressure treatment

Physical treatments such as high pressure (1000-3000 atm), hyperbaric (> atmospheric pressure) and hypobaric (< atmospheric pressure) pressure application to fruit and vegetables have been studied in recent years to minimize rot incidence and enhance quality of fresh produce. It has been shown that microbial contamination and undesirable enzymes of harvested fruit and vegetables can be inactivated without impairing the flavor and nutritional quality by applying pressure treatment. For example, high pressure treatment is capable of improving the bioavailability of micronutrients and phytochemicals such as lutein and carotenoids in fresh plant produce (McInerney et al., 2007). On the other hand, hypobaric (low pressure) treatment confirmed its effectiveness in controlling postharvest rots in strawberries (Romanazzi et al., 2001). Hyperbaric treatment was also effective in delaying the senescence of fresh produce as it reduces respiration rate and ethylene production. It also increased resistance to pathogens by increasing production of phytoalexins as a stress response to the pressure treatment and by increasing the activities of PAL and POD enzymes that induce resistance against pathogens (Baba et al., 1999; Hammerschmidt, 1999).

1.6 Role of UV-C irradiation as a postharvest treatment in horticulture

1.6.1 Uses of ultraviolet radiation in the horticulture industry

The ultraviolet region of the electromagnetic spectrum has been studied for its use as an alternative postharvest technology for chemical treatments in fresh produce. UV-C radiation is considered a good disinfectant (Gray, 2014). This disinfection phenomenon has been utilized in the horticulture industry as well as other industries that are involved in purification processes for example, fresh water and waste water industries. UV treatment requires a comparatively short treatment time and does not result in residual effects and unwanted by-products (Liu, 2009). Many studies have confirmed that ultraviolet radiation, especially the UV-C region, can be used as a postharvest treatment for fresh fruit and vegetables to prolong storage life, improve quality, induce defence mechanisms, and to enhance bioactive compounds. For example, UV-C treatment improved resistance to disease development in grapes primarily by inducing synthesis and accumulation of phytoalexins (Douillet-Breuil et al., 1999), as well as other defence mechanisms (Pombo et al., 2009). Some studies reported improvement of quality parameters such as colour, firmness, antioxidant compounds, and enzyme activities of broccoli as a response to stress exerted by UV-C irradiation (Costa et al., 2002; Costa et al., 2006). In addition, there is evidence that UV-C irradiation increased production of other secondary metabolites such as glucosinolates (GLSs) in broccoli (Sierra et al., 2014). Based on this evidence, it can be proposed that production and accumulation of secondary metabolites due to UV-C treatment may have an effect on attachment of pathogens to fresh produce surface and subsequent growth.

In addition to the UV-C (100 - 280 nm) portion of the electromagnetic spectrum (which is the only germicidal region), higher wavelengths including UV-B (280 – 315 nm) and UV-A (315 – 400 nm) have also been studied as potential postharvest treatments for fresh produce (Aiamla-or et al., 2009a; Aiamla-or et al., 2009b; Jansen & Bornman, 2012). Aiamla-or et al (2009b) studied the effect of UV-A and UV-B irradiation on broccoli branchlets and concluded that UV-B at a dose of 8.8 kJm^{-2} was more effective in retaining colour and reducing chlorophyll degradation than UV-A. However, most of the UV-A and UV-B studies have been focused on preharvest application rather than postharvest application (Costa et al., 2002; Wargent et al., 2011; Jansen & Bornman, 2012).

There is a lack of information on which UV wavelengths optimize quality retention and bioactive production of fresh produce. Most researchers have used a wavelength of 254 nm to treat fresh produce in their UV-C research while a wide range of doses from 0.15 to 40 kJm^{-2} has been used for irradiation of different produce with various results (Martínez-Hernández et al., 2015). Determination of optimum spectrum and dose range for each product in future studies would be beneficial to gain more benefits from UV radiation.

1.6.2 UV hormesis in horticultural crops

Although UV radiation is generally harmful to living organisms at high doses, it can induce disease resistance, delay ripening, improve quality attributes, and enhance phytochemicals in fresh produce at low doses (Charles & Arul, 2007). This is a useful phenomenon that has a potential to be utilized by the horticulture industry. Shama and Alderson (2005) reviewed the possibility of inducing a number of hormetic responses, including defence mechanisms and delayed senescence in a wide range of horticultural crops exposed to low doses of UV radiation. Studies have confirmed the hormetic effect of UV radiation, particularly UV-B and UV-C in improving quality of horticultural crops. For example, application of UV-B to nasturtium has increased the glucosinolate and phenol concentration adding additional health benefits to the produce (Schreiner et al., 2009). UV-C is the most effective treatment used in many studies and showed promising effects on storage life extension, quality attributes, reduction of decay, and enhancement of bioactives such as phenols, anthocyanins, and enzyme activities in broccoli (Costa et al., 2006; Lemoine et al., 2010a), strawberry (Erkan et al., 2008a), blueberry (Kossuth & Biggs, 1981), lettuce (Allende & Artés, 2003), and tomato (Stevens et al., 1997). These beneficial effects of UV-C irradiation as a postharvest treatment are discussed in detail in the following section. However, determining the hormetic dose of UV-C on individual types of product is important to attain optimum effectiveness of UV-C treatment as higher doses may result in deleterious effects on quality and storage life while lower doses may not deliver intended beneficial effects.

1.6.3 Effect of UV-C irradiation on fresh produce

Studies have confirmed the use of UV-C radiation as a postharvest treatment that effectively improves postharvest quality and controls microbial growth in a variety of

fruit and vegetables, suggesting a promising and effective technology for use in the horticulture industry. UV-C irradiation controls microbial growth directly by damaging the DNA of microorganisms and indirectly by inducing resistance against phyto-pathogens in fruit and vegetables through activated defence systems (Lemoine et al., 2007; Erkan et al., 2008b). As a result of defence systems induced by UV-C radiation, various bioactive compounds are produced in fruit and vegetables that may have direct impact on quality attributes and microbial safety. However, current literature shows that the effect of UV-C irradiation on inducing bioactive compounds varies depending on the product, which may subsequently affect the quality of products differently. For example, UV-C irradiation at a dose of 0.25 kJm^{-2} resulted in higher titratable acidity, anthocyanin content and firmness and lower respiration rate in fresh strawberries after treatment compared to untreated fruit. In addition, the low electrical conductivity of UV-C treated strawberry fruit suggested a delay in ripening and senescence (Baka et al., 1999). In broccoli, application of UV-C irradiation at a dose of 7 kJm^{-2} delayed yellowing and chlorophyll degradation at 20 °C, increased antioxidant capacity and reduced respiration rate in treated broccoli but adversely affected total phenol and flavonoid contents during storage (Costa et al., 2006).

UV-C irradiation is also effective in controlling pathogen growth and reducing attachment to fresh produce due to its direct germicidal effect and induced production of antimicrobial phytochemicals as a defence response against oxidative stress of UV-C irradiation. Application of UV-C irradiation on fresh and processed 'Lollo Rosso' lettuce (*Lactuca sativa*) at a dose of 8.14 kJm^{-2} significantly reduced growth of psychrotrophic bacteria, coliforms, and yeasts without compromising sensorial quality, due to its direct germicidal effect (Allende & Artés, 2003). Similarly, total microbial count in fresh cut apples was reduced by UV-C treatment at doses from 1.2 to 24 kJm^{-2}, but doses higher than 1.2 kJm^{-2} damaged cell structure, initiating oxidative reactions and dehydration of cells (Manzocco et al., 2011). Other than the direct effect, UV-C irradiation can induce resistance in plant tissues as a response to the mild stress of UV-C irradiation. For example, UV-C treatment significantly reduced incidences of grey mould in table grapes which were individually treated at doses of 0.125 to 0.5 kJm^{-2} and inoculated with *B. cinerea* 24 - 48 h after UV-C irradiation (Nigro et al., 1998). Decay caused by ripe rot disease in different varieties of blueberries was also significantly reduced by UV-C treatment at

a dose of 4 kJm^{-2} and this reduction may be due to increased total antioxidants as measured by total anthocyanin and total phenolics (Perkins-Veazie et al., 2008). These results suggest a possible correlation between increased production of anthocyanin and phenolic compounds by UV-C irradiation and decay control in fruit, but they did not demonstrate direct antimicrobial effect of these secondary metabolites against pathogens. As with the phyto-pathogens, growth of pre-existing human pathogens on fresh produce is also inhibited by UV-C irradiation. For example, populations of human pathogens such as *E. coli*, *S. enteritidis* and *L. monocytogenes* inoculated onto broccoli branchlets were effectively reduced by UV-C irradiation at a dose of 2.5 kJm^{-2} and remained unchanged during storage at 5 – 10 °C (Martínez-Hernández et al., 2015). However, current literature does not explain the mechanisms by which induced defence responses may control human pathogens inoculated onto fresh produce surface after UV-C treatment.

There is a plethora of research that evaluated the impact of UV-C irradiation at different doses on improved quality aspects and safety of fruit and vegetables. Most of the studies that involved in microorganisms dealt with controlling plant pathogens via induced defence responses and/or killing of human pathogens on fresh produce due to the germicidal effect of UV-C irradiation. However, the mechanism of inhibiting human pathogens inoculated onto a fresh produce surface after UV-C treatment is not known and needs to be elucidated.

1.7 UV-C radiation and its implications

1.7.1 UV irradiation

The energy of the sun that reaches earth is known as electromagnetic radiation. It consists of many forms of energy such as visible light, infrared, ultraviolet, and X-rays that occupy different wavelengths of the electromagnetic spectrum (Figure 1.1). Most of the solar radiation energy is in the infrared and visible region, representing approximately 49.4% and 42.3% of the total energy respectively, and covers a broad range of wavelengths from about 100 nm to 400 nm. UV light is in the range of wavelengths shorter than that of visible light, but longer than X-rays (< 100 nm) and it is in the non-ionizing region of the spectrum representing only about 8% of the total energy of the sun that reaches the surface of the earth (Gibson, 2003). The UV spectrum is divided into UV-A (315~400 nm), UV-B (280~315 nm), and UV-C

(100~280 nm) and long term exposure is considered harmful to living organisms. Fortunately, most of the detrimental radiation, including UV-C, which is approximately 0.5% of the total energy and extremely harmful to organisms, is absorbed by the ozone layer (Koutchma et al., 2009). UV-B which may cause photochemical damage to cellular DNA, resulting in erythema, cataracts, and skin cancer, is partly absorbed and represents only 1.5% of the total energy (Gibson, 2003). UV-A which can cause tanning in human skin, is capable of penetrating through the atmospheric protection to reach the earth and it represents approximately 6.3% of the solar energy (Gibson, 2003; Olds & Kimlin, 2006). Apart from their detrimental effects, both UV-A and UV-B are responsible for synthesis of vitamin D in human body (Hollosy, 2002; Olds & Kimlin, 2006; Liu, 2009).

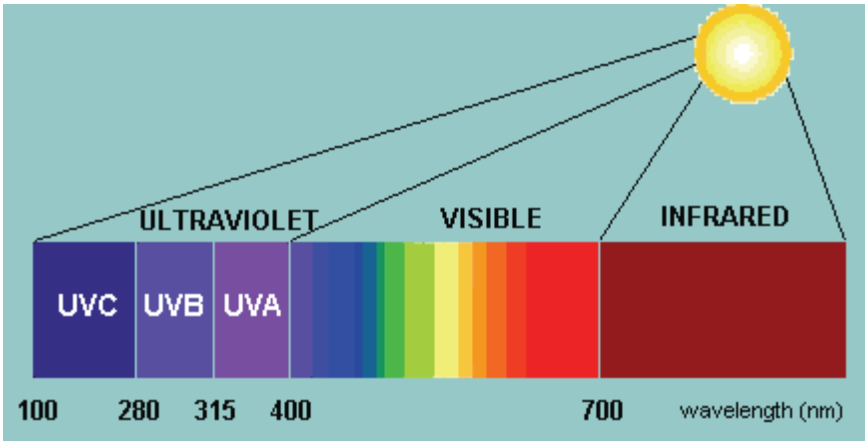

Figure 1.1 Solar spectrum (Photo: http://www.arpansa.gov.au/is_sunys.htm)

1.7.2 Effect of UV radiation on humans, biology and environment

Even though most of the wavelengths of UV radiation are absorbed by the ozone layer, there is a certain amount of radiation reaching the earth's surface. Prevailing atmospheric factors such as gases, air molecules, particulates, and clouds, and geophysical parameters such as solar elevation, latitude, altitude, and the type of the vegetation cover determine the intensity of the UV radiation that reaches the earth's surface (Olds & Kimlin, 2006). After filtering the detrimental radiation of wavelength shorter than 280 nm, including UV-C by the stratospheric ozone, approximately 0.5% of the total solar flux of UV-B reaches the Earth's surface. However, it is highly energetic and it may have considerable impact on organisms

(Xenopoulos & Schindler, 2001). The impact can be as severe as damaging macromolecular structures leading to possible alterations in primary productivity and structure and function of ecosystems (Xenopoulos & Schindler, 2001). The higher the energy and the intensity of the irradiance, the greater the damage to organisms. In many studies, the effect of UV radiation on the lives of humans, marine biology, microbes, amphibians, and coral communities, has been assessed (Cockell & Blaustein, 2001).

Ultraviolet radiation, in solar or artificial form, is responsible for causing damage to the human skin, eye diseases, and several autoimmune diseases. Dangerous diseases such as cutaneous melanoma, non-melanocytic skin cancer, and cancer of the lip may also be caused by UV radiation (Gallagher & Lee, 2006).

1.7.3 Effect of UV radiation on plant cells

UV radiation causes alterations in physiological and biochemical processes as well as in plant morphology (Wargent et al., 2011). Both UV-B and UV-C irradiation may result in a large amount of DNA photoproducts causing mutations during replication (Hollosy, 2002). Proteins have strong absorption of UV radiation at about 280 nm and at higher wave lengths of the UV-B region. Aromatic amino acids such as phenylalanine, tryptopane and tyrosine also absorb UV in this region (Hollosy, 2002). Lipids with isolated or conjugated double bonds can be photochemically modified by UV absorbance. Phospho- and glyco-lipids, which are the main components of the plant cell membrane and are fatty acids, may be damaged by UV-B radiation in the presence of oxygen. Pigments of the photosynthetic apparatus can also be destroyed by UV radiation subsequently reducing the photosynthetic capacity (Jordan et al., 1994). For example, chlorophylls and carotenoids may be adversely affected by relatively large amounts of UV-B radiation. It had been reported that UV-B irradiation has shown greater reduction in the amount of *chl b* than *chl a* and may cause selective destruction of *chl b* biosynthesis or degradation of precursors (Marwood & Greenberg, 1996).

1.7.4 Microbial inactivation mechanism of UV light

Upon exposure to direct UV radiation, DNA of microorganisms absorbs radiation energy resulting in formation of pyrimidine dimers that may develop mutations. For example, microorganisms absorb photons of UV light in the range of 200 to 310 nm

and become inactivated due to disruption of their DNA or RNA structures. UV radiation induces formation of pyrimidine dimers that form bonds between adjacent pairs of thymine or cytosine in the same DNA or RNA strand preventing replication (Koutchma et al., 2009). In addition to formation of pyrimidine dimers, nucleic acids (DNA/RNA) of the microbe can be damaged by formation of photoproducts of nucleic acids and nucleic acid lesions that also inhibit replication and transcription preventing cell multiplication (Allende et al., 2006). UV sensitivity of microorganisms basically depends on several factors such as exposure time (dose), physiological state, type of strain, repair mechanism and cell wall structure (Koutchma et al., 2009). As explained above, mechanisms of direct inactivation of microorganisms by UV radiation are well known and documented. However, indirect inactivation mechanisms on microorganisms by UV radiation are not fully known, e.g. mechanisms of controlling growth of human pathogens inoculated onto fresh produce surface after UV-C irradiation is not known (Figure 1.2). One possible mechanism could be the induced production of different types of phytochemical compounds by pre or postharvest UV treatments that may inactivate or damage post inoculated pathogens. Another possibility could be altering the surface of fresh produce to make it less supportive for the growth of microorganisms by UV-C irradiation. These possibilities need to be confirmed through future studies, in order to enhance the potentially beneficial effects of UV irradiation and minimize the potentially harmful effects.

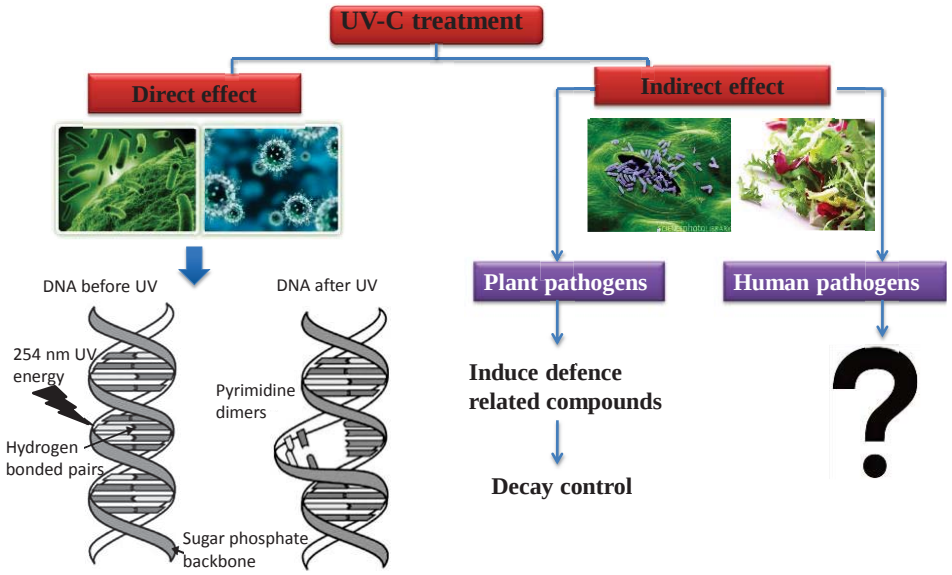

Figure 1.2 Conceptual mechanisms of inactivating microorganisms on fresh produce by UV-C irradiation. Direct inactivation of microorganisms that are on the surface during exposure to UV-C and indirect inactivation of microorganisms due to induced defence mechanisms are known to occur in the case of plant pathogens. Inactivation mechanisms for human pathogens contaminated after UV-C irradiation are yet to be elucidated.

1.8 UV induced protection against plant pathogens

Plants have developed defence mechanisms in order to protect against most phyto-pathogens and adverse environmental conditions: commonly referred to as biotic and abiotic stress respectively (Wang et al., 2006). Being in a vulnerable environment, plants are always exposed to a variety of pathogens. The presence of a pathogen can generate defence responses in plant tissues by inducing production of signal molecules such as salicylic acid (SA), methylsalicylic acid (MeSA), ethylene, and azelaic acid leading to systemic expression of pathogenesis-related genes (PR genes) of uninfected tissues at a distance (Durrant & Dong, 2004). This pathogen induced defence mechanism in plants is often called Systemic Acquired Resistance (SAR)(Fu & Dong, 2013). Similarly plants can respond to abiotic stresses such as heat, UV light, and pressure by expression of resistance genes, increasing production of antioxidant enzymes, lignification of cell wall, and production and accumulation of antimicrobial metabolites (Wang et al., 2006).

The main criticisms against UV-C irradiation as a postharvest treatment are inability to penetrate to inner tissues and/or the homogeneity in irradiating uneven surfaces in order to induce intended benefits of the treatment (Shama & Alderson, 2005; Allende et al., 2006). With elucidating the concept of SAR, it was possible to explain how non irradiated cells at a distance may get the signal and react to the stress by inducing relevant defence responses. Therefore, UV-C irradiation as an abiotic stress may induce production of signal molecules locally and subsequently induce expression of genes that are responsible for production of defence related compounds locally as well as systemically (Durrant & Dong, 2004).

1.9 Regulating the application of UV irradiation in the food industry

USFDA has established a code of regulations for irradiation of foods and food packaging using ionizing and non-ionizing radiation. In addition, there are defined regulations based on regional, international, and trade specified requirements.

Regulations related to non-ionizing radiation have also been coded by the USFDA emphasizing the safety limitations and intended use of UV radiation for processing and treating food and food products, potable water, and juice products (U.S. FDA, 2010). The recommended irradiation level for food and food products is 0.46-0.93 Jcm^{-2}. Health Canada has approved UV treatment to treat apple juice and cider products to achieve a significant reduction in the microbial load. However, there are no human food safety concerns associated with apple cider and juice treated with UV irradiation, hence no objections for selling UV irradiated apple cider and juice in the market (Koutchma et al., 2009).

1.10 Broccoli; a potential vegetable for research

Cruciferous vegetables are recognized for their beneficial health promoting properties including high nutrient content, antioxidant, and antimicrobial activities, and potential role in cancer prevention. The family Cruciferae comprises vegetables such as broccoli, cabbage, Brussels sprout, kale, and horse-radish. Broccoli originated in the Mediterranean coastal area and was introduced to other parts of the world as a vegetable around 6 B.C. (Maggioni et al., 2010). Broccoli is marketed as fresh or processed; mostly frozen as intact heads or florets and eaten raw or cooked (Boriss & Brunke, 2012). Due to recent educational and promotional campaigns

aimed at increasing awareness of healthy eating, broccoli consumption has increased (Warriner et al., 2009). For example, in the US broccoli consumption has increased fourfold from 1986 to 2010 (Boriss & Brunke, 2012). China and India are the main producers of broccoli in the world sharing 43% (90, 30,900 tonnes) and 32% (67, 45,000 tonnes) of the world production respectively. New Zealand also produced about 68,000 tonnes of Brassicas including broccoli (16,000 tonnes), cabbage (33,000 tonnes), and cauliflower (19,000 tonnes) in 2012, mostly for domestic consumption valued at 80.3 million dollars ("Fresh Facts," 2012). With the increase in consumption of vegetables including broccoli, there is an increased risk of transmitting foodborne pathogens to humans. Particularly the nature of broccoli, which comprises very tightly arranged florets on a compact bunch of branchlets, may provide protective surfaces for foodborne pathogens to grow and survive once contaminated. Under such situations washing and cleaning with disinfectants such as chlorinated water may not be effective (Wang et al., 2009). As a result, consumption of raw broccoli may raise concerns of microbial safety. Postharvest treatments that can effectively control foodborne pathogen growth on fresh cut broccoli are necessary to improve microbial safety.

1.10.1 Secondary metabolites of broccoli

Cruciferous vegetables contain a wide range of phytochemicals that are considered as secondary metabolites (Bennett & Wallsgrove, 1994). Many of the plant secondary metabolites are believed to play an important role in plant defence as well as prevention of human diseases such as cancer and cardiovascular diseases (Moreno et al., 2006; Variyar et al., 2014). Broccoli is rich in secondary metabolites and other nutrients such as vitamins, minerals, phenolics, carotenoids, and glucosinolates (GLSs). For example, vitamins such as vitamin A and K, minerals such as Se, phenolics such as quercetin and kaemferol, carotenoids such as β-carotene and lutein, and glucosinolates such as glucoraphanin and sinigrin were identified in broccoli (Fernandez-Leon et al., 2012). Glucosinolates and phenolics appear to be responsible for most of the health promoting properties of broccoli (Vallejo et al., 2002). GLSs and their hydrolysis products such as sulforaphane (derived from glucoraphanin), phenethyl isothiocyanate (derived from gluconasturtiin), allyl isothiocyanate (derived from sinigrin), and indole-3-carbinol (derived from glucobrassicin) have been evaluated for their fungicidal, bactericidal, nematocidal and allelopathic properties

(Moreno et al., 2006; Redovnikovic et al., 2008). The chemical structure of GLS consists of a β-D-thioglucose group, a sulphonated oxime group and a side chain derived from methionine, phenylalanine, tryptophan or branched chain amino acid (Figure 1.3) (Moreno et al., 2006; Kissen et al., 2009). An enzyme called myrosinase which exists in the plant cell vacuole hydrolyses GLSs into a number of compounds having different biological activities, once the enzyme and substrate come in contact through cutting, wounding, or mastication (Redovnikovic et al., 2008). When a pathogen tries to invade a leaf surface of a cruciferous vegetable, it induces production of GLSs, especially aromatic and indole GLSs locally and systemically. Some studies have demonstrated that abiotic stresses such as pressure, atmospheric gas changes, and UV-C irradiation also induced production of GLSs in broccoli (Jia et al., 2009; Van Eylen et al., 2009; Sierra et al., 2014). In addition to GLSs, other secondary metabolites such as phenolic compounds and flavonoids can also be induced in broccoli by applying both biotic and abiotic stresses (Costa et al., 2006; Lemoine et al., 2007).

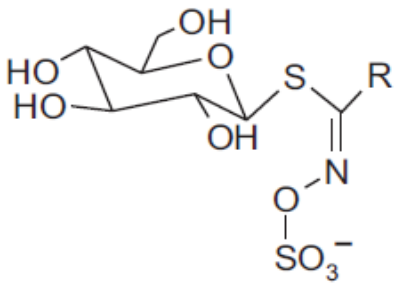

Figure 1.3 General structure of glucosinolates. R is the variable side chain derived from amino acids.

1.10.2 Measuring quality attributes of broccoli (Hue, Lightness, and Chroma)

Colour is one of the main attributes along with the texture that characterises freshness of most fruit and vegetables. Colour of fruit and vegetables changes with storage time because of different biochemical processes including chlorophyll degradation and browning (Oms-Oliu et al., 2010). Understanding the three dimensions of colour such as lightness (value), hue (colour), and chroma (saturation) is important to understand the colour change of UV-C treated broccoli over time. 'Lightness' or value refers to the degree of lightness or darkness of the colour along the vertical axis of the colour sphere; with white at the top and black at the bottom (Figure 1.4). 'Hue' is the natural colour we see: there are five principal hues from red to yellow, to green, to blue, to purple (Figure 1.4). 'Chroma' is the level of saturation or richness of a colour and is measured radially from the central grey axis. For example, pure, rich green is further away from the grey central axis than pale green (Figure 1.4) with reference to broccoli colour.

1.11 Impact of UV-C irradiation on broccoli after harvest

1.11.1 Quality and storage life of broccoli

Visual quality of broccoli degrades rapidly after harvest, as broccoli starts to senesce as supply of nutrients, water and other resources are terminated after detaching from the plant. In order to prevent quality losses, many postharvest treatments have been studied and UV-C irradiation was one of the promising postharvest treatments that improved quality aspects of broccoli including colour and firmness. Senescence of green vegetables involves chlorophyll degradation and break down of tissues. These processes increase production of free radicals which are toxic for tissues at high concentrations (Lemoine et al., 2010a). Superoxide dismutase (SOD) is one of the enzymes responsible for catalysing transformation of superoxide radical (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Higher SOD activity was reported in the broccoli cultivars which maintained green colour for comparatively longer time during storage (Toivonen & Sweeney, 1998) showing a relationship between SOD activity and delayed senescence in broccoli mediated by scavenging superoxide radicals (Lemoine et al., 2010a). Lemoine et al.(2007) also confirmed those observations where fresh cut broccoli irradiated at 8 kJm^{-2} and stored at 4 °C for 21 days showed delayed yellowing and chlorophyll degradation compared to untreated

broccoli . Minimal processing of broccoli may induce some physiological activities such as respiration and electrolyte leakage along with senescence (Costa et al., 2006). UV-C irradiation was effective in reducing the rate of respiration and electrolyte leakage, while increasing the phenolic and ascorbic acid content resulting in higher tissue integrity and antioxidant activity in minimally processed (fresh cut) broccoli (Lemoine et al., 2007). These results confirm that UV-C irradiation at moderate doses can be used as a postharvest treatment for extending storage life of broccoli without affecting quality.

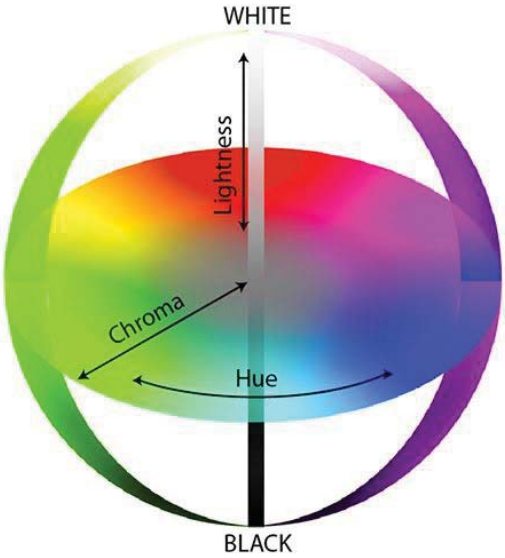

Figure 1.4 Dimensions of colour (Source: http://2.bp.blogspot.com/)

1.11.2 Antioxidant activity

UV-C irradiation of fresh produce including broccoli triggered responses that induced production and accumulation of antioxidant compounds and increased antioxidant enzyme activity through activation of relevant gene expression (Erkan et al., 2008a; Lemoine et al., 2010a; Liu et al., 2012). Many studies suggested that this may be the reason for effectiveness of UV-C irradiation on improving quality aspects and delayed senescence in fresh produce. As with senescence, plant tissues tend to increase production of reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals under adverse environmental conditions as a part of defence mechanism. However accumulation of excessive ROS in plant tissues may cause oxidization of nucleic acids, proteins, and lipids thus

affecting normal functioning of cellular processes and cell wall integrity (Lemoine et al., 2010a). In order to prevent oxidative damage plant cells have developed a mechanism to convert ROS into less toxic products utilizing plant secondary metabolites such as phenols, flavonoids, carotenoids, lignans, ascorbate, glutathione, and some enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase (Gonzalez-Aguilar et al., 2010). Here ROS play an important role as signalling molecules in tissues to initiate production of antioxidants which are vital for oxidative stress tolerance (Gonzalez-Aguilar et al., 2010). One of the main ROS synthesised during stress is hydrogen peroxide from O_2^- due to SOD activity, which may responsible for activating gene expression of antioxidant enzymes including phenylalanine lyase (PAL) and chalcone synthase (CHS) (Gonzalez-Aguilar et al., 2010). PAL is a key enzyme in phenylpropanoid metabolism through which phenylalanine is deaminated to produce trans-cinnamic acid. PAL can be induced by biotic or abiotic stresses resulting in production of phenylpropanoids such as phenolic acids and flavonoids (Lemoine et al., 2010a). In addition, there is evidence of UV-C inducing other secondary metabolites such as phytoalexins and GLSs in broccoli (Nadeau et al., 2012; Sierra et al., 2014). These findings suggest that UV-C irradiation is capable of inducing production of ROS in broccoli tissues which is the first step in triggering the production of antioxidant enzymes, antioxidants such as phenolic acids, flavonoids, and other secondary metabolites as a response to mild stress. These secondary metabolites may have a direct or indirect effect on suppressing growth of human pathogens if production and accumulation of these compounds is triggered after exposure to UV-C irradiation.

1.11.3 Gene expression: senescence and defence

1.11.3.1 Chlorophyll degradation

In order to understand the genetic involvement in UV-C dependent senescence, some researchers have studied the expression of senescence associated genes in general and as a result of UV-C treatment in particular. Senescence of broccoli is regulated by many genes associated with degradation of chlorophyll, proteins, lipids, and many other cellular structures (Chen et al., 2008). Pheophorbide a oxygenase (PaO) is one of the key enzymes in chlorophyll catabolism which catalyses the opening of the

porphyrin ring of the pheophorbide which is the catabolized product of chlorophyll by chlorophyllase or pheophytinase followed by removal of Mg^{2+} by Mg chelatase (Gomez-Lobato et al., 2012a). Gomez-Lobato et al. (2012a) identified expression of a gene encoding PaO (*BoPaO*) associated with broccoli senescence. They reported a five-fold increase in expression of *BoPaO* gene in broccoli inflorescence after 4 days of storage and observed a negative correlation between chlorophyll content and gene expression during that time. Interestingly, UV-C treatment at a dose of 10 kJm^{-2} controlled expression of *BoPaO* gene significantly for up to three days but it became higher than the untreated broccoli at day 5 at 22 °C, although no significant difference was observed in chlorophyll content (Gomez-Lobato et al., 2012a). They assumed that increased expression of *BoPaO* gene may possibly be due to the use of immature broccoli inflorescences detached from the branchlets causing accelerated senescence. In addition to PaO, chlorophyllase (CHL) and pheophytinase (PPH) are important enzymes involved in chlorophyll catabolism, and are involved in catalysing chlorophyll and pheophytin degradation respectively (Buechert et al., 2011). Major genes that encode CHL in broccoli have been identified as *BoCHL1*, *BoCHL2*, and *BoCHL3*. A recent study on broccoli senescence revealed that CHL activity increased with senescence while UV-C irradiation delayed the increment of CHL activity significantly resulting in delayed yellowing (Costa et al., 2006). Buechert et al. observed only *BoCHL1* and *BoCHL2* genes accumulated in fresh and stored broccoli. However, they argued that these genes did not have a direct correlation with chlorophyll degradation and were not manipulated by postharvest treatments (UV-C and heat) whereas a newly identified PPH enzyme encoding gene *BoPPH* expression was correlated with postharvest UV-C irradiation and chlorophyll degradation during broccoli senescence. They concluded that UV-C irradiation has a greater effect on expression of PPH than CHL genes that contributed to delayed senescence in broccoli after harvest (Buechert et al., 2011). These observations suggest that the mechanisms and genes responsible for delaying senescence in UV-C irradiated broccoli are not fully understood and need to be elucidated by further studies.

1.11.3.2 Cell membrane degradation

As with chlorophyll degradation, cell membrane degradation due to lipid oxidation by lipoxygenase (LOX) enzymes or ROS is one of the symptoms of senescence.

Lipoxygenase (LOX) enzymes catalyse peroxidation of poly-unsaturated fatty acids (PUFAs) causing cell membrane break down and loss of compartmentation of the tissue (Zhuang et al., 1995). In addition, these enzymes catalyse oxygenation of PUFAs into hydroperoxides and further transformation into oxylipins which can be also involved in cell membrane degradation (Gomez-Lobato et al., 2012b). Gomez-Lobato et al (2012b) observed an increased expression of this gene during senescence of broccoli. UV-C treatment at a dose of 10 kJm^{-2} delayed increment of the gene expression significantly until day 3, which then increased compared to untreated broccoli. However, chlorophyll content and LOX activity were not affected by the UV-C treatment (Gomez-Lobato et al., 2012b). Lack of correlation between lipoxygenase activity and chlorophyll retention with expression of *BoLOX1* gene in UV-C treated broccoli suggests that other genes might be involved in LOX activity that are regulated by UV-C treatment and further studies are needed to evaluate the effects of UV-C irradiation in regulating the expression of those candidate genes. Such knowledge is important in determining biomarkers for breeding new broccoli varieties with high quality and long storage life.

1.11.3.3 Pathogenesis related responses

Very little literature is available on pathogenesis related gene expression of broccoli by UV-C irradiation although there are many studies related to other fresh produce including tomatoes (Charles et al., 2009) and strawberries (Pombo et al., 2011) which can be used as basic guides to understand defence mechanisms of broccoli. For example, five *de novo* synthesised proteins were identified in tomato while three (a basic β-1,3-glucanase and two acidic chitinases) were assumed to be pathogenesis-related proteins as they were also induced after inoculation with *Botrytis cinerea.* In the same study they also identified UV-C induced expression of several constitutive proteins including one acidic β-1,3-glucanase, three acidic chitinases and three basic chitinases while reducing expression of ripening related proteins (Charles et al., 2009). On the other hand resistance to *B. cinerea* was also partly associated with the accumulation of sequiterpenoid phytoalexin rishitin and structural reinforcement by lignin, suberin, and other phenolics of tomatoes that have been enhanced as a result of UV-C irradiation (Charles et al., 2008a; Charles et al., 2008b). In strawberry UV-C irradiation increased the transcription and activity of enzymes such as PAL and PPO and proteins such as chitinases and β-1,3-glucanase which are involved in

defence against *B. cinerea* (Pombo et al., 2011). Chitanases and glucanases are basically important in breaking down fungal cell wall (van Loon et al., 2006). Similarly UV-C irradiation increased PAL, PPO, phenolics, and flavonoids in fresh cut broccoli suggesting a possible induction of the defence responses due to UV-C irradiation (Costa et al., 2006; Lemoine et al., 2007; Lemoine et al., 2010a). All these changes due to UV-C irradiation and resulting defence related compounds are produced and accumulated inside the tissue and targeted to surface penetrating phyto-pathogens. The potential use of these beneficial outcomes of UV-C irradiation for suppressing human pathogen growth on fresh produce surface is unknown. Therefore, studying such mechanisms will contribute to improve microbiological safety of fresh produce and reduce risk of foodborne disease outbreaks in the future.

1.12 Aim and research objectives

This chapter has reviewed the severity of foodborne diseases that occur every year due to consumption of food contaminated by human pathogens, and the importance of controlling growth and survival of foodborne pathogens on fresh produce after harvest in order to reduce the risk of foodborne illnesses. The seriousness of the disease caused by *L. monocytogenes* in humans and the virulence factors of the pathogen that challenge the food industry to innovate better solutions in preventing attachment and subsequent growth of the pathogen was discussed. Different methods used to achieve safer products have been evaluated briefly whereas UV-C irradiation as a postharvest treatment in controlling phyto-pathogens and human pathogens was discussed in detail. The current literature suggests a need for a technology that can prevent growth of human pathogens that may contaminate fresh produce and/or remain after basic washing. UV-C irradiation is known for its germicidal effect and bioactive enhancing ability in fresh produce suggesting a potential use of this technology for controlling *L. monocytogenes* on fresh cut broccoli without compromising product quality.

Therefore the objectives of this research were:

1. To determine the hormetic dose and effectiveness of UV-C irradiation as a postharvest treatment to retain quality of fresh cut broccoli

2. To evaluate the effectiveness of UV-C irradiation on growth and survival of post inoculated *L. monocytogenes* on fresh cut broccoli

3. To determine whether extracts of UV-C treated broccoli also control the growth of *L. monocytogenes in vitro*

4. To determine which bioactive compounds may be responsible for efficacy of UV-C irradiation in suppressing *L. monocytogenes*

2 EFFECT OF UV-C IRRADIATION ON PHYSIOLOGICAL PROPERTIES OF FRESH CUT BROCCOLI [*]

2.1 Introduction

Horticultural produce is highly perishable after harvest as it continues to maintain the internal physiological activities by performing most of the metabolic processes that existed when they were attached to the plant. So, it is a living biological body which respires, transpires and matures using its own reserves of water, carbohydrates and minerals which are non-renewable after being detached from the plant. As a result, nutritional and visual quality of vegetables deteriorates after harvest (Wills et al., 2007). This natural process is called senescence and leads to a loss of membrane integrity, and solute leakage from the cytosol (Lemoine et al., 2007). Natural disease resistance of fresh commodities is reduced during senescence and the product becomes more susceptible to tissue break down (Wills et al., 2007). Also stress caused by harvesting and processing accelerates senescence symptoms and microbial growth in fresh-cut broccoli (Lemoine et al., 2008). Tissue damage caused during processing increases respiration and electrolyte leakage in fresh-cut broccoli during storage resulting in accelerated senescence (Lemoine et al., 2007).

Several postharvest technologies have been studied to delay senescence symptoms in broccoli. For example, heat treatment either as hot water or hot air has been used successfully to delay degreening of broccoli (Forney, 1995; Tian et al., 1997; Costa et al., 2005b; Lemoine et al., 2009). UV-C irradiation has also been studied as a postharvest treatment to delay senescence symptoms in fresh-cut broccoli and many other horticultural products (Costa et al., 2006; Lemoine et al., 2007; Lemoine et al., 2008; Artes-Hernandez et al., 2009; Pombo et al., 2009; Lemoine et al., 2010b; Obande et al., 2011). In addition, UV-C has been studied and utilized as a sanitizing treatment for fresh products. But the main objective of this research was to alter the surface of fresh cut broccoli by using UV-C irradiation as a postharvest treatment and to study the subsequent effect on growth and survival of a human pathogen, *L. monocytogenes* on the surface of that fresh cut broccoli.

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[*] Materials of this chapter are included in the publication: Gamage, G.R., Wargent, J., Palmer, J.S., Heyes, J.A. (2013). Effect of UV-C irradiation on growth of post inoculated *L. monocytogenes* on fresh-cut broccoli. Acta Horticulturae, 1012, pp. 1031-1036

To our knowledge no study has been done to study the growth and survival of *L. monocytogenes* when inoculated onto fresh-cut broccoli after UV-C treatment has been completed. However it is important to ensure that the UV-C doses applied to control the growth of *L. monocytogenes* do not adversely affect the postharvest quality of fresh-cut broccoli. Therefore, this chapter focuses on evaluating the effect of UV-C irradiation on basic quality attributes such as weight loss and colour of fresh-cut broccoli to ensure the appropriateness and acceptance of UV-C irradiation as a postharvest treatment in fresh-cut broccoli.

2.2 Materials and Methods

2.2.1 Plant materials

Freshly harvested whole broccoli heads (*Brassica oleracea* L. italica) were obtained from a local producer (Kapiti Green Ltd, Levin, New Zealand). The particular variety of broccoli used in experiments was 'Serenity' which was cultivated during March to September in three consecutive years. These broccoli heads were transported to the laboratory in a car in which internal temperature was controlled at 20 °C within 2 hours after harvest and cut into branchlets, approximately 40 mm in diameter.

2.2.2 Storage containers (Clam shells)

Amorphous polyethylene terephthalate (APET) clamshells (Flight Plastic Packaging, Wellington, New Zealand) were used to store UV-C treated and untreated broccoli branchlets (see figure 2.3). Size of a clamshell was 123×112×72 mm and there were 8 punched holes on upper and bottom sides.

2.2.3 UV-C treatment

Broccoli branchlets were mounted upright on plastic racks and placed under a bank of four germicidal UV-C lamps (Philips ultraviolet TUV 30W/G30T8, Holland) which were designed to emit short wave ultraviolet radiation at 253.7 nm. Broccoli branchlets were irradiated for 2 min, 5 min or 10 min at a distance of 50 cm. Lamps were switched on half an hour before the treatment and briefly extinguished while loading the chamber. UV radiation exposure of broccoli branchlets was measured by an Optronics OL756 spectroradiometer (Optronics Laboratories, FL, USA). Five UV-C treated broccoli branchlets from the same treatment were put into a plastic

clamshell randomly and the weight of the clamshell was measured. Three similar samples were used for each treatment. After weighing all samples they were stored in an incubator at 15 °C and under 80-85% relative humidity with moderate light for seven or eight days depending on the experiment. Relative humidity inside the storage chamber was recorded by Grant Squirrel loggers (1200 series grant Instrument, Cambridge, UK) at the beginning of the study and assumed thereafter. Three punnets from each treatment and from the control were evaluated at different time intervals.

2.2.3.1 Measuring the UV-C dose inside the UV box

The bottom surface of the UV chamber was divided into 25 numbered blocks (Table 2.1). The measuring probe of the OL756 spectroradiometer (Optronics Laboratories, FL, USA) was placed in the centre of each block at the height of broccoli branchlet surface. Irradiation was recorded as $Wcm^{-2}s^{-1}$. UV-C irradiance varied with the position inside the box; i.e. irradiance was lower around the edges (Table 2.1). For this reason, plastic racks containing broccoli branchlets were placed at only position 2 - 4 in each direction while irradiating. Average values of UV-C doses in this middle area were calculated as 1.3 kJm^{-2} (2 min), 2.6 kJm^{-2} (5 min) and 5.2 kJm^{-2} (10 min).

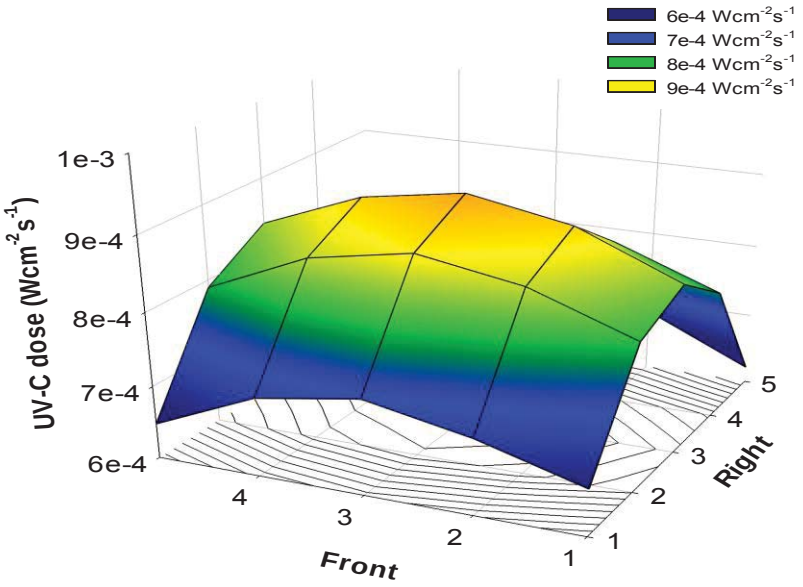

Figure 2.1 UV-C dosage mapping inside the UV chamber, measured by spectroradiometer and expressed as $Wcm^{-2}s^{-1}$. Positions were numbered from 1 - 5 from front to back and right to left.

2.2.4 Weight loss

Weight loss was calculated by subtracting the weight of the broccoli in each punnet from its initial weight at day 0, 2, 4, and 7 and recording the difference as a percentage of the initial weight of the broccoli.

2.2.5 Colour change

Surface colour was measured by a Konica Minolta reflectance spectrophotometer (model CM-2600d, Konica Minolta Sensing, Inc., Osaka, Japan) which was set up for the observer at 10° and illuminant D65. Data for L*, H* and chroma values were selected with SCI (spectral component included) for broccoli colour assessment. Three punnets from each treatment and from the control were assessed just after treatment and at day 2, 4, 7, or 8 depending on the experiment. Three SCI measurements from each branchlet were taken and averaged as one reading and average SCI value of five branchlets in a single punnet (one replicate) was calculated.

2.2.6 Statistical analysis

A complete randomized design was used in this study. Analysis of variance (ANOVA) was performed to analyse the effect of treatments using General Linear Model of Minitab (Minitab Inc., State College, PA, USA). Means of different treatments were compared using Tukey's Test at level 5%. Honest significant difference (HSD) values for repeated measures were calculated at each point of measurement.

2.3 Results and Discussion

2.3.1 Weight loss

Three different doses of UV-C; 1.3, 2.6 and 5.2 kJm^{-2}, were applied on fresh-cut broccoli which were then stored for 7 days at 15 °C. Weight loss increased up to 8% in all broccoli samples irrespective of the treatment by the end of day 7 (Figure 2.2).

Fresh products reduce weight after harvest because of water loss through transpiration and break-down of substrates during respiration (Charles & Arul, 2007). Lemoine and others (2007; 2010b) have observed approximately 4% weight

loss and no significant difference between UV-C treated and untreated broccoli samples when they treated fresh-cut broccoli florets with 8 kJ/m^{-2} and stored them for 3 weeks at low temperatures (0 °C and 4 °C). Even at higher doses (14 kJm^{-2}), UV-C treatment did not adversely affect broccoli heads stored at 20 °C for 5 days compared to control heads (Costa et al., 2006). When UV-C irradiation was used as a postharvest treatment to retain quality of other fresh produce such as blueberry (Perkins-Veazie et al., 2008) and tomato (Maharaj et al., 1999) there was also no significant difference in weight loss reported compared to untreated controls. However, Bal and Kok (2009) found a significant reduction in weight loss in UV-C treated kiwifruit at low temperatures (0 - 1 °C) compared to non-treated kiwifruit. Results of the current study demonstrated that UV-C irradiation at these low doses has no adverse effect on weight loss of fresh-cut broccoli during storage.

2.3.2 Colour change

2.3.2.1 Lightness

Lightness (L) of all samples increased slowly until day 2 irrespective of the UV dosage applied and a rapid increase was observed in untreated broccoli afterwards (Figure 2.4A). Even though broccoli branchlets were allocated to different treatments in a complete randomized way, lightness of samples treated with 5.2 kJm^{-2} irradiation was significantly different ($p < 0.05$) to other samples just after the treatment. This is presumed not to be a treatment effect. Data analysis of percentage lightness change showed that there was a significant difference between treatments only at day 2 (Table 2.1) where higher UV-C dose increased lightness of broccoli. However this increase was recovered by day 4 and there was no significant difference between treatments at the end of the experiment. Percentage increases of lightness of broccoli irradiated at 2.6 kJm^{-2} increased at a very slow rate initially (by about 1.7% and 7% by day 2 and 4 respectively), compared to other treatments and became equal to lightness of other samples by day 7 (15%). According to these data, visual quality of fresh-cut broccoli has not been adversely affected by UV-C irradiation compared to non UV-C treated broccoli during storage. Different studies have suggested that higher doses of UV-C, for example 8 and 10 kJm^{-2}, reduced the increase in lightness in broccoli compared to untreated broccoli or broccoli treated with low doses (Costa et al., 2006; Lemoine et al., 2010b). In this current research

the same experiment was repeated at 10 °C and a significant ($P < 0.05$) decrease in lightness was observed in fresh cut broccoli irradiated at 2.6 and 5.2 kJm^{-2} compared to untreated and 1.3 kJm^{-2} irradiation at day 4 (data not shown). However lightness of all samples increased up to 5% at 10 °C by day 7 except for 2.6 kJm^{-2} treatment. At the higher temperature (15 °C) L value increased up to 20% in untreated broccoli and about 14 % in 5.2 kJm^{-2} irradiated broccoli by day 7 though the difference was not significant at $P = 0.05$. Similarly a higher increase in lightness was reported when UV-C treated broccoli were stored at 20 °C rather than at 4 °C (Costa et al., 2006; Lemoine et al., 2007). This lightness increment may be attributed to senescence related yellowing of broccoli branchlets that could be accelerated at higher temperatures compared to lower temperatures (see 2.3.3.2) (Jones et al., 2006).

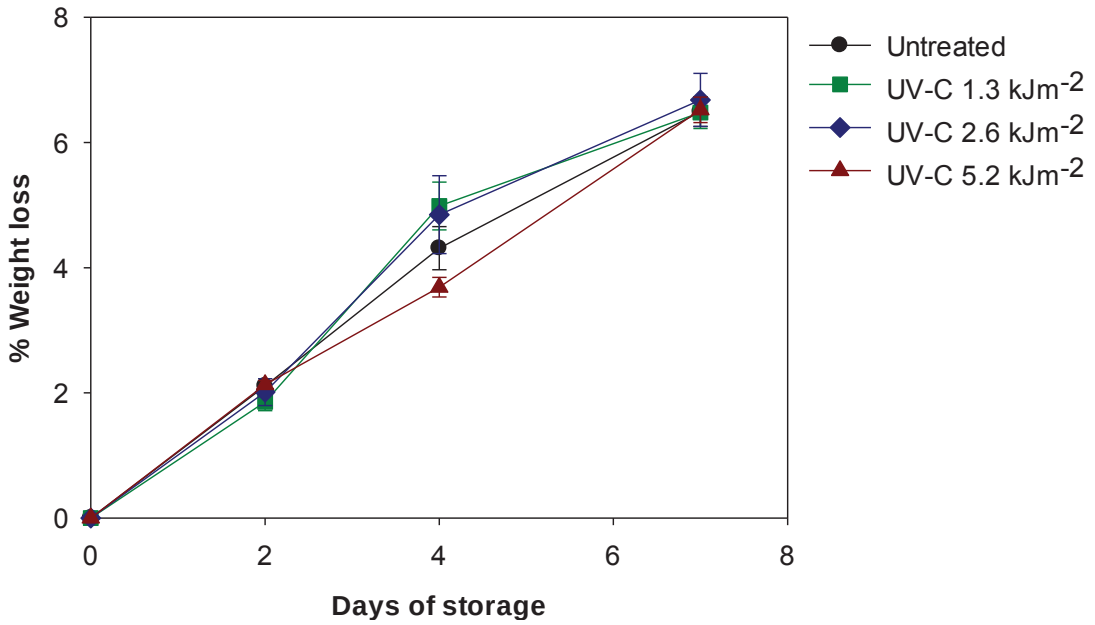

Figure 2.2 % weight loss of UV-C treated fresh cut broccoli during storage at 15 °C depending on different doses applied. Each data point represents an average of three replicates and error bars indicate standard error of means.

2.3.2.2 Hue

Hue value continuously decreased in all broccoli samples during storage irrespective of the treatment, representing a colour change from green to yellow. Initial hue was around 126 which dropped to 101 at day 7 (Figure 2.4B). Colour of green vegetables depends on the chlorophyll content, and with onset of senescence, chlorophyll starts to degrade. Costa and others (2006) have reported a correlation between hue (colour)

and chlorophyll degradation. They have observed a higher hue and delay in chlorophyll 'a' and chlorophyll 'b' degradation at day 6 at 20 °C when broccoli heads were exposed to 10 kJm^{-2} UV-C irradiation. In contrast, an inverse correlation was observed between chlorophyll content and activity of chlorophyll degrading enzymes - chlorophyllase and Mg-dechelatase resulting delayed yellowing. On the other hand, ethylene also stimulates chlorophyllase activity in broccoli (Tian et al., 1995).

2.3.2.3 Chroma

Fresh-cut broccoli irradiated with 5.2 kJm^{2} increased chroma at a slower rate than other broccoli samples, and retained the green colour compared to control and other treatments (Figure 2.3). The highest chroma value was recorded in broccoli treated with 1.3 kJm^{-2} at day 7 followed by untreated broccoli (Figure 2.4C).

2.3.3 Colour change in fresh-cut broccoli over time

Results of this study demonstrated that UV-C irradiation at doses of 1.3, 2.6, and 5.2 kJm^{-2} did not adversely affect colour of fresh-cut broccoli branchlets during 7 days of storage at 15 °C. Chlorophyll content is often used to evaluate the sensory quality of green fruit and vegetables (Jin-hua, 2007). Chlorophyll degrades rapidly during senescence as a result of chlorophyll catabolism by which chlorophyll is transformed into chlorophyllide by chlorophyllase (Figure 2.5). Subsequently chlorophyllide is converted into pheophorbide by Mg-dechelatase and oxidized to colourless compounds later on (Toivonen & Brummell, 2008). Yellowing of florets is one of the main symptoms of senescence of broccoli which occurs due to chlorophyll degradation and accumulation of pheophytin which is olive brown in colour (Costa et al., 2005a). Pheophytin is also a catabolised product of chlorophyll when Mg-dechelatase directly react on chlorophyll and subsequently turn into colourless pheophorbide by chlorophyllase (Toivonen & Brummell, 2008).

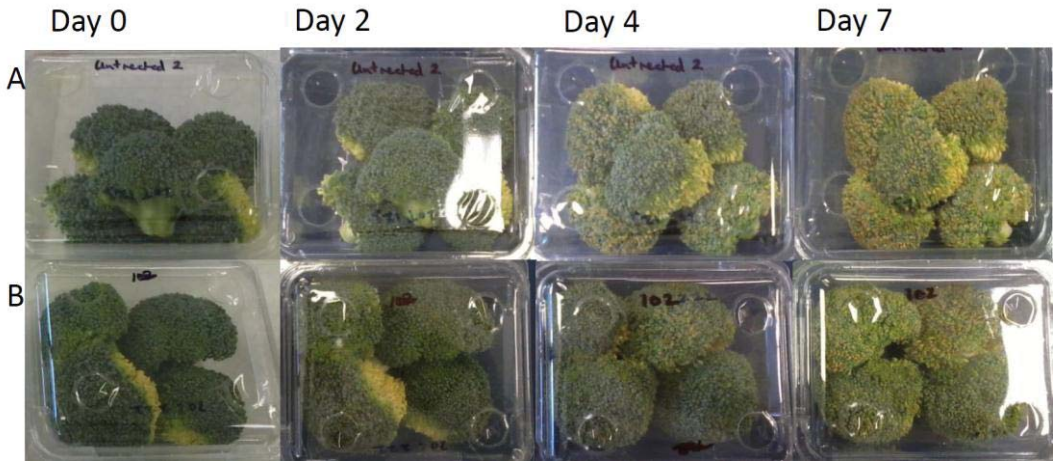

Figure 2.3 Visual colour change of fresh-cut broccoli during storage at 15 °C (A) untreated control and (B) UV-C treated (5.2 kJm^{-2}).

Table 2.1 Percentage lightness increase by UV-C treatment at day 2, 4, and 7

	% Lightness increase from day 0		
Treatment	Day 2	day 4	day 7
Untreated	2.61 ± 0.54^{AB}	15.28 ± 2.47	20.45 ± 2.5
1.3 kJm^{-2}	5.64 ± 1.72^{AB}	13.31 ± 1.75	19.9 ± 1.99
2.6 kJm^{-2}	1.66 ± 0.89^{B}	6.87 ± 2.02	15.03 ± 2.04
5.2 kJm^{-2}	6.78 ± 1.02^{A}	12.88 ± 0.81	14.06 ± 0.29
P value	0.037	0.058	0.096

Each data point represents mean ± SE (n = 3). Means with different superscript letters were significantly different ($P < 0.05$) following analysis of variance and honest significant different (HSD) mean separation procedures.

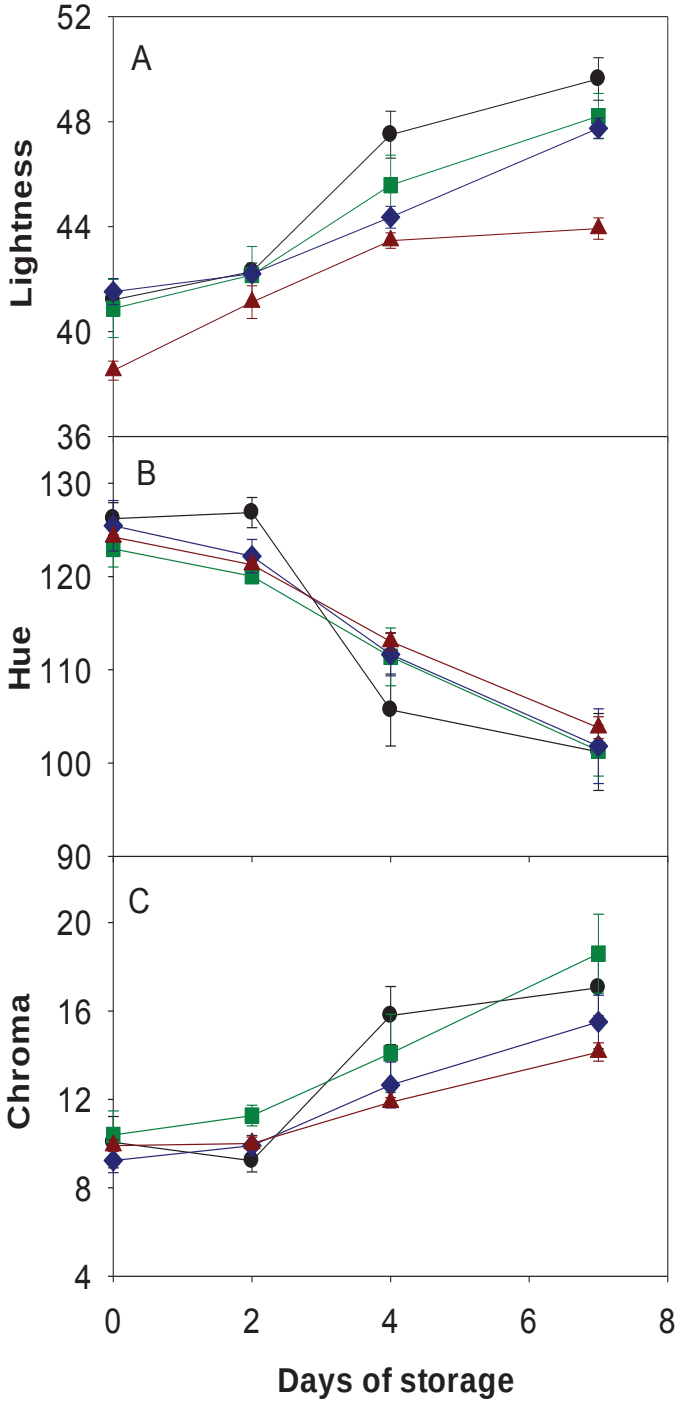

Figure 2.4 Colour change in UV-C treated fresh cut broccoli during storage at 15 °C depending on dosage applied. (A) Lightness, (B) Hue, and (C) Chroma. Symbols represent UV-C doses, ● = untreated, ■ = 1.3 kJm^{-2}, ♦ = 2.6 kJm^{-2}, ▲ = 5.2 kJm^{-2}. Each data point represents average of three replicates and error bars indicate standard error of means.

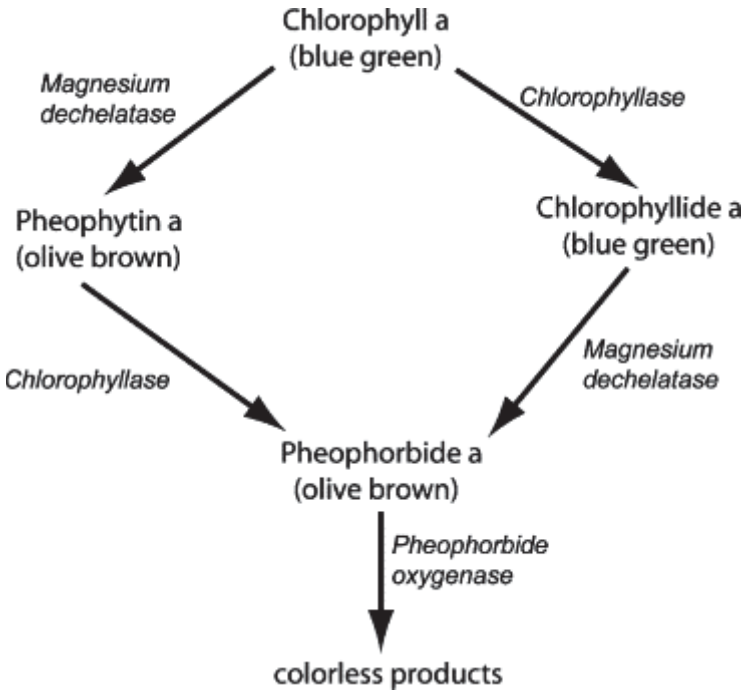

Figure 2.5 Hypothesised Type I chlorophyll breakdown pathways in green plant tissues. Adapted from Toivonen et al. (2008)

With mechanical damage or low temperature storage, tissues and cell membranes that separated the phenol compounds and PPO enzyme in the cell are disrupted and as a result allow the phenol oxidation reaction resulting in browning of tissues (von-Mollendorff, 1996). Abiotic or biotic stress factors including pathogen attack or postharvest treatments may induce stress condition in fresh produce and modify their phenolic metabolism and antioxidant activity as a defence mechanism to protect against such stresses (Lemoine et al., 2010a). Many postharvest treatments (heat, low temperature) including UV-C irradiation have inhibited activity of PPO and oxidation reactions of phenol compounds inside cells. Higher concentrations of phenolic and ascorbic acid were found in UV-C treated broccoli resulting in higher antioxidant activity and less production of reactive oxygen species (ROS) during senescence. (Lemoine et al., 2010a), thus, retaining the green colour of broccoli by delaying senescence.

2.4 Conclusion

Results of this preliminary study confirmed that UV-C irradiation at a dose of 1.3, 2.6, and 5.2 kJm^{-2} did not alter visual appearance and weight loss of fresh cut

broccoli significantly over time compared to the non UV-C treated control. However, there was a tendency (even though it was not significant) for UV-C irradiation at a dose of 5.2 kJm^{-2} to retain the colour of broccoli at day 7, where percentage lightness increase was 14% in broccoli irradiated at 5.2 kJm^{-2} compared to 20% in non UV-C treated broccoli. More importantly UV-C treatment at these doses did not show adverse effects on physiological properties of fresh-cut broccoli during storage at 15 °C. Therefore, it can be concluded that UV-C irradiation can be considered as a successful postharvest treatment for fresh-cut broccoli in terms of physiological qualities and further studies are needed to investigate the UV-C stress related impacts in changing bioactive content and pathogen control potential.

3 EFFECT OF UV-C IRRADIATION ON GROWTH AND SURVIVAL OF POST INOCULATED *LISTERIA MONOCYTOGENES* [*]

3.1 Introduction

As stated in Chapter 2, there has been a good deal of interest in the use of UV-C irradiation as a postharvest treatment aimed at delaying senescence of fresh produce and as a sanitizing step in food processing. The use of UV-C irradiation as a germicidal treatment in fresh produce primarily aims at inactivating microorganisms present on or near the surface (Obande et al., 2011). Fresh vegetables may be contaminated by a variety of microorganisms through direct contact with soil, insects, rain water, wind action, and/or irrigation water (Beuchat, 2002). Natural flora in fresh produce may consist of a variety of microorganisms including spoilage microorganisms that cause diseases and rots in plants and even microorganisms pathogenic to humans. Human pathogens such as *L. monocytogenes*, *E. coli*, and *Salmonella spp*, among many others cause serious foodborne diseases in humans such as salmonellosis and listeriosis. These pathogens are widely spread in nature especially in agricultural lands, sediments, and in decaying plant materials (Ells & Lisbeth, 2006). Fresh produce may be exposed to microbiological contamination during handling, processing, transportation, and storage. Since fresh products may be consumed raw or following minimal processing, risk of ingestion of viable pathogens is high (Ells & Lisbeth, 2006). Proper postharvest management practices should be followed to minimize microbiological contamination of fresh produce (Doménech et al., 2013).

Many postharvest treatments including surface sanitizing and packaging systems have been studied to control human pathogens in fresh produce (Olaimat & Holley, 2012; Severino et al., 2014). Among other pathogens *L. monocytogenes* is one of the important human pathogen that causes foodborne listeriosis in humans (McLauchlin et al., 2004). The number of reported cases of listeriosis related to fresh produce tend to increase in recent past even though there are fewer reported cases compared to other ready-to-eat products. Minimally processed or fresh vegetables could support

[*] Materials of this chapter are included in the publication: Gamage, G.R., Wargent, J., Palmer, J.S., Heyes, J.A. (2013). Effect of UV-C irradiation on growth of post inoculated L. monocytogenes on fresh-cut broccoli. Acta Horticulturae, 1012, pp. 1031-1036

the growth of *L. monocytogenes* while on display or refrigerated before consumption (Ells & Lisbeth, 2006; Luber et al., 2011). On the other hand natural microflora residing on the surface of fresh produce may also influence growth of secondary contaminated pathogens (Mukhopadhyay et al., 2014). At the consumer's end, washing lettuce pieces by dipping in chlorinated water and washing under running tap water may not adequately reduce *L. monocytogenes* population if the initial contamination is more than 10^3 cfu per gram of lettuce (Doménech et al., 2013). *L. monocytogenes* is reported to be resistant to sanitizers commonly used in fresh produce industry such as chlorine (Zhang & Farber, 1996) and attached more strongly to the cut edges of lettuce than *Salmonella* and *E. coli* (Takeuchi et al., 2000). Therefore Luber et al. (2011) highlighted in their review the necessity of processes that reduce the number of bacteria present in all ready-to-eat food, especially pathogens such as *L. monocytogenes* that could grow during refrigerated storage. This experiment aims at determining the effect of UV-C irradiation as a postharvest technology in minimizing growth and survival of *L. monocytogenes* inoculated onto fresh cut broccoli at different times after treatment. Artificial inoculation simulates potential contamination of fresh-cut broccoli after harvest.

In Chapter 2, the effect of three different doses of UV-C irradiation (1.3, 2.6 and 5.2 kJm^{-2}) on physiological properties of fresh-cut broccoli was studied. It was decided to use the two higher doses (2.6 kJm^{-2} and 5.2 kJm^{-2}) for subsequent microbiological and phytochemical analyses, as they caused no deleterious effects on fresh cut broccoli and were in keeping with previous doses for plant pathogens.

3.2 Materials and method

3.2.1 Important notice

It is highly recommended to follow all safety procedures when working with *L. monocytogenes* in the laboratory. The laboratory should be at least a Physical Containment level 2 (PC 2) and a class II laminar flow biosafety cabinet is recommended to be used. All the surfaces and equipment should be sanitized before and after use and all media or any contaminated items should be autoclaved before disposal. Pregnant women and immunocompromised persons should not be allowed to enter the laboratory while *L. monocytogenes* activity is in progress. For further health and safety guidance refer to the laboratory guide book of Food Safety and

Inspection Service, USDA (USDA, 2013). All specified safety procedures were followed for this project.

3.2.2 *L. monocytogenes* culture

Stock cultures of *L. monocytogenes* (NCTC7973) were maintained on beads at -80 °C. A single bead was transferred into 10 mL tryptic soy broth (TSB) and incubated for 24 h at 30 °C under aerobic conditions, in accordance with local laboratory practice. Serial dilution with maximum recovery dilution (MRD: 0.1% peptone water + 0.85% NaCl solution) solution was used to reach a concentration of 1×10^6 cells per mL.

3.2.3 Microbial assay

All fresh cut broccoli branchlets previously irradiated with UV-C (see 2.2.1, 2.2.2 and 2.2.3) and placed in a single clamshell were inoculated with 200 µL of prepared *L. monocytogenes* culture at 0, 6, or 24 h after UV-C treatment. The inoculum (200 µL) was spot inoculated over 5 branchlets. Three clamshells from each treatment were examined destructively for the number of colony forming units at day 0, 2, 4, 6 and 8 after irradiation. Untreated broccoli branchlets inoculated as mentioned above were used as the control. All samples were stored at 15 °C (apart from the preliminary experiment in which branchlets were stored at 20 °C) in an external environment of 80-85% relative humidity.

3.2.4 Enumerating *L. monocytogenes* cells

Broccoli florets of the branchlets of each clamshell were aseptically shaved with a sterilized knife and transferred into a stomacher bag and stomached for 1 min in 100 ml of MRD. After serial dilution with MRD, 0.1 ml aliquot from each dilution was spread plated onto Oxford agar plates (produced by Ford Richard, Auckland New Zealand) which is a selective medium and incubated for 48 h at 30 °C. The number of colony forming units (CFU) was counted using a colony counter on two successive dilutions at ca. 30 - 300 colonies per plate.

3.2.5 Enumerating mesophilic bacteria cells

Mesophilic bacteria extraction was done as mentioned in 3.2.4. After a serial dilution of the stomached solution with MRD, 0.1 ml aliquot from each dilution was spread

plated on to tryptic soy agar (TSA) plates (produced by Ford Richard, Auckland, New Zealand) and incubated for 48 h at 30 °C and CFU were counted using a colony counter.

3.2.6 Statistical analysis

A complete randomized design was used in this study, usually three replicates with samples prepared for each destructive measurement. Analysis of variance (ANOVA) was performed to analyse the effect of treatments using General Linear Model of Minitab (Minitab Inc., State College, PA, USA). Means of different treatments were compared using Tukey's Test at level 5%.

3.3 Results and Discussion

3.3.1 Growth and survival of *L. monocytogenes*

Preliminary study of UV-C treatment in controlling the growth of *L. monocytogenes* on fresh-cut broccoli revealed that UV-C irradiation at a dose of 5.2 kJm^{-2} significantly reduced ($P < 0.05$) the population of *L monocytogenes* inoculated onto fresh cut broccoli after treatment compared to untreated broccoli (Figure 3.1). A comparatively high UV-C dose (5.2 kJm^{-2}) was used to treat fresh-cut broccoli in this preliminary experiment and initial inoculum was about 1.25×10^6 cfu/mL for both treated and untreated broccoli. Here, growth data (CFU) of *L. monocytogenes* was obtained at day 4, and day 7 only. Broccoli samples were stored at 20 °C after inoculation in order to observe a treatment effect within a short period of time by stimulating growth at an elevated temperature. Even though the ideal temperature for *L. monocytogenes* to grow is between 30 - 37 °C, this is not a suitable storage temperature for fresh cut broccoli, as it starts to senesce rapidly at higher temperatures. Number of *L. monocytogenes* cells was reduced significantly ($P < 0.05$) in UV-C treated broccoli compared to the untreated broccoli at day 4 (Figure 3.1). However by day 7 the number of *L. monocytogenes* cells recovered from UV-C treated broccoli had returned to initial level by day 7 while a continuous increase was observed in untreated broccoli. In all experiments *L. monocytogenes* was inoculated onto fresh cut broccoli after UV-C treatment; hence *L. monocytogenes* suppression was not a direct inhibition by UV-C irradiation. It can be hypothesised that UV-C treatment may alter the surface of broccoli and as a result the surface is less attractive

for *L. monocytogenes* to attach and grow. This may be due to presence of antimicrobial compounds (Griffiths et al., 2001) on the surface of broccoli or altering the surface properties such as hydrophobicity (Wang et al., 2012) of broccoli surface to make it undesirable for attachment and growth of *L. monocytogenes*.

In the second experiment two different UV-C doses (2.6 and 5.2 kJm^{-2}) were applied to fresh cut broccoli and untreated branchlets were used as the control. All samples were inoculated at three different times after UV-C treatment: immediately after treatment, 6, and 24 h after treatment. Initial inoculum was approximately 1×10^6 cfu/mL. The number of viable *L. monocytogenes* cells was counted by dilution plating (see section 3.2.3) at day 3, 4, 6, and 8. The third experiment was a repetition of the second experiment and additionally, in the third experiment, growth data (CFU) of *L. monocytogenes* were taken immediately after inoculating the samples (at 0, 6, and 24 h after UV-C treatment) and day 2, 4, 6, and 8 after UV-C treatment. Untreated control samples were also inoculated together with treated samples: immediately after treatment, 6 and 24 h after treatment and stored for 8 days. All samples were stored at 15 °C in order to simulate temperature abuse during transportation, retail and consumer storage which could allow *L. monocytogenes* to grow to harmful levels. Results of both experiments showed the same trend (Figure 3.2) in controlling the growth of *L. monocytogenes* and confirmed the results observed in the first experiment (Figure 3.1).

In the third experiment, both UV-C doses (2.6 and 5.2 kJm^{-2}) restrained the growth of *L. monocytogenes* on fresh-cut broccoli samples inoculated 24 h after treatment throughout the storage period. Similar results were observed in the broccoli samples treated with 5.2 kJm^{-2} and inoculated 6 h after treatment (Figure 3.2 E&F). An unusual increase in *L. monocytogenes* numbers was observed at day 6, only in samples treated with 2.6 kJm^{-2} and inoculated 6 h after treatment, which then decreased to the same level as broccoli treated with 5.2 kJm^{-2}, by day 8 (Figure 3.2E). However, the number of *L. monocytogenes* cells increased significantly after day 4 in all untreated broccoli and broccoli inoculated immediately after treatment (Figure 3.2D). The number of *L. monocytogenes* cells recovered from broccoli branchlets inoculated 24 h after treatment was approximately 1 and 2 log cycles less than the untreated broccoli initially and at day 8 respectively (Figure 3.2F). These results imply that UV-C treatment is more effective in suppressing growth and

survival of *L. monocytogenes* on fresh-cut broccoli if contaminated 24 h after UV-C exposure than 6 h.

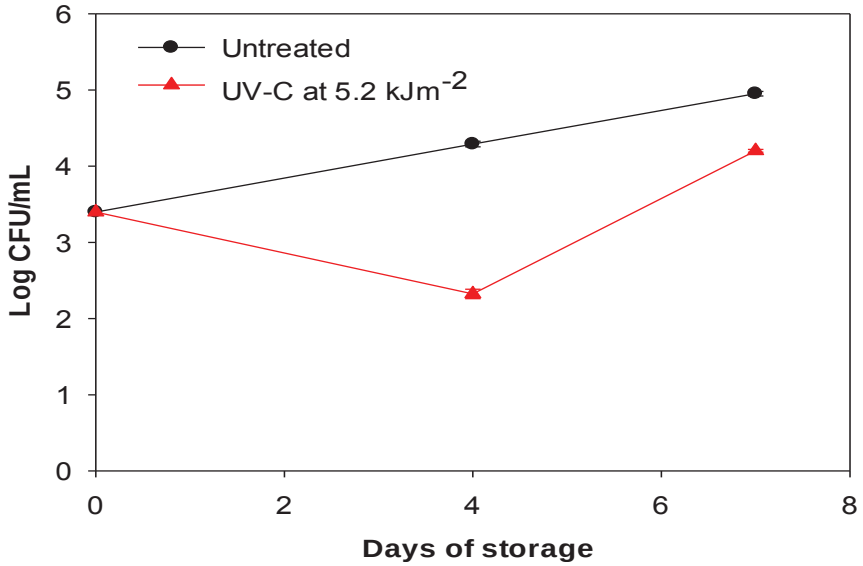

Figure 3.1 Growth of *L. monocytogenes* on UV-C treated fresh cut broccoli branchlets at a dose of 5.2 kJm^{-2} and untreated broccoli stored at 20 °C (Preliminary experiment). Each data point represents mean of three replicates and error bars represent standard error. (Error bars are not visible as they are smaller than symbols).

In fact these results confirmed that UV-C treatment restrained the number of *L. monocytogenes* that could be recovered from fresh cut broccoli when they were inoculated 24 h after treatment. On the other hand, an increase in *L. monocytogenes* numbers in untreated broccoli and broccoli inoculated immediately after treatment indicated that the surface of broccoli was not as unfavourable for *L. monocytogenes* as the surface after 24 h of the treatment and was similar to the non UV-C treated broccoli. Previous studies have suggested that mild stress produced by UV-C irradiation can induce the expression of defence-related genes (Pombo, et al., 2011) and consequently stimulate the synthesis of defence related phytochemicals in broccoli (Lemoine, et al., 2010a) such as phenolics, glucosinolates, and the derivatives of GLSs (Aires et al., 2011; Björkman et al., 2011; Shen et al., 2013) which may also offer a variety of health benefits. Particularly, volatile compounds such as hydrolysis products of GLSs may accumulate on the surface of broccoli branchlets (Griffiths et al., 2001) affecting the growth and/ or attachment of *L.*

monocytogenes cells. To our knowledge this is the first report of prior UV-C treatment that affects the subsequent development of less supportive surfaces for the growth of a human pathogen that may contaminate fresh cut broccoli during processing. It can be suggested that volatile compounds may accumulate on the surface with time and/or alter the surface morphology of UV-C treated broccoli affecting the growth and survival of *L. monocytogenes*. However these hypotheses need to be tested by phytochemical analyses.

3.3.2 Mesophilic bacteria growth

Fresh produce naturally harbours numerous microorganisms. Total number of mesophilic bacteria naturally present on UV-C treated and untreated fresh cut broccoli branchlets over the storage time was studied in the second and third experiments. Results of experiments 2 and 3 showed a similar trend, in that the total mesophilic bacteria count increased over time (Figure 3.3). The number of total mesophilic bacteria increased by about one log cycle by day 8 in all samples irrespective of treatment and the time of inoculation with *L. monocytogenes* after UV-C treatment.

Total mesophilic bacteria count decreased on the surface of broccoli by about 0.25, 0.5, and 0.75 log cycles when measured 0, 6, and 24 h after UV-C treatment respectively (Figure 3.3D, E, & F). However by day 2, all populations increased and equalled the numbers observed in untreated broccoli, except for the UV-C treated broccoli inoculated with *L. monocytogenes* 24 h after treatment (Figure 3.3F). Mesophilic bacteria growth of those samples increased by about 0.5 log cycle by day 4 and then was unchanged until day 8 whereas an increase above 7 log was observed in all other samples. Even though the mesophilic bacteria count of UV-C treated and untreated broccoli is significantly different, it is within a half of log range (Figure 3.3F). The initial reduction of mesophilic bacteria observed in the experiment may be due to direct bactericidal effect of UV-C irradiation on the existing bacteria on the surface of UV-C treated broccoli and/or due to competition by newly introduced *L. monocytogenes* cells. However mesophilic bacteria seemed to have recovered from the UV-C damage and started to grow again as normal after day 2. The influence of indigenous flora on broccoli on the growth of *L. monocytogenes* is not clear from these results and a correlation analysis between the growth of two bacteria may help in explaining the possible relationship.

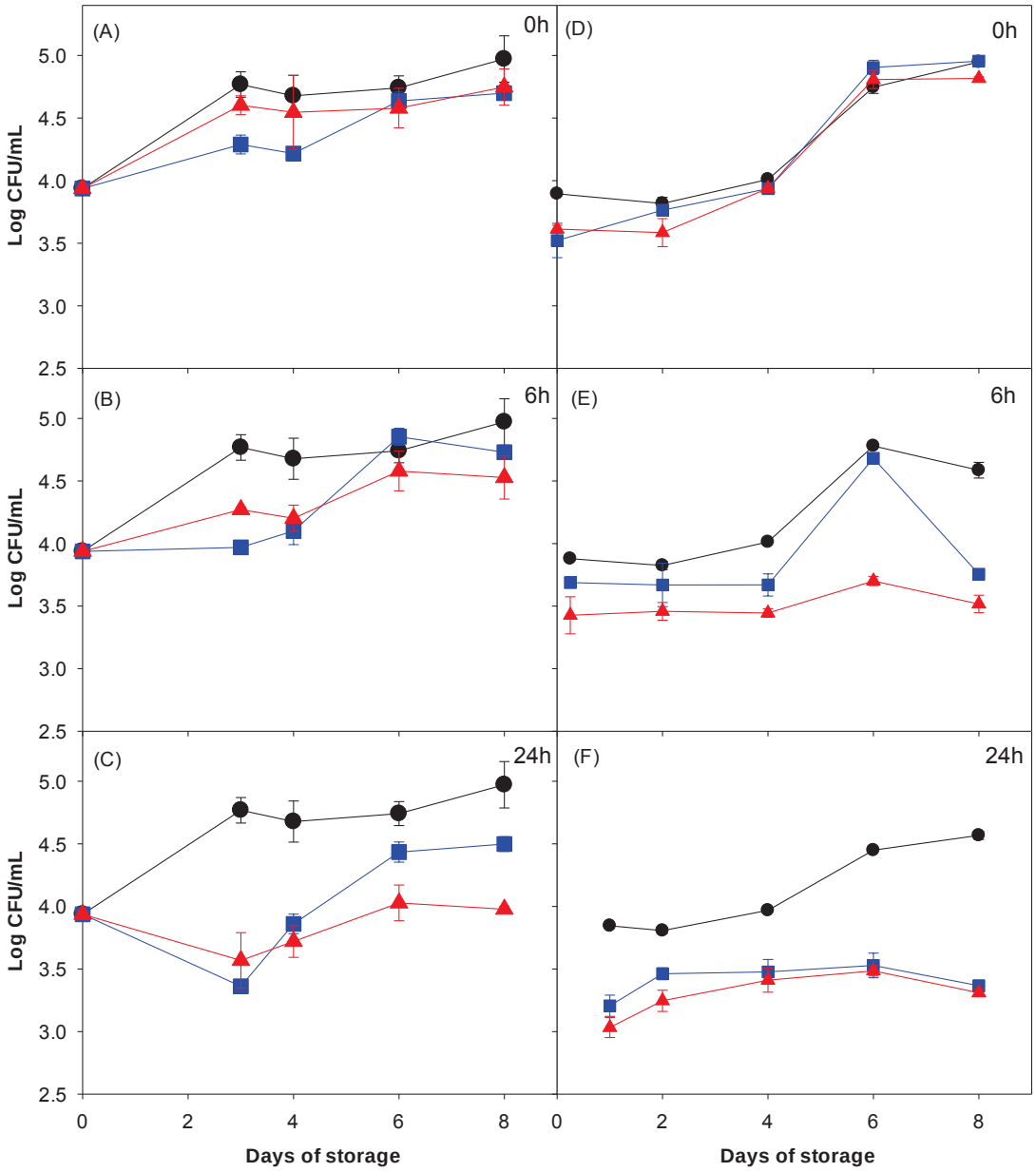

Figure 3.2 Growth of *L. monocytogenes* inoculated onto untreated and UV-C treated fresh cut broccoli branchlets at different times (0, 6, and 24 h) after UV-C treatment and stored at 15 °C. UV-C irradiation was at doses of ● = 0 kJm^{-2}, ■ = 2.6 kJm^{-2}, and ▲ = 5.2 kJm^{-2}. (A), (B), and (C) from experiment 2 results and (D), (E), and (F) from experiment 3 results. Each data point is an average of three replicates and error bars represent standard error.

3.3.3 Correlation between growth of *L. monocytogenes* and mesophilic bacteria

A positive correlation was observed when growth of *L. monocytogenes* was plotted against total mesophilic count (Figure 3.4), but correlation coefficients (R^2) were low for many of the treatments (Table 3.1). Therefore, it is not possible to establish a good correlation between the growth of mesophilic bacteria and *L. monocytogenes*. However the slopes of regression lines of different treatments and inoculation times show a trend in preferential suppression of *L. monocytogenes* by UV-C treatment. The highest slopes were generally observed in samples irradiated with 2.6 and 5.2 kJm^{-2} and inoculated 6 and 24 h after treatment. It indicates that mesophilic bacteria tend to grow well on the broccoli surface even though the growth of *L. monocytogenes* was restrained by the UV-C treatment. That may be because mesophilic bacteria may have become comparatively adapted to broccoli surface and may be more tolerant to surface changes induced by UV-C treatment than *L. monocytogenes* which may be more vulnerable to the same changes than mesophilic bacteria.

Different types of bacteria species live together as dynamic colonies in the natural environment and may obtain mutual benefits from nutrients and defence without adversely affecting each other (Lee & Lee, 2010; Aires et al., 2011). For example, different initial microflora levels on iceberg lettuce did not influence the growth of *L. monocytogenes* (Carrasco et al., 2008). Mesophilic bacteria naturally on the surface of broccoli may form biofilms (Ukuku et al., 2005) or combine with pathogenic bacteria to form more stable biofilms. It has been revealed that *L. monocytogenes* can form either monoculture biofilms or mixed culture biofilms together with other bacteria. For example, Bremer et al (2001) observed a significantly higher number of *L. monocytogenes* cells attached to stainless steel surfaces forming biofilms and an ability to survive for a longer time when in a mixed culture with *Flavobacterium* than in a monoculture. Seo and Frank (1999) also studied the effect of biofilms produced by *Pseudomonas fluorescens,* a common bacterium causing deterioration in vegetables, on attachment of *E. coli* O157:H7 to lettuce leaf surfaces. They reported that *E. coli* did not necessarily attach to the biofilms produced by *Pseudomonas* (Seo & Frank, 1999). UV-C irradiation is capable of inducing host defence responses by way of inducing production of defence related compounds (Terry & Joyce, 2004) which may diffuse to or be volatilized from the surface of broccoli. Therefore, microorganisms on the surface of broccoli may face adverse

conditions. Results of this current study suggest that *L. monocytogenes* could not sustain its natural growth on UV-C treated broccoli when inoculated 6 h after UV treatment whereas natural microflora continued to grow without interruption.

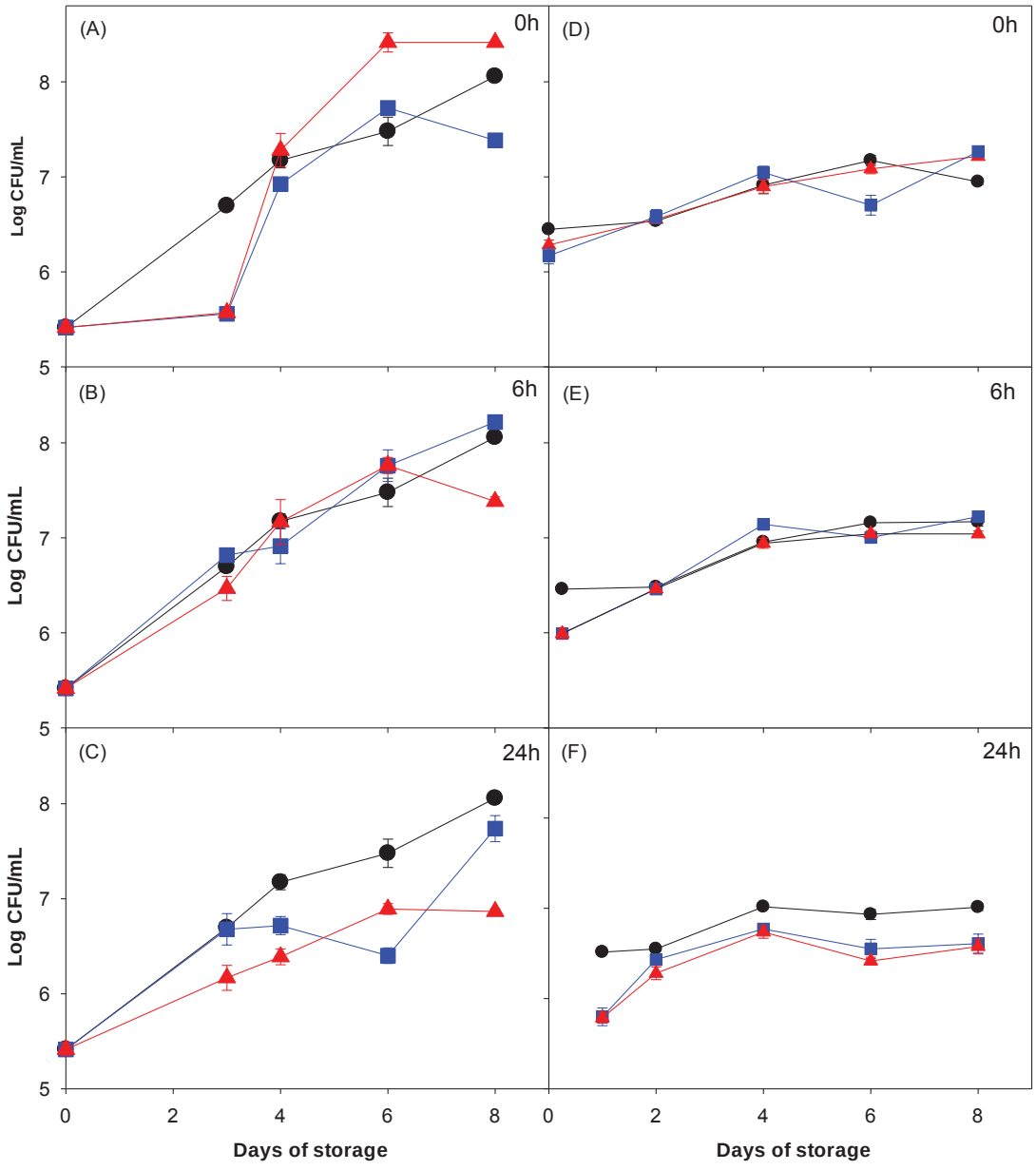

Figure 3.3 Growth of total mesophilic bacteria on untreated and UV-C treated fresh cut broccoli branchlets at different times (6, and 24 h) after UV-C treatment and stored at 15 °C. UV-C irradiation was at doses of ● = 0 kJm^{-2}, ■ = 2.6 kJm^{-2}, and ▲ = 5.2 kJm^{-2}. (A), (B), and (C) from experiment 2 results and (D), (E), and (F) from experiment 3 results. Each data point is an average of three replicates and error bars represent standard error.

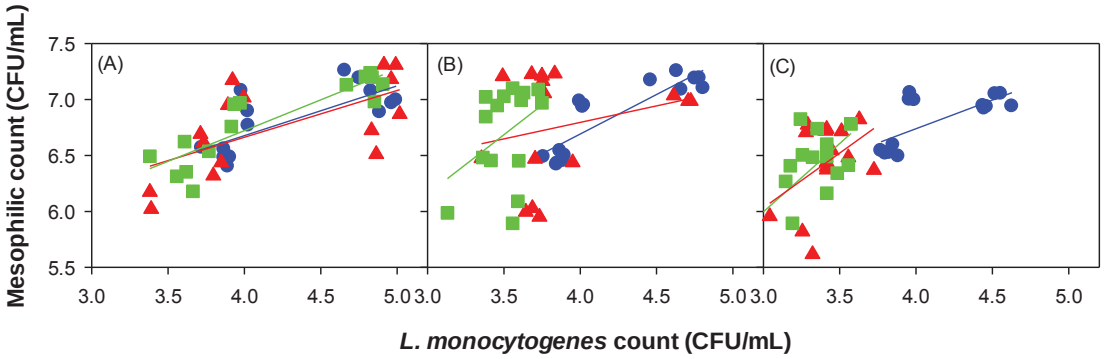

Figure 3.4 Correlation between growth of *L. monocytogenes* and total mesophilic count in untreated and UV-C treated fresh cut broccoli branchlets at (● = untreated, ■ = 2.6 kJm^{-2}, ▲ = 5.2 kJm^{-2}) stored at 15 °C. (A) Bacteria counts taken immediately after, (B) 6 h and (C) 24 h after UV-C treatment

Table 3.1 Correlation coefficient values and their *P* values associated with growth of *L. monocytogenes* and mesophilic bacteria counts on untreated and UV-C treated fresh cut broccoli at different UV-C doses and different times of inoculation after UV-C treatment

Inoculation time	Treatment	R^2	*P*
0h	0 kJm^{-2}	0.56	0.0014
	2.6 kJm^{-2}	0.41	0.0102
	5.2 kJm^{-2}	0.77	<0.0001
6 h	0 kJm^{-2}	0.76	<0.0001
	2.6 kJm^{-2}	0.07	0.347
	5.2 kJm^{-2}	0.16	0.1462
24 h	0 kJm^{-2}	0.52	0.0026
	2.6 kJm^{-2}	0.19	0.1021
	5.2 kJm^{-2}	0.45	0.0063

3.4 Conclusion

UV-C irradiation as a postharvest treatment is capable of suppressing the growth of *L. monocytogenes* in fresh cut broccoli under simulated contamination 6- 24 h after UV-C treatment. The reduction in the growth of *L. monocytogenes* on UV-C treated broccoli may be due to induction of secondary metabolic pathways and/or altered surface characteristics which may be unfavourable for growth and survival of *L. monocytogenes.* UV-C irradiation can stimulate accumulation of stress-induced defence related compounds that may be harmful for *L. monocytogenes*, at appropriate intensities. Therefore it is important to study the inhibitory effect of UV-C treated

broccoli extracts and the phytochemical compounds that may be altered by UV-C in order to confirm the method of suppressing the growth of *L. monocytogenes* by UV-C treated broccoli.

4 ANTIMICROBIAL EFFECT OF EXTRACTS OF UV-C TREATED BROCCOLI

4.1 Introduction

Brassicaceae vegetables including broccoli, cauliflower, Brussels sprouts, and cabbage contain a variety of secondary metabolites such as polyphenolic compounds and GLSs. Plant secondary metabolites may have nutritional and health promoting properties in addition to close association with the defence system against insects and plant pathogens (Björkman et al., 2011). Glucosinolates and their hydrolysis products are mainly associated with particular biological properties of Brassicaceae vegetables including characteristic odour and taste (Fahey et al., 2001). There are about 120 GLSs identified in plants and GLSs and their break-down products have been studied extensively for their fungicidal, bactericidal, herbivore and pathogenesis related properties (Moreno et al., 2006). Cellular break-down upon mechanical damage, insect attack or infection exposes GLSs to a degradative enzyme, myrosinase, which is stored in vacuoles and produces a range of hydrolysis products such as isothiocyanates (ITCs), thiocyanates (TCs), nitriles, and various indole compounds. Recent studies have demonstrated the antimicrobial effect of these bioactive products against plant pathogens as well as human pathogens (Hu et al., 2004; Aires et al., 2009; Lee et al., 2011).

Production of GLSs and other secondary metabolites in plants can be induced by both biotic and abiotic stresses. Once plants are disturbed by an insect or a pathogen, signalling molecules such as jasmonic acid (JA) and salicylic acid (SA), are produced and subsequently induce production of defence related compounds such as phenolics, flavonols, and GLSs (Redovnikovic et al., 2008). Similarly, postharvest abiotic stresses such as cutting, pressure processing, UV light, other radiation treatments, and modified atmospheres can be effective in inducing defence responses in plants (Cisneros-Zevallos, 2003). Stress produced by the moderate doses of UV-C irradiation applied in this study (chapter 3) may have induced production of secondary metabolites in broccoli that are harmful for *L. monocytogenes*.

Objectives of this study were to determine whether the changes caused by the UV-C treatment in secondary metabolites suppress the growth of *L. monocytogenes*, to

separate the secondary metabolites by solvent extraction into four different groups of varying polarity, and to find out which groups are more suppressive for *L. monocytogenes*. In principle, the most polar compounds will appear in the aqueous extracts and the least polar (like waxes) will appear in hexane. An *in vitro* assay was conducted to determine the antimicrobial effect of each crude extract against *L. monocytogenes.*

4.2 Materials and Methods

4.2.1 Sample preparation

In a new experiment, florets of UV-C treated (at a dose of 2.6 or 5.2 kJm^{-2}) and untreated broccoli branchlets (treated in the same way as 2.2.1, 2.2.2, and 2.2.3 but not inoculated with *L. monocytogenes*) were shaved and frozen in liquid nitrogen, just after UV-C treatment, 6 and 24 h after treatment. All the samples were kept at -70 °C until extraction.

4.2.2 *L. monocytogenes* culture

Stock cultures of *L. monocytogenes* (NCTC7973) were maintained on beads at -80 °C. A single bead was transferred into a 10 mL tryptic soy broth (TSB) (Difco Laboratories, Bacto Dickinson and Company, USA) and incubated for 24 h at 30 °C. Serial dilution with maximum recovery dilution (MRD: 0.1% peptone water + 0.85% NaCl solution) solution was used to reach a concentration of 1×10^6 cells per mL.

4.2.3 Solvents

Different solvents having a range of polarities were used for extraction using solvent-solvent partition method (Figure 4.1). Solvents used were distilled water, n-butanol (Ajax Chemicals, Australia), 10% methanol (Merck KGaA, Germany), 80% ethanol (Sigma Aldrich, USA, n-Hexane (Merck KGaA, Germany), and chloroform (BDH PROLABO, EU).

4.2.4 Plant material extraction

Frozen florets (10 g) of UV-C treated broccoli were mixed with dry ice and ground in a coffee grinder to a smooth powder. The samples were combined with pre-warmed 80% ethanol (100 ml) in a Schott bottle and placed in a water bath at 80 °C for 5 minutes.

After keeping the samples at 5 °C for an hour, supernatant was filtered through a vacuum filter (0.45 µm filter paper) and transferred into a round bottom flask. The extraction process of Mahasneh and El-Oqlah (Mahasneh & El-Oqlah, 1999) was followed. Briefly, ethanol extract (80% ethanol) was evaporated to dryness at 40 °C under reduced pressure using a rotary evaporator (Buchi Rotavapor R-215, Switzerland) and the resulting gummy residue was partitioned as in the diagram (Figure 4.1). Firstly, residue was partitioned between chloroform and water (1:1 V/V): solvent-solvent partitioning. Then the crude chloroform extract was partitioned again between n-hexane and 10% aqueous methanol, and butanol extract was fractionated from aqueous extract. All the solvents were evaporated under reduced pressure at 40 °C. Final crude extracts of different solvents were dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) (DMSO- less than 1.5% in wells) to provide a consistent and moderately compatible solvent for use in microbial assay.

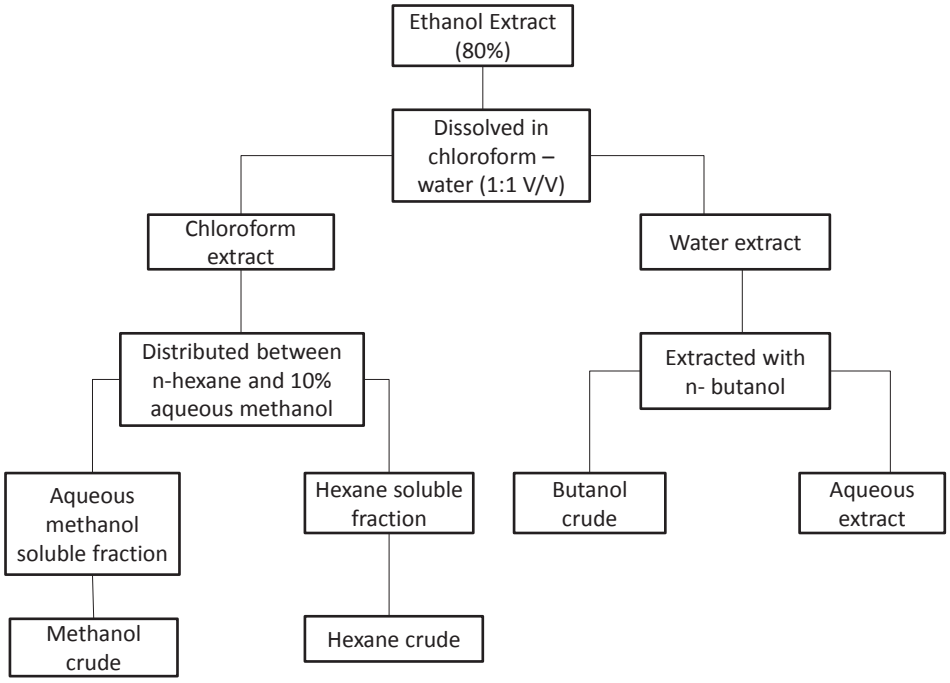

Figure 4.1 Extraction procedure of broccoli using different solvents. 80% ethanol extracts of UV-C treated and non-treated broccoli were partitioned and fractionated using chloroform, water, n-butanol, 10% aqueous methanol and hexane. Final extracts were evaporated under reduced pressure at 40 °C to obtain crude extracts.

4.2.5 Microbial assay

4.2.5.1 Establishing L. monocytogenes *growth curve*

A single bead of the stock culture of *L. monocytogenes* (NCTC7973) maintained on beads at -80 °C was transferred into 10 mL TSB (Difco Laboratories, Bacto Dickinson and Company, USA) and incubated for 24 h at 30 °C. Overnight culture was transferred into a centrifuge tube and centrifuged at 6000 g for 20 min at 4 °C. Supernatant was discarded and remaining cells were washed twice with distilled water. Ten millilitres (10 mL) of TSB was added to the tube and vortexed for 2 minutes. An aliquot of 100 µL was pipetted out into each well of the first 3 rows of 96 micro-well plate followed by 50 µL of TSB. Three replicated plates were used and all plates were incubated at 30 °C for up to 22 h. Optical density (OD) of the wells was measured regularly using a plate count reader (Spectro star-Nano, BMG LABTECH, Germany).

4.2.5.2 DMSO concentration assay

The same procedure was followed as in 4.2.5.1 to evaluate the effect of different concentrations of DMSO on the growth of *L. monocytogenes*. An aliquot of 50 µL of the *L. monocytogenes* culture was pipetted into all the wells of 96 micro-well plate and 50 µL of different concentrations of DMSO (0, 0.125, 0.25, 1.25, 2.5 %) were added to each well (a separate row was used for each concentration) (Figure 4.2). Three replicated plates were used and all the plates were incubated for 24 h at 30 °C. Optical density was measured regularly during the incubation time.

4.2.5.3 L. monocytogenes *assay*

Each extract residue was dissolved in a measured volume of pure DMSO and TSB depending on their weight, to achieve twice the desired initial concentration (1 mg) of the crude extract and to make sure final concentration of DMSO in the wells was less than 2%. Fifty microliters (50 µL) of TSB was added to the wells of first four rows (A-D) of the 96 micro-well plate (Figure 4.2) except the first well of each row. One hundred microliters (100 µL) of broccoli extract was put in the first well of each row and then two-fold serial dilution was done up to 6 times (6 different concentrations of the extract). Then 50 µL of *L. monocytogenes* culture (1×10^6 CFU/mL) was added to each well except for the first row (solvent control). Micro well plates were incubated at 30 °C for 20 h and OD was measured at every 2 h using a plate reader (2^{nd} OD reading

was taken after 8 h of the first reading: initial experiments showed a very slow OD change during first 8 h). Each 96-micro well plate had three different treatments (Figure 4.2) from a single solvent extract and the experiment was replicated on three different plates. Three replicated samples were used for each treatment at different extraction times (6 h and 24 h after treatment). Change in optical density of each well was calculated by subtracting corresponding initial OD from the final OD at each time point.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Solvent control (5.2 kJm^{-2} treatment)												
B	5.2 kJm^{-2} treatment, i.e.; Aqueous												Rep1
C	2.6 kJm^{-2} treatment, i.e.:Aqueous												Rep1
D	Untreated, i.e.; Aqueous extract												Rep1
E													
F													
G													
H													

Figure 4.2 Diagram showing the arrangement of aqueous extracts from treatments in a 96-microwell plate. Row A: solvent control (5.2 kJm^{-2} treatment), row B: 5.2 kJm^{-2} treatment, aqueous extract, row C: 2.6 kJm^{-2} treatment, aqueous extract, row C: untreated, aqueous extract

4.2.5.4 Experimental schedule

Replicates of different solvent extracts of UV-C treated and untreated broccoli obtained at different times after UV-C treatment were assayed against *L. monocytogenes* in sequential manner (table 4.1) in order to avoid any effect of extract storage time on experiment results. A total of three months elapsed between the first and the last analysis.

4.2.6 Statistical analysis

A complete randomized design was used in this study. Analysis of variance (ANOVA) was performed to analyse the effect of treatments using General Linear Model of Minitab Version 16 (Minitab Inc., State College, PA, USA). Means were separated by Tukey's test at significance level of 0.05. HSD values for repeated measures were calculated at each point of measurement.

Table 4.1 Experiment schedule of extracting UV-C treated and untreated broccoli depending on replicate, time after treatment and solvent

Replicate	Extraction time after treatment	Fraction	Order
1	24 h	Aqueous	1
1	24 h	Butanol	2
1	24 h	Hexane	3
1	24 h	Methanol	4
2	24 h	Aqueous	5
2	24 h	Butanol	6
2	24 h	Hexane	7
2	24 h	Methanol	8
1	6 h	Aqueous	9
1	6 h	Butanol	10
1	6 h	Hexane	11
1	6 h	Methanol	12
2	6 h	Aqueous	13
2	6 h	Butanol	14
2	6 h	Hexane	15
2	6 h	Methanol	16
3	24 h	Aqueous	17
3	24 h	Butanol	18
3	24 h	Hexane	19
3	24 h	Methanol	20
3	6 h	Aqueous	21
3	6 h	Butanol	22
3	6 h	Hexane	23
3	6 h	Methanol	24

4.3 Results and Discussion

4.3.1 Weight of crude extracts from different solvents

The weight of crude extracts measured after evaporation under reduced pressure was different depending on the solvent used for extraction (Table 4.2). A higher proportion was found in the aqueous fraction compared to other extracts. The weights of butanol and hexane crude extracts were almost similar while 10% methanol crude extracts were the lowest. However, no effect was observed on the weight of crude extracts due to UV-C treatment and time of extraction.

Table 4.2 Weight (mg) of different solvent-crude extracts according to time of extraction

Time after treatment	Aqueous (mg)	n-butanol (mg)	10% methanol (mg)	n-hexane (mg)
6 h	325.85 ± 6.02	158.75 ± 6.80	19.22 ± 2.92	145.15 ± 9.82
24 h	304.0 ± 22.3	159.5 ± 11.0	18.53 ± 2.08	158.8 ± 13.2

4.3.2 Establishing *L. monocytogenes* growth curve

Understanding the growth behaviour of *L. monocytogenes* in advance is important to understand the behaviour of the bacteria during the assay. It also helps to make sure that the bacteria are viable and growing well under experimental conditions. Therefore the growth of *L. monocytogenes* in Tryptic Soy Broth was studied (TSB) and established the growth curve (Figure 4.3). A clear lag phase (0 - 12 h), an exponential phase (12 - 20 h), and a stagnant growth phase (after 20 h) could be observed in the growth curve. Similar growth pattern for *L. monocytogenes* has been reported in other studies also (Francois et al., 2006; Mytilinaios et al., 2012). In these preliminary studies a slow change in OD was observed during the lag phase of the growth curve. Therefore, it was decided to measure OD after 8 h of the initial reading in broccoli extract assays.

The final crude extracts were a gummy smear attached to the round bottom flask after evaporation under low pressure in the rotary evaporator. In order to dissolve all the crude extract, more volume of DMSO was needed. In this study the concentration of DMSO was 1.25% in the final mix. In general, DMSO concentration used to dissolve plant materials is usually about 1% (Talib & Mahasneh, 2010). Since the concentration of DMSO was higher than normally used, the effect of different concentrations of DMSO against *L. monocytogenes* was tested. Results showed that there was no significant inhibitory effect up to 2.5% DMSO application and the same growth pattern was observed as in cultures with 0% DMSO (Figure 4.4). This confirmed that at this concentration DMSO did not adversely affect the growth of *L. monocytogenes* and followed the normal sigmoidal shape observed in the previous experiment (Figure 4.3) during 24 h incubation period.

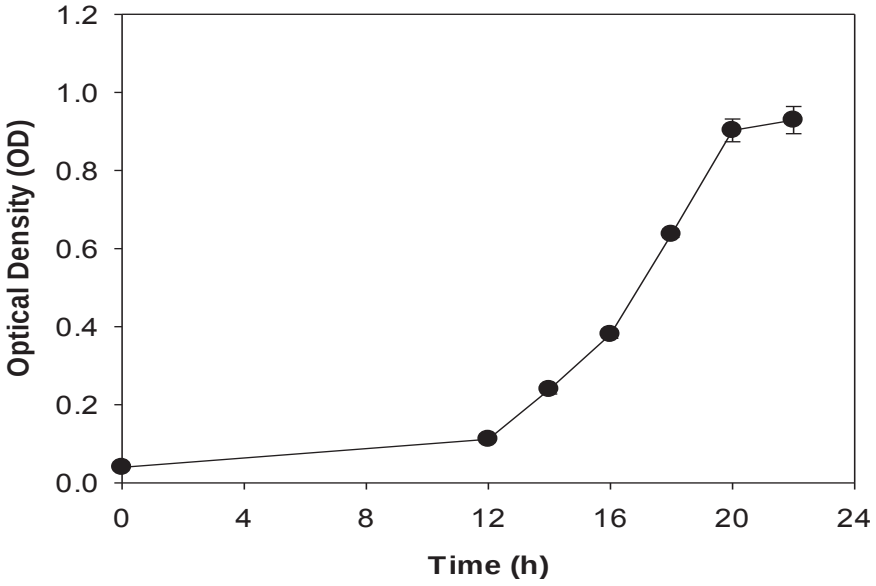

Figure 4.3 Growth curve of *L. monocytogenes* at 30 °C. Optical density (OD) of the 96-micro well plates was measured at 0, 12, and then every 2 h. Each data point is an average of three biological replicates and error bars represent standard error of means.

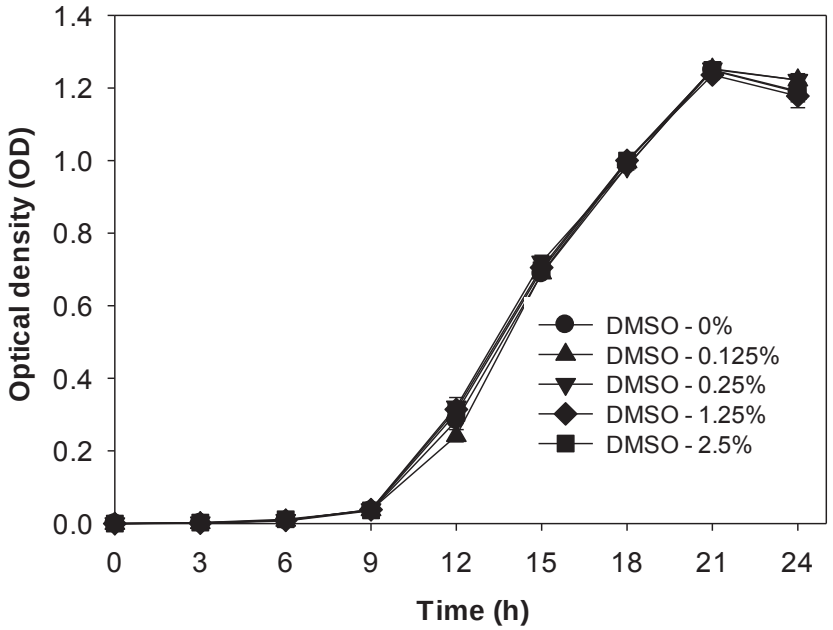

Figure 4.4 Effect of different concentrations of DMSO on growth of *L. monocytogenes* at 30 °C. Optical density (OD) was measured every 3 h up to 24 h. Final DMSO concentrations in a range from 0 to 2.5% were used. Each data point is an average of three biological replicates and error bars represent standard error of means.

4.3.3 Effect of UV-C treated broccoli extract in controlling the growth of *L. monocytogenes*

A single two-fold dilution (second concentration) of the four solvent extracts was selected for comparison throughout the experiment. The first concentration (1 mg extract per well) showed a very high inconsistency in growth of *L. monocytogenes*. However, a two-fold dilution (0.5 mg extract per well) gave consistent *L. monocytogenes* growth. There was no significant difference between different replicates at this second dilution (data not shown). The antimicrobial effect in the third concentration and beyond became weaker as the extracts became more diluted. Therefore the treatment effect was not statistically significant ($P < 0.05$) in the third and fourth dilutions. OD measurements were taken immediately and 8 h after applying broccoli extracts into *L. monocytogenes* culture, and continued at 2 h intervals thereafter. *L. monocytogenes* population increased during this time in all treatments and started to decline after 18 h. So, OD data at 18 h were selected for statistical data analysis throughout the experiment.

Estimated length of time to complete the whole experiment was approximately 3 months. Therefore the experiment was conducted in a way that different replicates and post UV-C treatment extraction times (6 h and 24 h) were chosen in a logical manner in order to account for time delays between replicates. The experimental schedule is given in table 4.1. All treatments (control, 2.6 and 5.2 kJm^{-2}) of a particular solvent extract were assayed at the same time, as shown in Table 4.1 and Figure 4.2.

However, towards the end of the experiment an unusual reduction in growth of working culture of *L. monocytogenes* was detected. Average optical densities of later assays (i.e. the 3rd replicate of 24 h and 6 h extracts) were approximately 40 % and 20% of earlier ODs, respectively. In order to identify and find remedial measures for issues encountered with the culture, a pure *L. monocytogenes* assay was carried out (Figure 4.5). This assay showed that pure *L. monocytogenes* culture also suffered from the same problem and grew to approximately half the population found at the start of the experiment series (see Figure 4.3). Therefore a number of corrective measures were employed, for example, changing the growth medium and increasing the concentration of growth medium, in order to understand the possible causes for growth retardation of *L. monocytogenes,* but it was not possible to determine the exact reason for the failure. It was suspected that it may be due to a fault in the plate count reader at the time. There were higher standard errors in the third replicate because of this reduced growth of *L.*

monocytogenes. However statistical analysis excluding the 3rd replicate made no difference to treatment effects with regards to all extracts at 14, 16, or 18 h time points because of the fact that one replicate of all treatments was assayed each time a batch of experiments was run. Therefore all three replicates were taken into account in the data analysis.

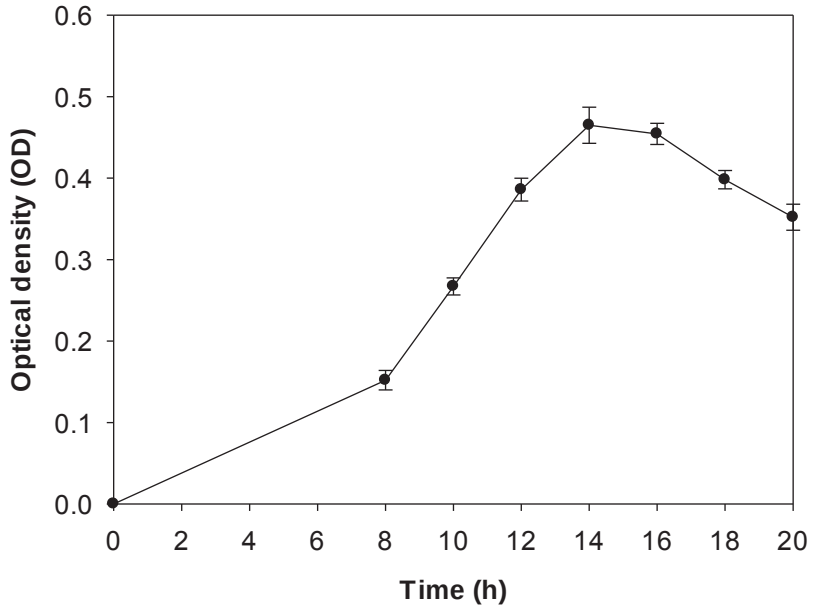

Figure 4.5 Growth curve of *L. monocytogenes* during third rep. of broccoli extract assays. Optical density (OD) of the 96-micro well plates was measured at 0, 8, and then every 2 h. Each data point is an average of three biological replicates and error bars represent standard error of means.

4.3.3.1 Aqueous extract

The aqueous extract of broccoli treated with UV-C at a dose of 2.6 or 5.2 kJm^{-2} and extracted 24 h after treatment (24 h aqueous extract) showed a significant ($P < 0.05$) antimicrobial activity compared to the control (Table 4.3). Optical density of *L. monocytogenes* culture incorporated with 24 h aqueous extracts of broccoli irradiated at a dose of 2.6 and 5.2 kJm^{-2} increased up to 0.5 and 0.4 respectively at 18 h, whereas the OD increased up to 0.65 after 18 h in 24 h aqueous extract of untreated broccoli (Figure 4.6). Unlike this inhibitory effect of aqueous extracts prepared 24 h after irradiation, aqueous extracts prepared 6 h after irradiation had no inhibitory effect on growth of *L. monocytogenes*.

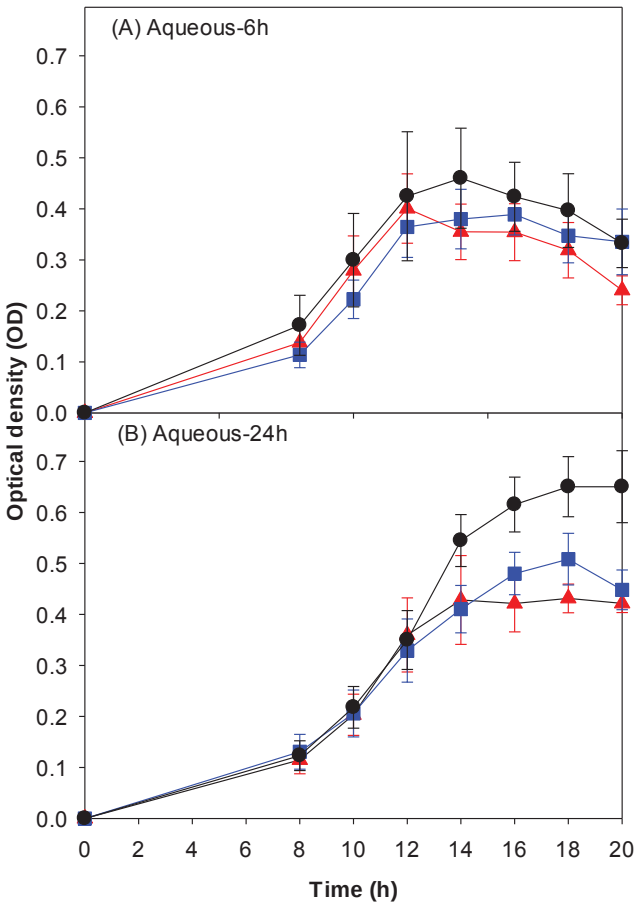

Figure 4.6 Growth suppression of *L. monocytogenes* by aqueous extracts of UV-C treated broccoli at a dose of ● = 0, ■ = 2.6 and ▲ = 5.2 kJm^{-2} extracted (A) 6 h after and (B) 24 h after UV-C treatment. Each data point represents a mean of three samples analysed as repeated measures. Error bars represent the standard error (n = 3).

4.3.3.2 Butanol extract

As with the aqueous extracts, the butanol fraction of the broccoli also appeared to suppress growth of *L. monocytogenes* (Figure 4.7), only when irradiated at high dose (5.2 kJm^{-2}) of UV-C and extracted 24 h after treatment (24 h butanol extract) but this effect was not statistically significant at $P = 0.05$. This extract restrained the bacteria population at 0.3 OD after 16 h while other treatments failed to inhibit bacterial growth and an increase up to 0.45 OD was observed at 18 h. Again, as for aqueous extracts, butanol extracts obtained 6 h after UV-C treatment (6 h butanol extract) did not show any significant antimicrobial activity against *L. monocytogenes.* Optical density of the

L. monocytogenes cultures incorporated with 6 h butanol extract increased up to 0.35 OD at 16 - 18 h, irrespective of the UV-C treatment.

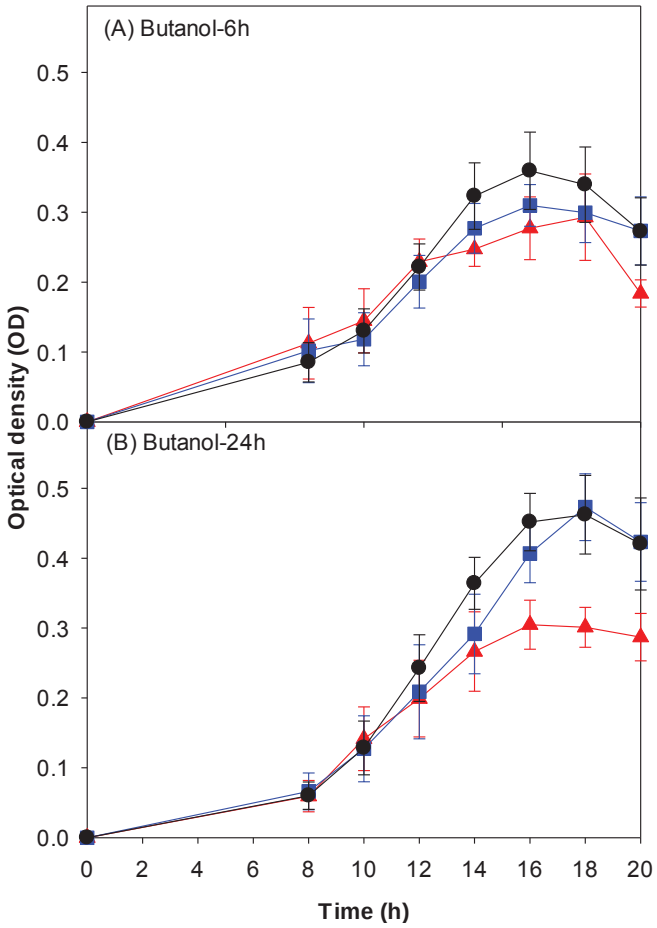

Figure 4.7 Growth suppression of *L. monocytogenes* by butanol extract of UV-C treated broccoli at a dose of ● = 0, ■ = 2.6 and ▲ = 5.2 kJm^{-2} extracted (A) 6 h after and (B) 24 h after UV-C treatment. Each data point represents a mean of three samples analysed as repeated measures. Error bars represent the standard error (n = 3).

4.3.3.3 Methanol extract

The methanol fraction of the UV-C treated broccoli also showed no antimicrobial activity against *L. monocytogenes* during the assay. Irrespective of the treatment and time of extraction after the treatment, optical density increased in all samples up to 0.4 OD and then dropped after 18 h (Figure 4.8). All the chlorophylls and waxes have been extracted into the chloroform fraction and thereafter into the hexane fraction leaving

very little material in the methanol fraction. This was confirmed by the weight of these extracts where hexane extract was about 3 fold higher than the methanol extract.

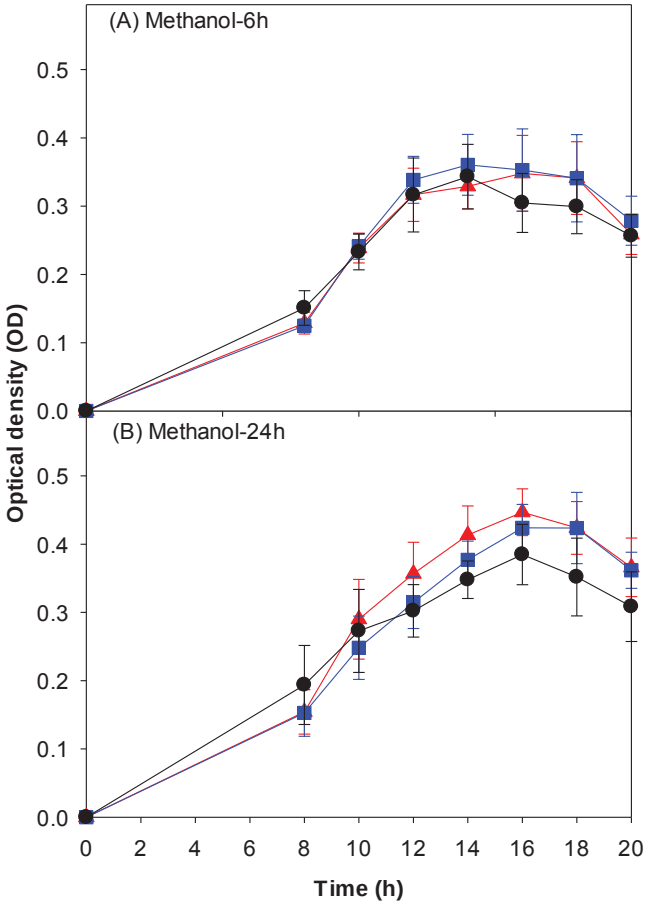

Figure 4.8 Growth suppression *of L. monocytogenes* by 10% methanol extract of UV-C treated broccoli at a dose of ● = 0, ■ = 2.6 and ▲ = 5.2 kJm^{-2} extracted (A) 6 h after and (B) 24 h after UV-C treatment. Each data point represents a mean of three samples analysed as repeated measures. Error bars represent the standard error (n = 3).

4.3.3.4 Hexane extract

Hexane extracts were waxy and dark green, presumably because of extraction of chlorophyll and waxy substances in the plant tissues by this non-polar solvent. Optical density of the cultures incorporated with untreated broccoli extracts increased up to 0.4 after 16 h in both 6 h and 24 h extracts (Figure 4.9). However optical density decreased in UV-C treated extracts after 12 h and remained below 0.3. Statistical analysis revealed that there was no significant difference between treatments at 6 h or 24 h extracts, but the trend shows both 6 and 24 h extracts were a little suppressive.

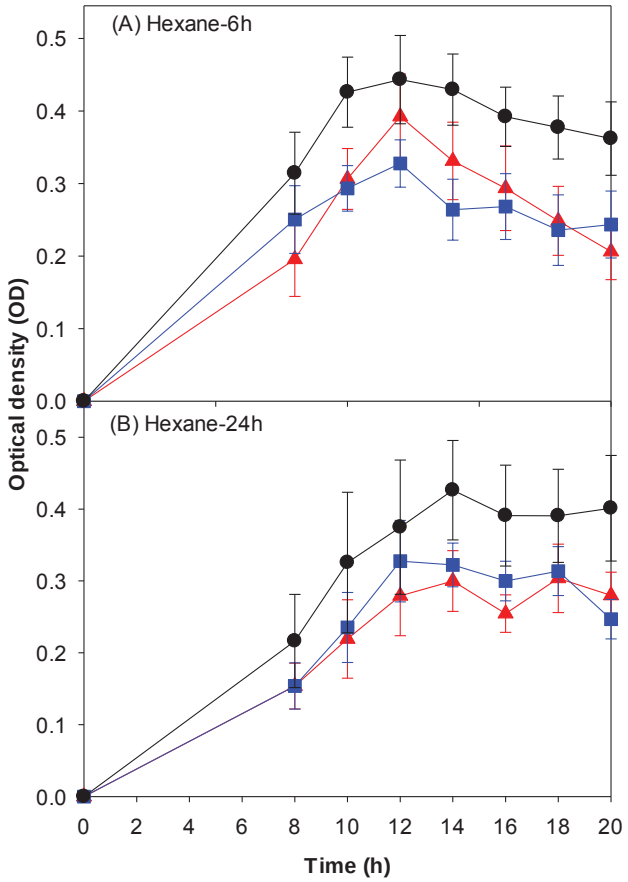

Figure 4.9 Growth suppression of *L. monocytogenes* by hexane extract of UV-C treated broccoli at a dose of ● = 0, ■ = 2.6 and ▲ = 5.2 kJm^{-2} extracted (A) 6 h after and (B) 24 h after UV-C treatment. Each data point represents a mean of three samples analysed as repeated measures. Error bars represent the standard error (n = 3).

Table 4.3 Effect of UV-C treated broccoli extracts on growth of *L. monocytogenes*

Treatment	Aqueous		n-butanol		10% methanol		n-hexane	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
0 kJm^{-2}	0.34 ± 0.10	0.65 ± 0.06^{A}	0.34 ± 0.10	0.46 ± 0.09	0.30 ± 0.08	0.35 ± 0.09	0.38 ± 0.06	0.39 ± 0.06
2.6 kJm^{-2}	0.35 ± 0.08	0.51 ± 0.05^{AB}	0.30 ± 0.08	0.47 ± 0.05	0.34 ± 0.08	0.42 ± 0.07	0.24 ± 0.03	0.31 ± 0.06
5.2 kJm^{-2}	0.32 ± 0.07	0.43 ± 0.02^{B}	0.30 ± 0.10	0.30 ± 0.05	0.34 ± 0.08	0.42 ± 0.01	0.25 ± 0.08	0.30 ± 0.05
p value	NS	$P < 0.05$	NS	NS	NS	NS	NS	NS

Each data point represents mean ± SE (n = 3). Means with different superscript letters were significantly different ($P < 0.05$) following analysis of variance and honest significant different (HSD) mean separation procedures. NS = Not significant

4.3.4 Suppressive activity of broccoli extracts

Antimicrobial activity of different extracts (ethanol, butanol, water, hexane, 10% methanol) of some medicinal plants against *E. coli, Salmonella typhimurium, Pseudomonas aeruginosa, Bacillus cereus and Candida albicans* has been reported. Butanol and aqueous extracts had the lowest minimum inhibition concentration (MIC) values against those pathogens, implying that antimicrobial compounds were preferentially extracted into high polarity solvents (Talib & Mahasneh, 2010). In this study also a similar antimicrobial activity against *L. monocytogenes* was observed in aqueous and butanol fractions of UV-C irradiated broccoli extracts. The hexane fraction of UV-C irradiated broccoli also suggested growth suppression of *L. monocytogenes* by both 6 h and 24 h extracts compared to the untreated control but the effect was not statistically significant (Figure 4.9). Results of this study suggest that the antimicrobial activity observed in these extracts may be due to presence of particular substances that have been produced as a result of UV-C treatment and need further investigation to identify the specific active compounds. Stronger antimicrobial activity demonstrated by the extracts obtained 24 h after UV-C treatment also suggests that any antimicrobial substance/s produced and accumulated within 24 h influence the growth and survival of *L. monocytogenes*. On the other hand, concentration of the substance/s accumulated over 6 h was not strong enough to control the growth of the pathogen.

Secondary metabolites present in Brassicaceae plants have been involved in herbivory, pathogen control and human nutrition and health (Bennett & Wallsgrove, 1994; Antonious et al., 2009; Rosa et al., 2010; Björkman et al., 2011). GLSs are some of the major secondary metabolites present in broccoli and are responsible for many of the biological properties including characteristic odour, taste and plant defence (Aires et al., 2009; Aires et al., 2011). There are three types of GLSs commonly found in Brassicaceae vegetables namely aromatic (derived from phenylalanine), aliphatic and alkanyl (derived from methionine), and indole/ indolyl (derived from tryptophan) and hydrolysis products of GLSs include mainly isothiocyanates, thiocyanates and nitriles (Jones et al., 2006; Fernandez-Leon et al., 2012). Most bioactive ITCs in broccoli are sulforaphane (derived from glucoraphanin), allyl isothiocyanate (derived from sinigrin), and indole-3-carbonyl (derived from glucobrassicin) (Jones et al., 2006). Aires et al. (2009) studied in detail the antimicrobial activity of three types of intact GLS; aliphatic (sinigrin and

glucoraphanin), aromatic (glucotropaeolin and gluconasturtiin) and indole (glucobrassicin) glucosinolates and their hydrolysis products on Gram positive and Gram negative bacteria. They found that intact GLSs were not inhibitory against any of the bacteria while some of the hydrolysed products were highly inhibitory. All isothiocyanates (ITC) (sulforaphane (SFN), benzyl isothiocyanate (BITC) and 2-phenylethyl-isothiocyanate (PEITC), except allyl isothiocyanate (AITC) showed high antimicrobial activity against Gram positive bacteria but only SFN and BITC were effective against Gram negative bacteria (Aires et al., 2009). Glucosinolate-derived indole hydrolysed products such as indole-3-acetonitrile (IAN) and indole-3-carbonyl (I3C) have also been tested against human pathogens. I3C showed high inhibitory effect against Gram positive bacteria; *Staph. aureus, Staph. epidermis, and Enterococcus faecium* but much less activity against Gram negative bacteria; *E. coli* and *P. aeruginosa* (Sung & Lee, 2007) whereas IAN was effective against Gram negative human pathogen/gastrointestinal bacteria (Aires et al., 2009). I3C is a naturally occurring hydrolysis product of GLSs found in Brassica vegetables at high concentrations (Sung & Lee, 2007). Lee et al. (2011) also studied the antimicrobial effect of five indole derivatives from cruciferous vegetables including IAN and found that IAN and Indole-3-carboxyaldehyde (I3CA) reduced biofilm formation of *E. coli* O157:H7 and *P. aeruginosa*. SEM analysis clearly showed that IAN inhibited curli production in *E. coli O157:H7* and thereby reduced biofilm formation whereas in *P. aeruginosa* it reduced the production of polymeric matrix (Lee et al., 2011).

Besides GLSs there are other types of important secondary metabolites in broccoli such as flavonols and carotenoids that are associated with defence mechanism (Jones et al., 2006). Lutein and β-carotene present in broccoli protect cellular membranes by scavenging free radicals (Fernandez-Leon et al., 2012) and may be included in the non-polar solvent fraction (hexane). Compounds such as quercetin and kaempferol which may be included in the more polar solvent fractions (aqueous and butanol) were highly correlated with antioxidant activity of broccoli (Lemoine et al., 2010a). However very little information is available on the type of substances that may be bacteriostatic against *L. monocytogenes*. Therefore, it is important to screen potential candidate compounds in broccoli that may be induced by postharvest treatments like UV-C irradiation in order to control human pathogen contamination of fresh produce. Since these broccoli extracts showed a suppressive effect against *L. monocytogenes* it is worthwhile to conduct appropriate phytochemical analyses to

identify potential antimicrobial compounds in broccoli that were induced by UV-C irradiation. There may be bioactive compounds (e.g. ITCs or their precursors, GLSs) present in the extracts as previously reported in the literature.

4.4 Conclusion

The aqueous fraction of fresh cut broccoli irradiated with UV-C at a dose of 2.6 or 5.2 kJm^{-2} and extracted 24 h after treatment was effective in suppressing the growth of *L. monocytogenes*. This observation suggests that dose and time after treatment are key determinants of the inhibition while type of solvent is also important in *in vitro* assays. Most of the antimicrobial substances in broccoli may have been extracted by polar solvents (aqueous and butanol) and they are more likely to be water soluble compounds. Antimicrobial effects of those extracts may be due to accumulation of antimicrobial substances in tissues over time as a result of UV-C treatment. Abiotic stress exerted by UV-C irradiation may alter production of substances that inhibit the growth of *L. monocytogenes*. GLSs and their hydrolysed products are the most potent candidates responsible for antimicrobial effect of broccoli extracts. Even though these substances have been studied extensively in relation to fungicidal, bactericidal, and anticarcenogenic properties, very little is known on their potential for control of human pathogens on Brassicaceae vegetables. Phytochemical analyses in determining volatile compounds, using GC-MS methods and LC-MS methods for identifying GLSs and other compounds would be important to determine the potential antimicrobial substances induced by UV-C irradiation in broccoli.

5 EFFECT OF UV-C IRRADIATION ON PHYTOCHEMICAL COMPOSITION OF FRESH-CUT BROCCOLI

5.1 Introduction

As discussed in chapter 4, extracts from UV-C treated broccoli showed a suppressive effect on growth of *L. monocytogenes.* This suppressive effect varied depending on UV-C dose and time after treatment. Although the exact mechanism behind that suppression is unknown, it can be suggested that UV-C irradiation has induced particular phytochemical/s that are harmful for growth of *L. monocytogenes*. This experiment aimed to identify candidate antimicrobial compounds in UV-C treated broccoli that may have a correlation with growth suppression of *L. monocytogenes*. Findings of many studies support the hypothesis that abiotic stresses such as UV-C irradiation promote production of secondary metabolites in plants which may be harmful for pathogens (Bennett & Wallsgrove, 1994; Kunz et al., 2006). Plant defence responses for hormetic doses of postharvest treatments may induce changes in volatile and non-volatile secondary metabolites as well as major plant nutrients (Mann et al., 2012). Phytochemical analyses and microbiological assays demonstrated the antimicrobial effect of some secondary metabolites of broccoli, particularly hydrolysis products of GLSs such as sulforaphane and IAN, in controlling human pathogens (Aires et al., 2009). In addition, some studies reported presence of volatile compounds such as dimethyldisulfide, dimethyltrisulfide and ITCs in cabbage extracts (another Brassicaceae vegetable) that may have antimicrobial activity against pathogens (Valette et al., 2006). Any volatile or non-volatile compound that is produced inside the tissues as a response to UV-C irradiation would have to diffuse to or evaporate from the surface of broccoli in order to suppress growth of *L. monocytogenes*.

Broccoli crude extract assays (chapter 4) revealed that different solvent extracts showed varying degrees of suppression against *L. monocytogenes*. For example, aqueous extracts obtained 24 h after UV-C treatment were suppressive. Chloroform-hexane extracts also showed a little effect (but was not significant), only after 6 h whereas chloroform-methanol was not effective at all. It was decided to use 80% ethanol as a mixed polarity solvent for extraction of both polar and non-polar phytochemicals of broccoli to be used in the chemical analyses. For profile analysis Liquid

Chromatography (LC) is a commonly used technique due to its simplicity and wide availability. LC coupled with different types of detectors has been used for characterization of these compounds. Mass spectrometry (MS) detectors are needed to confirm the presence of specific compounds when standards are not available. In order to identify volatile and non-volatile compounds that may be altered by UV-C irradiation supplementation of LC information with GC is required (Ares et al., 2013).

This chapter focuses on profiling phytochemicals of UV-C treated and untreated fresh cut broccoli extracts obtained 6 h and 24 h after treatment in order to identify treatment-dependent effects on phytochemical composition. Standard compounds were not used in phytochemical profiling so that LC-HRMS and GC-MS analyses together with published literature were used for identification of compounds.

5.2 Materials and Method

5.2.1 Sample preparation

5.2.1.1 Plant material

The same broccoli samples used in chapter 4 experiment were used for chapter 5 experiments. Broccoli branchlets treated with UV-C at a dose of 0, 2.6 or 5.2 kJm^{-2} and frozen immediately after UV-C treatment, 6 h and 24 h after treatment were used for phytochemical analyses using GC-MS and LC-HRMS.

5.2.1.2 GC-MS analysis

Volatile compounds from control and UV-C treated (at a dose of 2.6 or 5.2 kJm^{-2}) broccoli branchlets were extracted using a modified method of Matich et al. (2012). Shaved florets of broccoli branchlets, frozen in liquid nitrogen were ground using a mortar and pestle and extracted with 10 mL of preheated 80% ethanol to one gram of plant material (Matich et al., 2012). The mixture was held in a water bath for 5 minutes at 80° C (to inactivate myrosinase) and kept overnight at 5° C. The supernatant was filtered through a vacuum filter (0.45 µm filter paper) and extracts were kept at -20° C until analysis. Three replicates were used for each treatment.

5.2.1.3 LC-HRMS analysis

The same procedure was followed as in 5.2.1.1 to extract phytochemicals from broccoli for LC-HRMS analysis, but only 5 mL of preheated 80% ethanol was added instead of 10 mL.

5.2.2 Analysis of phytochemical compounds in UV-C treated broccoli.

5.2.2.1 GC-MS analysis

Volatile compounds were analysed using a gas chromatograph coupled with a mass spectrometer (Shimadzu QP-2010STechnologies, Japan). Two microlitre split (5:1) injections were made at 220 °C onto a 30 m × 0.25 mm × 0.25 ZB5 capillary column with a He flow of 0.9 ml min^{-1}. Oven temperature program was: 1 min at 35 °C, 3 °C min^{-1} to 100 °C, 7 °C min^{-1} to 240 °C, hold for 10 min. MS parameters included ion source temperature 200 °C, interface temperature 220 °C and solvent cut time 4 min. Volatile compounds were identified by comparing their mass spectra and retention time with the reference spectra in mass-spectra library (NIST05 LIB) and also with those reported in the literature.

5.2.2.2 LC-HRMS analysis

Broccoli phytochemical compounds were analysed using liquid chromatography high-resolution mass spectrometry (LC-HRMS), with a Dionex Ultimate 3000 Rapid Separation LC and a micrOTOF QII mass spectrometer (Bruker Daltonics, Bremen, Gremany) fitted with an electrospray ion source. Extracts were diluted (1:1 v/v) with naringenin 7-neolesperidoside solution (2 mg/mL) before the analysis. An UHPLC column; C 48, 150 mm ×2.0 mm × 1.8 µm (YMC Co. Ltd, Japan) was used to separate the compounds. Solvents were A = 0.2% formic acid and B = 100% acetonitrile. The gradient was as follows: 90% A, 10% B, 0-0.5 min, linear gradient to 65% A, 35% B, 0.5-15 min; linear gradient to 50% A, 50% B, 15-18 min; linear gradient to 100% B, 18-22 min; composition held at 100% B, 22-27min; linear gradient to 90% A, 10% B, 27-27.2 min, flush under initial conditions until 30 min. The injection volume was 1 µL.

5.2.2.3 LC-HRMS data analysis

MS data analysis software package consisting of different programs (Bruker Daltonics, Bremen, Germany) was used to analyse phytochemical data. All phytochemical

compounds present in all samples were extracted from raw MS data files by the 'Find Molecular Features' program. Then all the molecular features detected in all samples were combined into a bucket table using the 'Profile Analysis' program. All these combined data were then subjected to principal component analysis (PCA) to visualize separation and clustering of compounds depending on different treatments. To confirm possible treatment effects identified in the PCA, *t*- tests were done and results were presented as volcano plots where fold change and *P* values were given for each compound. Compounds having fold change higher than 2 and $P < 0.05$ were selected and accurate masses and retention times were used to obtain peak areas from exact ion chromatogram (EIC) using 'Quant Analysis' program of the Data Analysis Software.

5.2.2.4 Tentative compound identification

Previously selected compounds having fold change greater than 2 and $P < 0.05$ were subjected to identification procedure where the most likely elemental composition was determined using accurate masses and suggestive molecular formulae were generated. Depending on the mSigma and error Da values, the most likely formula for the mass was selected. Maximum mSigma level and error Da levels were set at 30 and 4 respectively (Chong et al., 2013). Finally, nomenclature of identified compounds used Chemspider database (www.Chemspider.com) and referring to the existing literature.

5.2.3 Statistical analysis

A complete randomized design was used in this study. For phytochemical compound analysis, peak area of the compounds obtained from GC-MS were analysed using PCA followed by analysis of variance (ANOVA) to analyse the effect of treatments using General Linear Model of Minitab Version 16 (Minitab Inc., State College, PA, USA). Means were separated by Tukey's test at significance level of 0.05. Data obtained from LC-HRMS were analysed by 'Profile Analysis' program (Bruker Daltonics, Bremen, Germany).

5.3 Results and Discussion

5.3.1 GC-MS analysis

About 40 peaks were generated by GC-MS, and 33 compounds were selected and used in the statistical analyses after removing noise in the data set (Table 5.1). Firstly,

principal component analysis (PCA) was done to find out whether different treatments separated depending on the area of the peaks. PCA of the phytochemicals showed a distinctive separation of fresh broccoli (untreated and extracted immediately after UV-C treatment) extract and extracts of UV-C treated broccoli obtained 24 h after UV-C treatment (Figure 5.1). Also a clear separation was observed between extracts of UV-C treated broccoli obtained 24 h after treatment (red ellipse) and untreated broccoli extracts obtained 24 h after UV treatment (green ellipse). However, they were clustered closely, indicating that the difference between UV-C (5.2 kJm^{-2}) treated and untreated broccoli 24 h after treatment may not be highly significant. Re-analysis using only UV-C treated (5.2 kJm^{-2}) and untreated broccoli data showed a clear separation (Figure 5.2) and allowed examination of the loading plot which revealed that the observed separation in the PCA was due to increase or decrease in abundance of a large number of compounds (Figure 5.3). The separation observed between fresh broccoli extracts and all extracts obtained after 24 h irrespective of the treatment was due to chemical composition changes accompanying natural senescence.

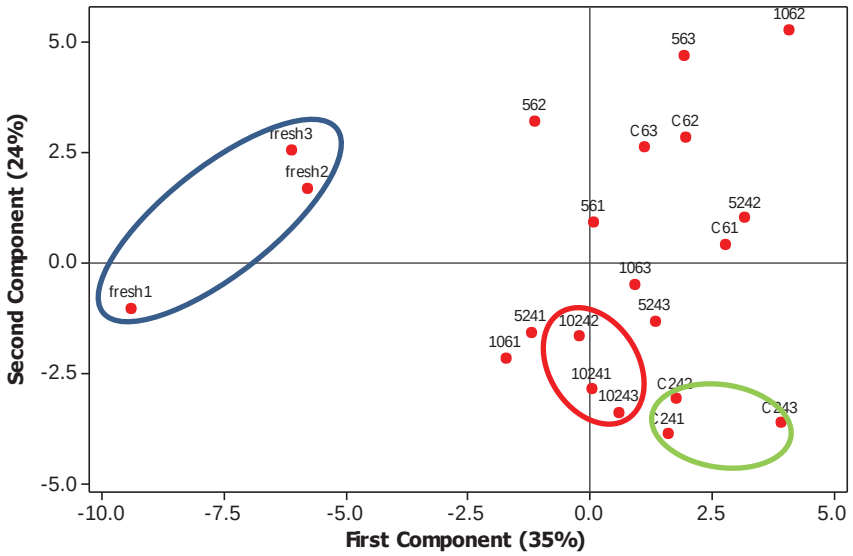

Figure 5.1 Principal component analysis (PCA) of 33 compounds found in the GC-MS analysis of extracts (80% ethanol) of UV-C treated (at 2.6 and 5.2 kJm^{-2}) and untreated broccoli obtained 0, 6 and 24 h after treatment. Blue ellipse = untreated broccoli extracted immediately after treatment, red ellipse = UV-C treated (5.2 kJm^{-2}) broccoli extracted 24 h after treatment and green ellipse = untreated broccoli extracted 24 h after treatment.

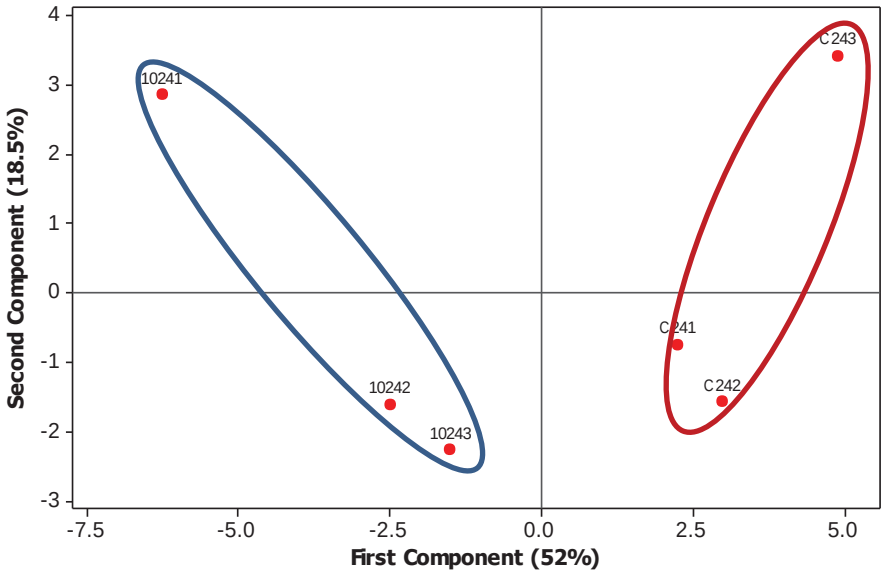

Figure 5.2 Principal component analysis (PCA) of 33 compounds found in the GC-MS analysis of extracts (80% ethanol) of broccoli treated with (5.2 kJm^{-2}) and untreated broccoli extracts obtained 24 h after treatment. UV-C treated (5.2 kJm^{-2}) broccoli clustered (in blue ellipse) separately from untreated broccoli (in red ellipse) along the first component axis.

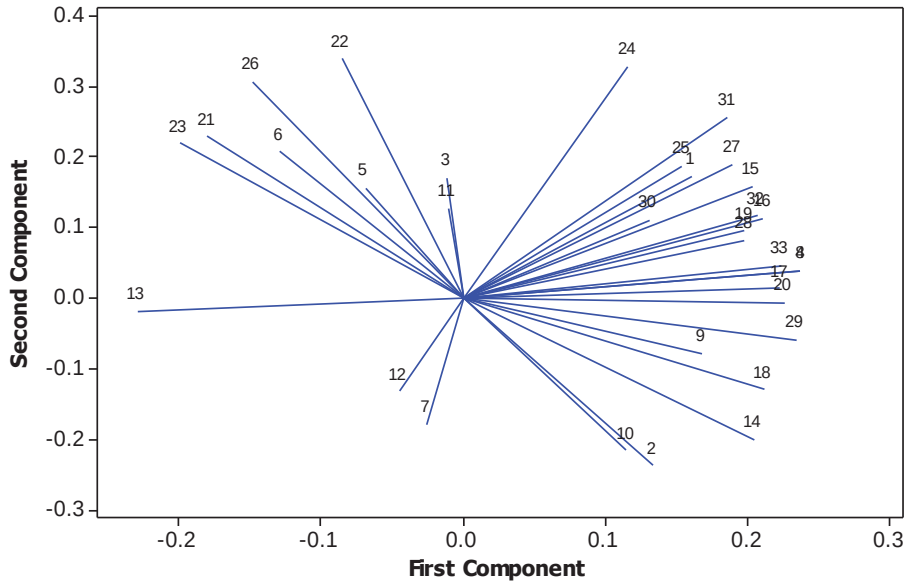

Figure 5.3 Loading plot of the 33 compounds found in the extracts (80% ethanol) of UV-C treated (5.2 kJm^{-2}) and untreated broccoli obtained 24 h after treatment.

The observed suppressive effect of UV-C treated fresh cut broccoli (*in vivo*) in chapter 3 and broccoli extracts (*in vitro*) in chapter 4 may be attributed to influence of UV-C irradiation on production of certain antimicrobial phytochemicals in broccoli. Four compounds showed greater than two fold change (> 2) in UV-C treated broccoli 24 h after irradiation compared to untreated broccoli (Table 5.2). Two were highly increased (21 & 23) and two were decreased (15 & 32) by UV-C treatment. In reference to growth suppression of *L. monocytogenes*, it can be suggested that compound 15 and 32 may have supported while compound 21 and 23 may be unfavourable for growth and survival of *L. monocytogenes* in the UV-C treated and untreated fresh cut broccoli respectively. However, the average peak area given by GC-MS of these four compounds (Figure 5.4), showed that abundance of compound 32 was not significantly different 24 h after treatment compared to non UV-C treated broccoli or any other treatment. This suggests that compound 32 may not have a key role in suppressing *L. monocytogenes* on fresh cut broccoli. On the other hand, abundance of compound 15 was significantly ($P < 0.05$) higher at two time points, 6 h and 24 h after UV-C treatment in untreated broccoli compared to fresh and treated broccoli. This difference suggests that UV-C treatment has suppressed a senescence related increase in compound 15.

Compound 23 and 21 slightly increased in abundance after UV-C treatment. This suggests a possible role of these two compounds in suppressing *L. monocytogenes* on fresh cut broccoli inoculated 24 h after UV-C treatment even though the changes here appear not significant. These results suggest that observed suppression of *L. monocytogenes* could be a combined effect of more than one compound, for example combined effect of compound 21 and 23. Results of the PCA analysis of phytochemical compounds of UV-C treated and untreated broccoli 24 h after treatment also support this hypothesis where a group of compounds including compound 21 and 23 were clustered into the UV-C treated side (Figure 5.3).

Other than the compounds that were changed by two fold or greater in UV-C treated broccoli, compounds that were changed by less than two fold could still be important if the concentration goes over minimum inhibitory concentration (MIC) value. But such small changes are hard to demonstrate with low number of replicates and adding more replicates in future studies would be advantageous.

Due to senescence of broccoli after harvest phytochemical composition changes during storage (Wills et al., 2007). UV-C irradiation altered the natural increment or decrement of phytochemicals occurring due to natural senescence. For example, compounds 17,

18, 26, 27, 28, and 33 increased overtime in both treated and untreated samples while UV-C reduced the increment of compound 17 and 18 in treated broccoli compared to untreated broccoli (Table 5.3). In contrast, UV-C treatment increased degradation of compound 1 and 14 over time compared to untreated broccoli. These compounds may be associated with the observed higher growth of *L. monocytogenes* in untreated fresh cut broccoli. However, irrespective of the treatment an increase was observed in compounds 27, 28, and 30 while compound 4 and 8 were degraded completely 24 h after treatment suggesting a senescence related change in these phytochemicals. Compound 13 also decreased over time and was not observed in untreated broccoli after 24 h but UV-C irradiation reduced the decrease in treated broccoli.

MS identification of compounds resulted in more than 80% match between library data and tentatively identified compounds in broccoli samples as listed in the Table 5.1. Present literature provides a wide range of compounds found in broccoli. Many of the studies have focused on identifying and quantifying common phenolics, flavonoids, and glucosinolates in broccoli depending on various factors including environment, cultivar, cultivation practices, processing, postharvest treatments, and packaging (Jia et al., 2009; Van Eylen et al., 2009; Aires et al., 2012; Fernandez-Leon et al., 2012; Matich et al., 2012), but the current research was focused on studying potential phytochemicals that may be induced or suppressed by UV-C treatment and influence directly or indirectly subsequent growth and survival of *L. monocytogenes*. Dimethyltrisulfide is a volatile compound found in Brassicaceae vegetables (de Pinho et al., 2009) which is known for its antimicrobial properties. Most of the studies of volatiles in broccoli have reported both dimethyltrisulfide and dimethyldisulfide (Forney et al., 1991; Derbali et al., 1998) which were observed in some of the samples in this experiment and in the preliminary experiment (data not shown). Dimethyltrisulfide content increased in all samples over 24 h while a 15% increase was observed in UV-C treated samples compared to untreated samples.

Table 5.1 List of compounds identified by GC-MS analysis in UV-C treated and untreated fresh cut broccoli

Comp #	Retention Time	Tentative compound identification	Similarity %	Mol wt	Formula
1	6.19	Butanoic acid ethyl ester	90	116	$C_6H_{12}O_2$
2	6.49	Pyruvic acid methyl ester	94	102	$C_4H_6O_3$
3	7.08	4-methyl pyrimidine	89	94	$C_5H_6N_2$
4	11.56	1,2-Cyclopentanone	82	98	$C_5H_6O_2$
5	12.95	Dimethyl trisulfide	96	126	$C_2H_6S_3$
6	13.85	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	91	144	$C_6H_8O_4$
7	19.62	2,10-dimethyl undecane	88	184	$C_{13}H_{28}$
8	22.11	Dihydroxy -6-methyl-2,3-dihydro-4H-pyran-4-one	89	144	$C_6H_8O_4$
9	24.41	3,7-Dimethyldecane	94	176	$C_{12}H_{26}$
10	27.75	4,4-Dimethylundecane	90	184	$C_{13}H_{28}$
11	28.31	2-Methoxy-4-vinylphenol	96	150	$C_9H_{10}O_2$
12	30.31	3,4,5,6-tetramethyloctane	87	170	$C_{12}H_{26}$
13	31.63	Unknown	*	*	*
14	31.90	Unknown	*	*	*
15	33.36	Sulforaphane nitrile	83	145	$C_6H_{11}NOS$
16	33.96	Unknown	*	*	*
17	34.41	Eicosane	93	282	$C_{20}H_{42}$

Table 5.1 continued.

Comp #	Retention Time	Tentative compound identification	Similarity %	Mol wt	Formula
18	35.41	Unknown	*	*	*
19	35.69	Unknown	*	*	*
20	37.11	9-Octadecanone	80	268	$C_{18}H_{36}O$
21	37.41	2-Amino-3-hydroxypyridine	83	110	$C_5H_6N_2O$
22	37.83	3,7-dimethyldecane	83	170	$C_{12}H_{26}$
22	37.829	3,7-dimethyldecane	83	170	$C_{12}H_{26}$
23	38.187	Unknown	< 80	114	$C_6H_{10}O_2$
24	38.507	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	89	296	$C_{20}H_{40}O$
25	38.886	Pentadecanal-	82	226	$C_{15}H_{30}O$
26	40.127	Linolenic acid	87	278	$C_{18}H_{30}O_2$
27	40.388	Palmitic acid	95	256	$C_{16}H_{32}O_2$
28	40.83	Palmitic acid ethyl ester	93	284	$C_{18}H_{36}O_2$
29	41.44	Eicosanoic acid	80	312	$C_{20}H_{40}O_2$
30	42.39	11,14,17-Ecosatrienoic acid ester	88	320	$C_{21}H_{36}O_2$
31	42.58	Phytol	95	296	$C_{20}H_{40}O$
32	43.24	Cis cis cis, 7,10,13-hexadecatrienal	89	234	$C_{16\ h26}O$
33	45.20	N1-Isopropyl-2-methyl-1,2-propane	88	130	$C_7H_{18}N_2$

* similarity is less than 80%

Table 5.2 Compounds identified by GC-MS with largest changes in peak area between UV-C treated (5.2 kJm^{-2}) and untreated, at 24 h after treatment

Comp #	Retention Time	Compound name	fold change (5.2 kJm^{-2} -24h /untreated-24 h)
32	43.24	Cis cis cis, 7,10,13-hexadecatrienal	0.23
15	33.36	Sulforaphane nitrile	0.52
23	38.19	Unknown	2.54
21	37.41	2-Amino-3-hydroxypyridine	2.76

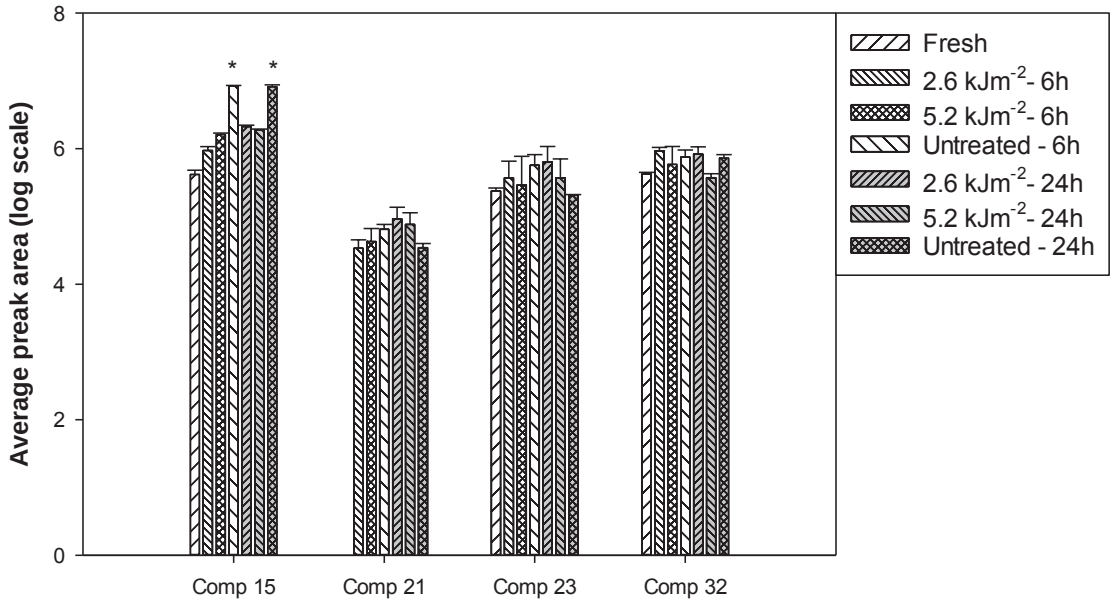

Figure 5.4 Average peak area (log scale) of compounds identified by GC-MS analysis that were modified by UV-C irradiation (as shown in table 5.2). Bars with asterisks are significantly different at $P < 0.05$ from other bars for that compound. Tentative identification for compound 15 is sulforaphane nitrile, 21 is 2-amino-3-hydroxypyridine, and 32 is cis, cis, cis, 7,10,13-hexadecatrienal. Compound 23 is unknown

Table 5.3 Compounds identified by GC-MS with largest changes in peak area between broccoli treated with UV-C at 5.2 kJm^{-2} at 24 h, untreated at 24 h and untreated at 0 h.

Comp #	Retention Time	Compound name	fold change (5.2 kJm^{-2}-24 h/ untreated-0h)	fold change (untreated-24 h/ untreated-0h)
1	6.19	Butanoic acid	0.44	>0.5
4	11.56	Cyclopentanone	*	0.00
8	22.11	Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	*	0.00
13	31.63	Unknown	0.26	0.00^
14	31.90	Unknown	0.45	>0.5
17	34.41	Eicosane	4.19	6.42
18	35.41	Unknown	4.74	7.44
26	40.13	Linolenic acid	2.96	2.69
27	40.39	Palmitic acid	2.08	2.33
28	40.83	Palmitic acid ethyl ester	2.05	2.34
33	45.20	N1-isopropyl-2-methyl-1,2-propane	< 2	2.04

*Not found in UV-C treated and untreated broccoli 24 h after treatment. ^Not found in untreated broccoli extracted 24 h after treatment

Sulforaphane nitrile content has also increased in both treated and untreated broccoli over 24 h. Untreated broccoli extracts contained nearly 48% higher sulforaphane nitrile content than that of UV-C treated samples after 24 h. Sulforaphane nitrile is a less bioactive compound than sulforaphane (4-(methylsulphinyl) butyl isothiocyanate) (Matusheski & Jeffery, 2001), which is a hydrolysis product of glucoraphanin (Van Eylen et al., 2009). In general, more than 80% of the glucoraphanin hydrolysis products found in broccoli is sulforaphane nitrile (Jeffery et al., 2003). Conversion of ITCs (sulforaphane) and nitriles as a result of glucoraphanin hydrolysis may also depend on environmental factors such as pH and plant cultivar. Nitriles are produced under low pH while ITCs are formed in high pH media (Bell & Wagstaff, 2014). UV-C irradiation reduced formation of sulforaphane nitrile. However, it was not clear from the results of the current study whether UV-C induced formation of sulforaphane instead in treated broccoli.

Many of the peaks generated by GC-MS with higher retention times represented long carbon chain fatty acids such as linolenic acid, palmitic acid and eicosanoic acid, and fatty acid esters such as palmitic acid ethyl ester and eicosatrienoic acid ester which are known to be components of leaf surface waxes (Baker, 1974). Constitutive compounds of the epicuticular wax layer including hydrocarbons, esters, and fatty acids were increased in UV-B treated 'waxless' line of pea seedlings (*Pisum sativum* L.) (Gonzalez et al., 1996) while no significant change in total waxes was reported for UV-B treated *Brassica napus* leaves (Ni et al., 2014). Interestingly, Ni et al.(2014) observed that UV-B treatment increased the amount of most wax constituents including ketones, aldehydes and esters in the first leaf of the *Brassica napus* seedlings in addition to a structural modification of epicuticular wax of the leaf surface.

5.3.2 LC-HRMS analysis

LC-HRMS analysis of extracts obtained from fresh broccoli at time zero and UV-C treated and untreated broccoli 6 h and 24 h after treatment identified more than 1200 molecular features across all samples that were tabulated into one 'bucket table'. Principal component analysis (PCA) of those compounds showed clustering depending on treatments confirming presence of treatment differences. Therefore, further analysis was done using *t*-tests to generate volcano plots and to calculate fold change and corresponding *P* values. Compounds with a fold change greater than 2 and $P < 0.05$ were selected for further investigation. In this phytochemical analysis, only untreated and fresh cut broccoli irradiated at 5.2 kJm^{-2} were compared. Comparing UV-C treated at 5.2 kJm^{-2} and untreated broccoli extracted 24 h after treatment using *t*-test, revealed that nine compounds have a fold change greater than 2 and $P < 0.05$ and these are illustrated in the volcano plot (Figure 5.5a). After removing noise in the data set only eight compounds were selected that showed an increase in extracts treated at 5.2 kJm^{-2} and extracted 24 h after treatment. Comparison between UV-C treated at 5.2 kJm^{-2} and extracts of untreated broccoli obtained 6 h after treatment showed only 2 compounds which met the cut-off criteria (Figure 5.5b) suggesting that synthesis and accumulation of compounds depend on the time after treatment. When UV-C treated broccoli samples extracted 6 h and 24 h after treatment were compared, there were 9 compounds that have fold change greater than 2 time and $P < 0.05$ (Figure 5.5c). Of these, only one compound decreased in abundance between 6 h and 24 h. Comparison between untreated broccoli extracted at 6 h and 24 h revealed that the abundance of 12

compounds changed significantly ($P < 0.05$) over time, three of which were reduced in 24 h extracts while an increase greater than 2 fold was observed at $P < 0.05$ in others (Figure 5.5d). Compounds that changed significantly overtime in untreated 24 h extracts represent a senescence related change in phytochemicals of fresh cut broccoli.

The main objective of this experiment was to identify the impact of UV-C irradiation on phytochemical compounds in fresh cut broccoli during 24 h that may be responsible for suppression of post inoculated *L. monocytogenes*. Interestingly, there were eight compounds that increased in abundance with UV-C treatment 24 h after treatment compared to untreated broccoli (Figure 5.5a). Two compounds out of the eight also increased in untreated 24 h extracts which suggests a senescence related increase. Therefore, only six compounds were assumed to be induced by UV-C irradiation during the 24 h (Table 5.4). The observed UV-C dependent change in phytochemical compounds in broccoli may be attributed to increased production of defence related phytochemicals as the mild stress produced by UV-C irradiation induces expression of defence related genes. For example, Charles and others (2009) have observed activation of gluconohydrolase enzymes which catalyse synthesis of PR proteins in tomatoes by UV-C irradiation and concluded that induction of the activity of defence hydrolases contribute to the long term resistance induced by UV-C in tomato fruit.

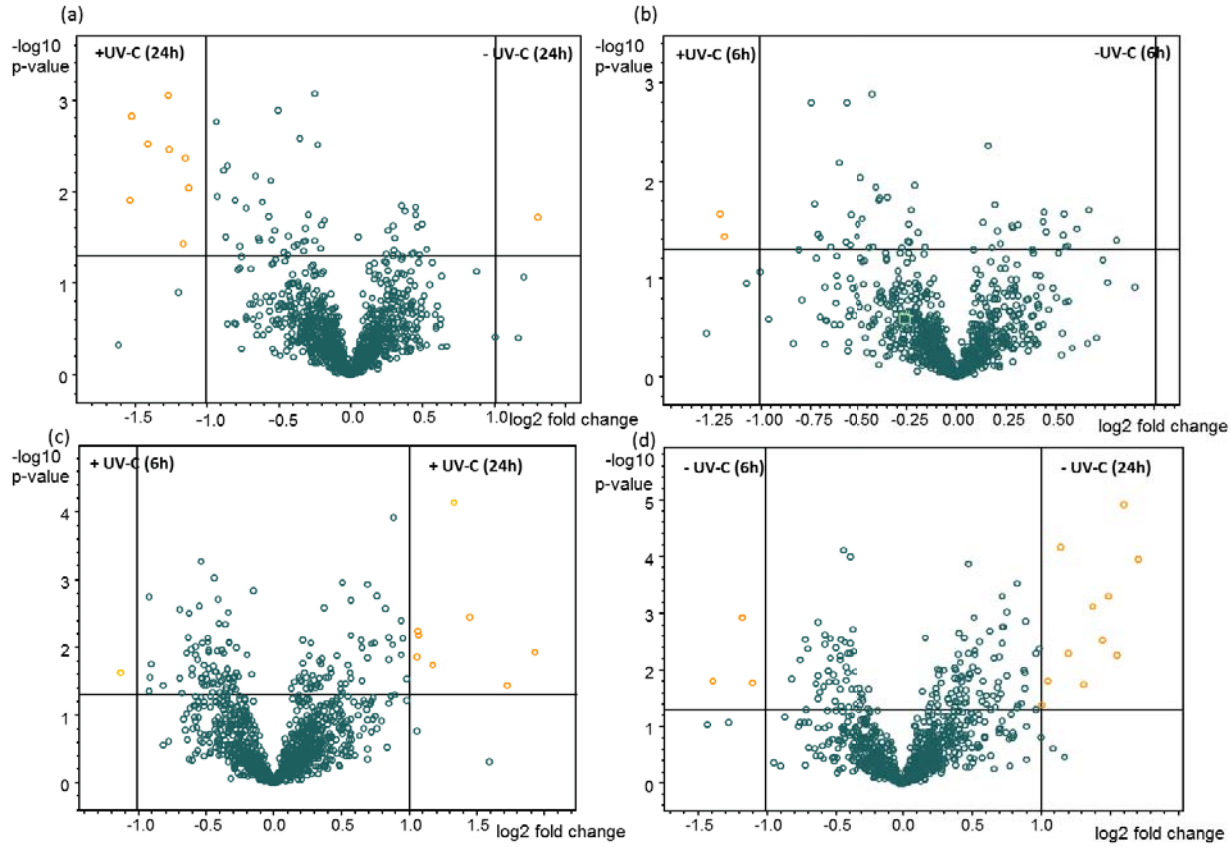

Figure 5.5 Treatment dependent differences of compounds determined by *t*-test between two treatments (plotting fold change versus *P* value) and illustrated by volcano plots; (a) untreated (24 h)/treated (24 h), (b) untreated (6 h)/treated (6 h), (c) treated (24 h)/treated (6 h) and (d) untreated (24 h)/untreated (6 h). Yellow circles represent compounds having > two fold change in respective samples at *P* < 0.05

It was a challenging task to identify the six compounds regulated by UV-C irradiation accurately (table 5.4). 'Quant Analysis' using isotope mass resulted in a few possible formulae matches which had mSigma values less than 30 and low error mDa values (< 4). However, it was not possible to identify the compounds with previous reports from Cruciferae studies by searching the Chemspider database. Very little research has focused on metabolite profiling after physical postharvest treatments, compared to the large number of studies on GLSs and phenolics. Only one study has been done recently using UV-B irradiation to treat lettuce plants (Wargent et al., 2014). They found 10 compounds regulated by UV-B irradiation and were able to name some of them. Wargent et al. (2014) reported one compound regulated by UV-B having a mass of 337.092 and RT of 7.01 min and identified it as *p*-coumaryl quinic acid ($C_{16}H_{18}O_8$). A compound having very similar mass of 337.089 but a different retention time (RT =

14.06), was identified in the current study and was increased by UV-C irradiation. If compound 5 is indeed *p*-coumaryl quinic acid, it belongs to hydroxycinnamic acid group of phenolic acids (Teixeira et al., 2013). *p*-coumaryl quinic acid has been identified in kale and may be associated with lignin metabolism and pathogenesis in plants (Ferioli et al., 2013). Another compound having a mass of 161.976 (RT = 2.24 min) ($C_4H_5NO_2S_2$) was tentatively identified as raphanusamic acid in Chemspider (www.chemspider.com). Bednarek et al. (2005) made the first report of raphanusamic acid as a break down product of indole GLSs in *Arabidopsis thaliana*. Raphanusamic acid is a possible derivative of cysteine and readily available for production of camalexin, which is a phytoalexin produced upon pathogen infection (Bednarek et al., 2005). Chemspider (www.chemspider.com) search resulted in one matching search for the compound having m/z ratio of 299.0746; RT 3.39 min and a suggestive formula of $C_{13}H_{16}O_8$. This compound is tentatively identified as salicylic acid β-glucoside which is a storage form of salicylic acid: a signalling molecule that is found in plant cells and plays a key role in cellular communication (Kawano et al., 2004). Synthesis and accumulation of this signalling molecule in plant tissues triggered by a biotic or abiotic stress leads to systemic expression of PR genes in distal tissues (Fu & Dong, 2013). This phenomenon is important for UV-C irradiation to be effective in fresh produce like broccoli since there can be shaded places where UV-C is unable to reach. Unfortunately, it was not possible to find possible matches for the other three compounds that were induced by UV-C treatment. The compounds which have been tentatively identified belong to the group of antioxidants (phenolic acid), signalling molecules (SA), and phytoalexins (raphanusamic acid) which fit into the plant defence scenario at different levels. Therefore, these results are consistent with published literature which reported hormetic doses of UV-C may induce expression of defence related genes and as a result induce defence related pathways such as the phenylpropanoid (Lemoine et al., 2010a) and camalexin pathways (Bednarek et al., 2005).

Table 5.4 LC-HRMS identification of chemical compounds that were increased by UV-C irradiation at 5.2 kJm^{-2} and extracted after 24 h of treatment.

Comp #	RT (min)	Mass (m/z)	Tentative Identification	Formula	Measured mass (m/z)	mDa diff	m (Sigma)	Peak area		Fold change
								Untrt-24 h	5.2 kJm^{-2}-24 h	5.2 kJm^{-2}-24h/Untrt-24h
1	1.89	306.116	Unknown	$C_{16}H_{21}NO_3S$	306.1169	0.7	25.5	7478.6	18527	2.48
2	2.24	161.968	Raphanusamic acid	$C_4H_5NO_2S_2$	161.968	-0.9	18.4	3897.3	12075	3.10
3	3.39	299.077	Salicylic acid-β-glucoside	$C_{14}H_{12}N_4O_4$	299.0786	3.7	8.6	50696	112094	2.21
4	5.6	349.103	Unknown	$C_{20}H_{18}N_2O_2S$	349.1061	-0.3	18.5	112694	247475	2.2
5	14.06	337.089	*p*-coumaryl quinic acid	$C_{16}H_{18}O_8$	337.0929	3.9	26.8	3668.8	17263	4.70
6	14.14	531.15	Unknown	$C_{26}H_{28}O_{12}$	531.1508	-2.1	5.6	326237	702096	2.16

5.3.2.1 UV-C regulation of phytochemicals in 80% ethanol and aqueous extracts

In the *in vitro* assay of broccoli extracts obtained using different solvents, aqueous extracts were the most effective in suppressing growth of *L. monocytogenes*. Aqueous extract of fresh cut broccoli treated with 5.2 kJm^{-2} and extracted 24 h after treatment significantly reduced growth and survival of *L. monocytogenes* compared to 6 h extracts. These results imply that accumulation of certain water soluble phytochemical/s during 24 h period in broccoli tissues due to UV-C irradiation was harmful for growth of *L. monocytogenes*. Talib and others also observed similar results where aqueous extracts were more effective in killing human pathogens than hexane and 10% methanol extracts (Mahasneh & El-Oqlah, 1999; Talib & Mahasneh, 2010). LC-HRMS analysis of ethanol extracts (80%) of UV-C treated broccoli (5.2 kJm^{-2}) revealed that six compounds were increased after 24 h that may have an effect on growth suppression of *L. monocytogenes*. The fate of these identified compounds was studied in the aqueous extracts of UV-C treated and untreated broccoli (prepared at 4.2.4) using the same method as of 5.2.2.2 and 5.2.2.3. Five of the compounds were identified in the aqueous extracts also whereas compound 2 was not detected. Being a mixed polarity solvent, ethanol may have extracted most of these compounds before fractionation whereas only water soluble compounds may be included in the aqueous extracts after fractionation (Figure 5.6). Only the compound three was increased significantly by UV-C irradiation in aqueous extracts and was found in similar abundance in ethanol extracts. It was tentatively identified as salicylic acid-β-glucoside and may be a potential candidate for the growth suppression of *L. monocytogenes* due to its role in induced defence responses and as an antimicrobial compound itself. Leaf extracts of *Delonix regia* were effective in controlling *E. coli* and chemical analysis revealed they contained high concentration of phenolic acids and flavonoids including salicylic acid. Salicylic acid was the third highest phenolic acid found in those leaves (Shabir et al., 2011). Presence of salicylic acid-β-glucoside in both ethanol and aqueous extracts at significantly higher amounts in UV-C treated samples 24 h after treatment compared to extracts of untreated broccoli confirms its role in growth suppression of *L. monocytogenes* in *in vitro* assays. But its role in *in vivo* assays could not be confirmed yet as mode of transportation to the surface is unclear. However, salicylic acid might be converted into more volatile form of methyl salicylate (MeSA)

(Durrant & Dong, 2004) that may be evaporated from the surface as reported in many herbivore studies (Mann et al., 2012; Niinemets et al., 2013).

5.3.2.2 Glucosinolates in UV-C treated fresh cut broccoli

GLSs are the most characteristic compounds in Brassicas that are responsible for a variety of biological processes including plant defence (Redovnikovic et al., 2008). Many studies focused on improving GLS content in Brassicaceae vegetables due to their health promoting and bioactive properties (Jones et al., 2006). Therefore, GLS content in UV-C treated and untreated broccoli 24 h after treatment was examined. Ten major glucosinolates in broccoli were identified by LC-HRMS analysis (Figure 5.7). Abundance of GLSs has not been affected by either treatment or time of extraction. Neoglucobrassicin was the most abundant GLS found in these broccoli samples followed by glucoiberin, glucobrassicin, and gluconusturtiin. In contrast, Sierra et al. (2014) reported that neoglucobrassin was the highest GLS found in broccoli after UV-C irradiation at a dose of 3.0 kJm^{-2} and stored at 4 °C (Sierra et al., 2014). In general, glucoraphanin is the predominant GLS reported in broccoli accounting for more than 50% of the total GLSs while sinigrin is found in low levels (Jeffery et al., 2003). Low levels of sinigrin observed in this current study compared to other GLSs agree with previous reports. However, abundance of glucoraphanin was also comparatively less than that of other GLSs. This low abundance may be due to conversion of glucoraphanin by UV-C irradiation into its hydrolysis form of sulforaphane or sulforaphane nitrile. GC-MS analysis of broccoli metabolites showed an approximately 2 fold decrease of sulforaphane nitrile in UV-C treated broccoli 24 h after treatment. These results suggest that UV-C irradiation may have decreased accumulation of sulforaphane nitrile in broccoli tissues by preventing conversion of sulforaphane into sulforaphane nitrile which is a less bioactive form (Tierens et al., 2001). Tierens et al. (2001) confirmed the antimicrobial effect of sulforaphane against *E. coli* and reported IC_{50} value of *E.coli* inhibition as 282 μM where they recovered 65 μg of sulforaphane from one gram of fresh *Arabidopsis* leaf extract. In addition to those GLSs, 4-methoxyglucobrassicin, glucobrassicicanapin, gluconapin, progoitrin were found in all broccoli samples which are the most common GLSs found in Brassicaceae vegetables (Moreno et al., 2006; Aires et al., 2009; Picchi et al., 2012; Bell & Wagstaff, 2014; Bell et al., 2015).

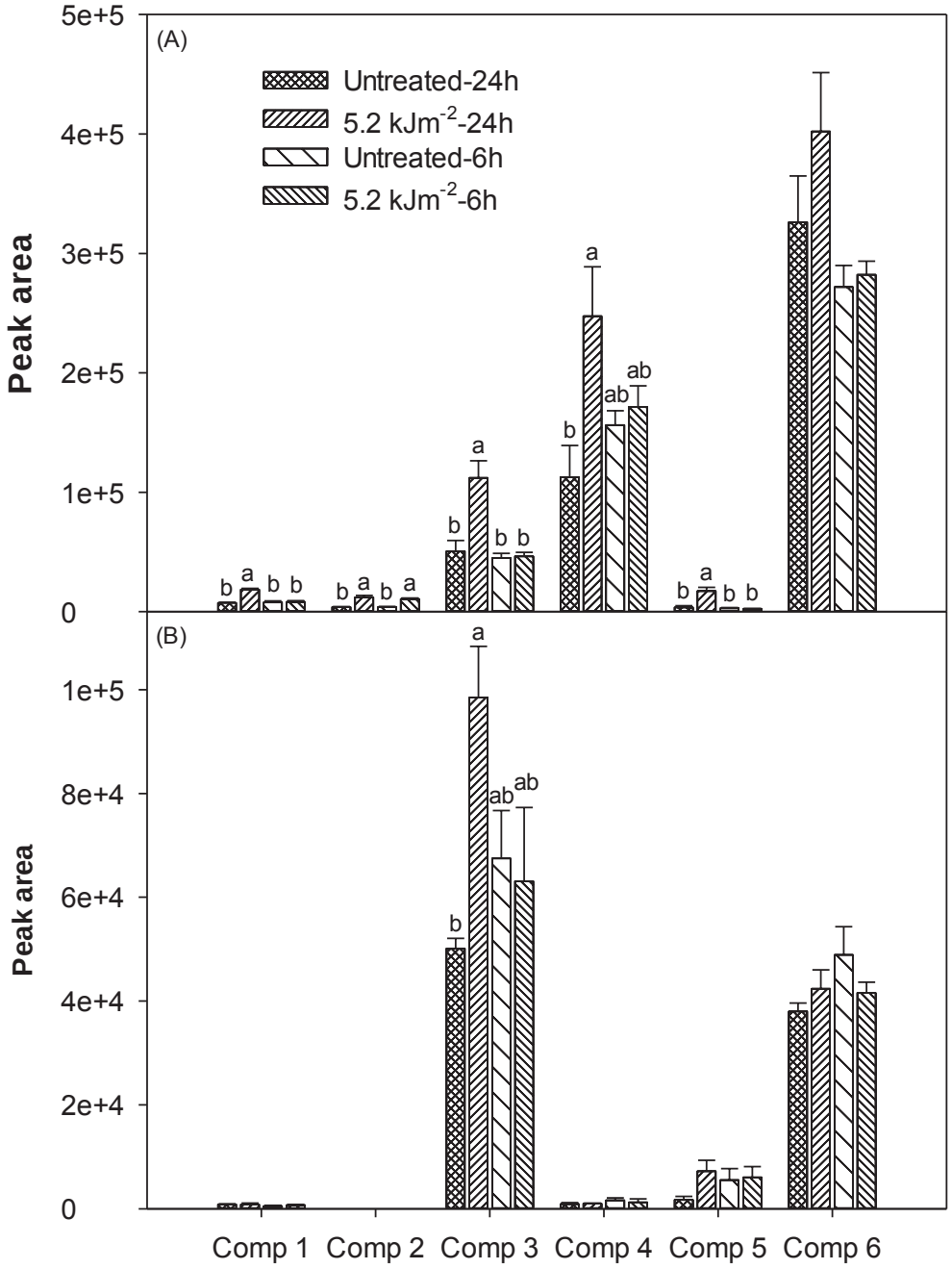

Figure 5.6 Chemical compounds increased by UV-C irradiation at 5.2 kJm^{-2} and extracted 6 h and 24 h after treatment, (A) solvent 80% ethanol and (B) aqueous extract. Data represent means of five replicates (n = 5 ± SE). Different lower case letters denote significant differences (*P* < 0.001 in A and *P* = < 0.05 in B) among treatments for same compound.

However, very few studies focused on evaluating the effectiveness of UV-C irradiation as a postharvest treatment in improving GLS content in broccoli. In one of the studies Nadeau et al. (2012) observed an increase in of 4-methoxyglucobrassicin,

4-hydroxyglucobrassicin and glucoraphanin in broccoli by UV-C irradiation at a dose of 1.2 kJm^{-2} while glucobrassicin content was depleted during storage at 4 °C for 14 days. According to Sierra et al. (2014) only neoglucobrassicin was increased as a response to UV-C irradiation. In the current study none of the GLSs were induced by UV-C irradiation (Figure 5.7) Therefore, the effectiveness of UV-C irradiation in inducing GLSs in broccoli should be studied further in future studies.

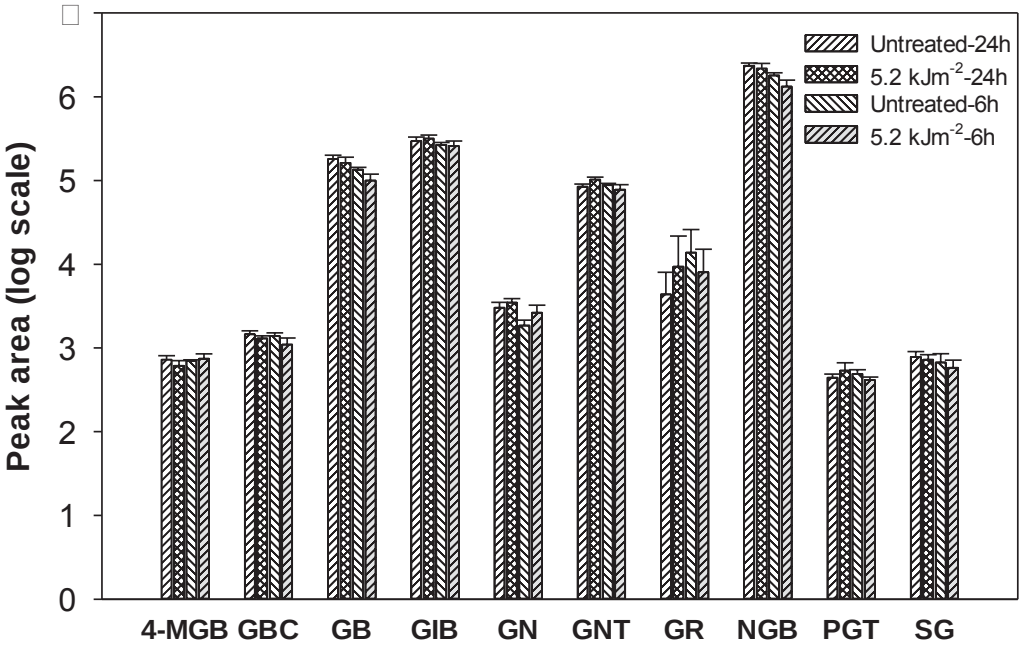

Figure 5.7 Glucosinolates identified by LC-HRMS in UV-C treated (5.2 kJm^{-2}) and untreated broccoli extracts obtained 6 h and 24 h after treatment. Error bars represent standard error (n = 5). 4-MGB = 4-methylglucobrassicin, GBC = glucobrassicanapin, GB = glucobrassicin, GIB = glucoiberin, GN = gluconapin, GNT = gluconasturtiin, GR = glucoraphanin, NGB = neoglucobrassicin, PGT = progoitrin, SG = sinigrin. Data represent means of five replicates (n = 5 ± SE).

5.4 Conclusion

LC-HRMS analysis of broccoli extracts obtained 24 h after treatment revealed that the concentration of six phytochemical compounds was increased by UV-C irradiation at a dose of 5.2 kJm^{-2} compared to untreated broccoli. Even though it was not possible to assign formulae and accurate nomenclature for all of the compounds perfectly, compound 2, 3, and 5 were tentatively identified as raphanusamic acid, salicylic-β-glucoside, and *p*-coumaryl quinic acid and all three compounds are

potentially involved in plant defence mechanism against plant pathogenesis. Being increased by abiotic stress like hormetic doses of UV-C irradiation indicate that these compounds can be induced as a defence response and may be responsible for growth suppression of *L. monocytogenes* upon accumulation during 24 h after treatment to a harmful level. However, the expected influence of UV-C irradiation on changing GLSs content in treated broccoli were not observed in the study and missing data of volatile compounds cannot be ruled out: a head space gas analysis of UV-C treated fresh cut broccoli would be needed to identify volatile compounds.

6 OVERALL DISCUSSION

6.1 Introduction

The overall goal of this research was to determine the effectiveness of prior UV-C irradiation as a postharvest treatment in controlling growth and survival of *L. monocytogenes* on the surface of fresh cut broccoli. In order to achieve this goal: (1) UV-C irradiation at different doses was evaluated for its impact on fresh cut broccoli quality, (2) UV-C irradiation was evaluated for its ability to control growth and survival of *L. monocytogenes* inoculated onto fresh cut broccoli surface after treatment, (3) The suppressive effect of extracts of broccoli treated with different doses of UV-C against *L. monocytogenes* was studied *in vitro*, and (4) phytochemical compounds of UV-C treated and untreated broccoli were analysed using GC-MS and LC-HRMS to identify potential treatment dependent changes in phytochemical composition that may be associated with the growth suppression of *L. monocytogenes*.

6.2 Summary of results

UV-C irradiation at a dose of 2.6 and 5.2 kJm^{-2} did not adversely affect the visual quality of fresh cut broccoli stored at 15 °C compared to untreated samples, confirming that UV-C irradiation can potentially be used as a postharvest treatment for fresh cut broccoli without compromising visual quality. Growth suppression of *L. monocytogenes* on fresh cut broccoli was achieved using these hormetic doses of UV-C irradiation. UV-C treatment at a dose of 5.2 kJm^{-2} suppressed the growth of *L. monocytogenes* particularly when inoculation took place 24 h after treatment. This suppression was evident in the *in vitro* assay of aqueous extracts of broccoli treated with UV-C at doses of 2.6 and 5.2 kJm^{-2} and extracted 24 h after treatment. UV-C dependent changes in phytochemicals were also observed in the phytochemical analysis by GC-MS and LC-HRMS. Six compounds that have been significantly increased by UV-C were identified in the LC-HRMS analysis. Some of those compounds were tentatively identified as defence related compounds in the plant defence system that are induced or synthesised during biotic or abiotic stress conditions in plant tissues.

6.2.1 UV-C irradiation and human pathogen control

Pathogen contamination is a major concern for the fresh produce industry, which has to deal with an increasing number of foodborne illness outbreaks related to fresh produce every year. The main objective of this study was to investigate the effect of UV-C irradiation on controlling growth and survival of the human pathogen, *L. monocytogenes* inoculated onto fresh cut broccoli after treatment. Three inoculation regimes (immediately after UV-C treatment, 6 h, and 24 h after treatment) were used to mimic the situations where postharvest contamination of fresh produce can occur. Subsequent growth and survival of the pathogen was observed during eight days of storage at 15 °C. This temperature was used to study growth behaviour of the pathogen where temperature abuse occurs during storage. Fresh broccoli should normally be stored at 0 - 4 °C to retain quality for the longest period, but during transportation or retail storage, temperature abuse can occur allowing favourable conditions for the pathogen to grow. Therefore, employing an effective postharvest technology that controls these foodborne pathogens upon contamination of fresh produce after harvest is important. Many postharvest treatments including UV-C irradiation have been studied for their use as a surface disinfectant to remove microorganisms and to improve other physiological quality parameters whereas this research focused on controlling *L. monocytogenes* inoculated onto the surface of broccoli after UV-C treatment. UV-C irradiation employed in this research successfully suppressed growth of *L. monocytogenes* at a dose of 2.6 and 5.2 kJm^{-2} when inoculation occurred 24 h after UV-C treatment and 5.2 kJm^{-2} was effective when *L. monocytogenes* was inoculated 6 h after treatment. Efficacy of the UV-C treatment depended on the irradiation dose and the time of inoculation of the pathogen after treatment, implying that post-irradiation changes were time and UV-C dose dependent and may have contributed to the creation of a surface of broccoli unfavourable for pathogen growth.

When extracts of broccoli irradiated at the above doses (2.6 and 5.2 kJm^{-2}) and extracted at different times (0, 6, and 24 h) after treatment were assayed against *L. monocytogenes*, the data showed that aqueous extracts of broccoli treated with UV-C at 2.6 and 5.2 kJm^{-2} and obtained 24 h after treatment were effective in controlling *L. monocytogenes* growth *in vitro* while butanol extract of broccoli irradiated at 5.2 kJm^{-2} and obtained 24 h after treatment also showed a trend in suppressing growth of *L. monocytogenes*. However extracts obtained immediately and 6 h after treatment

did not have significant suppressive effects. These results imply that particular compounds which tend to partition into polar solvents may be responsible for growth suppression of *L. monocytogenes*. Similarly, Mahasneh et al. (1999) reported a preferential extraction of antimicrobial compounds into more polar solvents such as water and butanol. Tierens et al. (2001) also reported that water extracts of *Arabidopsis thaliana* leaves showing the highest antimicrobial activity against *E. coli* compared to 70% methanol, ethanol and acetone. The possible mechanisms of growth suppression of *L. monocytogenes* by aqueous or butanol extracts were studied in this research by analysing phytochemical changes in treated broccoli during the 24 h after UV-C treatment.

6.2.2 Suggested inactivation mechanisms of post-inoculated *L. monocytogenes* by UV-C treatment

Freshly harvested broccoli can be treated with UV-C irradiation for a distinct time period as explained in this research (i.e. 5.2 kJm^{-2}), which improved safety, by controlling *L. monocytogenes* on fresh cut broccoli inoculated 6 h after treatment and onwards. Figure 6.1 illustrates a conceptual model of UV-C irradiation in inducing plant resistance by producing defence related phytochemical compounds and systemic acquired resistance in distant cells. Evidence presented here suggests that suppression of *L. monocytogenes* growth on the broccoli surface is potentially achieved by this same process. Application of UV-C irradiation around harvest time may deliver three beneficial outcomes: 1. sanitization of pre-existing contamination from the field, 2. increased production and/or modification of leaf surface waxes and, 3. initiation of systemic induced resistance in broccoli. The first beneficial effect is due to direct germicidal effect of UV-C radiation. This research has contributed evidence for the second beneficial effect: i.e. making the surface of broccoli less favourable for growth and survival of *L. monocytogenes* by inducing production of defence related phytochemicals that are harmful for *L. monocytogenes* or modifying surface morphology. Due to the direct germicidal effect of UV-C irradiation, any existing contamination of the broccoli surface can be reduced, resulting in sanitization. The indirect benefits of UV-C irradiation probably depend on ROS production by univalent reduction of oxygen molecules. The primary ROS produced in the tissue is $O_2^{\cdot -}$ followed by a series of reactions forming a variety of ROS, and

induction of antioxidant enzymes including SOD (Gonzalez-Aguilar et al., 2010). One of the main ROS synthesised during stress is hydrogen peroxide from $O_2^{\cdot-}$ due to SOD activity which may be responsible for activating gene expression of PRPs and HSPs (Gonzalez-Aguilar et al., 2010). On the other hand, signalling molecules such as SA and JA induced by UV-C stress are also believed to contribute to induction of defence related genes under stress conditions (Durrant & Dong, 2004). These different mechanisms are all involved in production of different defence related compounds in plants as a response to stress. A few such compounds were identified in this current study, and their role in defence has been discussed in chapter 5. Accumulation of antimicrobial compounds induced by UV-C irradiation on the surface of broccoli through diffusion and/or evaporation may affect growth of *L. monocytogenes.* For example, accumulation of compounds such as salicylic acid-β-glucoside, *p*-coumaryl quinic acid and raphanusamic acid was observed 24 h after UV-C irradiation; these may have diffused to the surface while volatile bioactive compounds such as dimethyl trisulfide may have evaporated. In addition, slightly higher levels of linolenic acid, a constitutive compound of leaf waxes, in UV-C treated broccoli 24 h after treatment may also indicate altered wax deposition, reducing both attachment, and subsequent growth of *L. monocytogenes.* Increased deposition of surface waxes may increase the hydrophobicity of the surface, reducing ease of attachment of *L. monocytogenes* onto the surface of broccoli. UV-C irradiation induces defence responses not only in the immediate contact cells, but at a distance as well, i.e. systematically. Many studies have confirmed the effectiveness of postharvest treatments including UV-C irradiation in inducing acquired systemic resistance responses in fresh produce (Terry & Joyce, 2004). The presence of salicylic acid-β-glucoside (stored form of SA) at a significantly higher level in UV-C irradiated broccoli suggests a possible induction of SAR. If this mechanism is activated in broccoli tissues as a response to UV-C irradiation, it would help to minimize concerns about treatment efficacy; inconsistent irradiation of the complex surface of a broccoli head may not matter, as long as some tissues receive adequate UV-C dose to induce systemic changes that propagate throughout tissues.

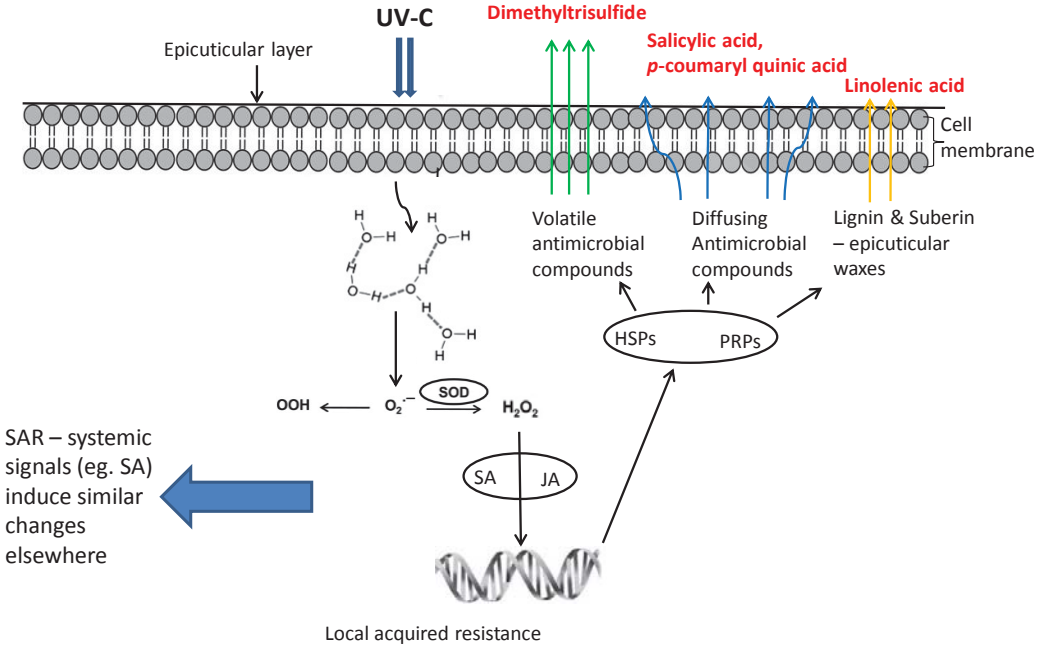

Figure 6.1 Conceptual model of prior UV-C treatment inducing defence responses in fresh cut broccoli. UV-C irradiation may modify epicuticular wax layer and suppress growth of *L. monocytogenes* by inducing production and accumulation of compounds (in red) as a defence response. OOH = peroxyl radical, $O_2^{\cdot-}$ = superoxide, SOD = superoxide dismutase, H_2O_2 = hydrogen peroxide, SA= salicylic acid, JA = jasmonic acid, HSPs = heat shock proteins, PRPs = pathogenesis related proteins.

6.2.3 Induced resistance of fresh cut broccoli by UV-C irradiation

The growth suppression of *L. monocytogenes* by prior UV-C irradiation observed in the current research may be attributed to mechanisms such as: 1. induced production of antimicrobial compounds, 2. modification of broccoli surface properties, and 3. up-regulating expression of genes that induce defence responses, as a response to the mild oxidative stress exerted by UV-C irradiation. In the current study, only the first mechanism was explored by analysing phytochemicals that may have changed due to application of UV-C irradiation using GC-MS and LC-HRMS. However, part of the second mechanism could also be explained by the altered production of linolenic acid, which is a constitutive compound of epicuticular waxes. However, the effect of UV-C irradiation on changing the composition and/or morphology of waxes was not

studied further in this research. If UV-C irradiation changes the composition and/or morphology of the waxes on surface of broccoli, those changes could affect attachment and growth of microorganisms in many ways. For example, epicuticular waxes play a key role in preventing fungal attacks on fruit by blocking cracks and natural openings (Fallik, 2004). Hot water rinsing and brushing of sweet bell peppers showed a rearrangement of natural waxes and covered natural cracks and stomata (Fallik et al., 1999). Similar surface wax modification was observed in leaves of *Brassica napus* seedlings where different forms of waxes such as plates with irregular edges, short and long rod crystals, fused together and edges of crystals became unclear after exposure to UV-B radiation (Ni et al., 2014). In addition, a newly formed layer of lignin-like compounds was observed on citrus peel after irradiation with UV-C (Ben-Yehoshua, 2003).

The role of the wax layer in suppressing growth of human pathogens is not clear. On one hand, microorganisms may not get adequate nutrients from the inside of the cell if the wax layer becomes thicker and covers natural openings. On the other hand, UV-C irradiation may increase the hydrophobicity of the surface of broccoli by increasing waxes and make the surface unfavourable for attachment and subsequent growth of human pathogens like *L. monocytogenes* that have cell membranes of hydrophilic nature. The attachment strength and mechanism of *L. monocytogenes* onto prior UV-C treated broccoli surface was not explored in the current study and could form the basis of a future study. Increasing the hydrophobicity of the surface of broccoli which may impair *L. monocytogenes* attachment raises the risk of promoting attachment of other types of pathogens that have hydrophobic cell membranes. For example, *Salmonella* spp. have more hydrophobic cell membranes than *L. monocytogenes* and *E. coli* (Ukuku & Fett, 2002). Further studies are needed to evaluate the effect of prior UV-C irradiation on controlling both Gram positive and Gram negative human pathogens in addition to *L. monocytogenes*.

Identification of putative compounds such as salicylic acid-β-glucoside and *p*-coumaryl quinic acid that are derivatives of shikimate pathway (Jahangir et al., 2009) and that have been induced by UV-C irradiation, suggests a possible correlation between these compounds and pathogen suppression. Synthesis of salicylic acid from chorismate which is an intermediate of shikimate pathway is catalysed by the isochorismate synthase enzyme (Dixon et al., 2002). *Arabidopsis thaliana* contains two isochorismate synthase (ICS) encoding genes; of which one gene is responsible

for inducing plastid targeted enzyme during fungal and bacteria attack (biotic stress). Therefore, it can be hypothesised that UV-C stress (abiotic stress) may also induce expression of the ICS encoding gene and production of salicylic acid in UV-C treated fresh cut broccoli as a part of the defence mechanism induced by UV-C irradiation. GC-MC analysis of phytochemical compounds of UV-C treated fresh cut broccoli indicated presence of linolenic acid which is an important fatty acid constituent of membranes of most plant cells and precursor for jasmonic acid (JA). Jasmonic acid is synthesised via the octadecanoid pathway which can be activated by ultraviolet radiation (Conconi et al., 1996; Niinemets et al., 2013). Conconi et al.(1996), working with UV-C irradiated tomato leaves, observed expression of several plant defensive genes (proteinase inhibitor I and II) that are normally activated through the octadecanoid pathway after wounding. Phytoalexins such as brassinin and camalexin were also synthesised in Brassicaceae plants while many GLSs were induced as a defence response (Jahangir et al., 2009). However, results of the current study did not show any treatment dependent change in GLS content (Figure 5.7) suggesting that GLSs may not have influenced the pathogen control. Results of this current research revealed that prior UV-C irradiation controlled growth and survival of post inoculated *L. monocytogenes* on fresh cut broccoli and confirmed presence of suggestive bioactive compounds that may have a bacteriostatic effect. In order to effectively suppress growth and survival of *L. monocytogenes*, bioactive compounds produced as a response to UV-C irradiation that are of antimicrobial nature should diffuse or evaporate from the surface. Therefore, head space gas analysis accompanied by extract analysis would be a better approach to measure such volatiles and the compounds that may be lost during extraction: since hydrolysis products of GLSs are volatile, they may be lost while heating the samples. Importantly, these diffusible or volatile compounds could be suppressive for a wide range of human pathogens. Identification of a particular compound (or compounds) that is induced by UV-C irradiation in broccoli and that suppresses *L. monocytogenes* would allow the use of that compound as a biomarker in future studies to define an action spectrum or to develop new broccoli varieties with enhanced resistance to human pathogen attachment. There is no guarantee that the 254 nm wavelength used in most studies has the greatest efficacy. Therefore, antimicrobial activity of the identified compounds that have been induced by UV-C irradiation in fresh cut broccoli against *L. monocytogenes* should be studied in future research and perhaps

against other types of human pathogens as well in order to confirm the effectiveness of those compounds in controlling other pathogens. In addition, these compounds could also be used for rapid screening of improved treatment regimes.

6.3 Practical implications of the research

UV-C irradiation is currently being used mostly as a surface sanitizer or treatment for water purification used for food processing. Some commercial usage in the horticulture industry is also reported; for example, fresh cut apples and peppers (fresh-appeal, USA, Inc.). Findings of this research suggest that UV-C irradiation at a dose of 5.2 kJm^{-2} is effective in controlling growth and survival of *L. monocytogenes* when inoculated 6 - 24 h after treatment and this suppression may be due to induced production of phytochemicals and/or surface modification. Based on this findings, some preharvest and postharvest applications of this technology can be suggested. The first option is to apply UV-C as a preharvest treatment in the field one day before harvest if processing is undertaken immediately after harvest: washing, floreting, packing and sending to the retail market. Advantage of employing UV-C irradiation as a hurdle technology here, together with washing is that UV-C irradiation will reduce the number of pathogens already attached to surface and further washing will remove some of the remaining microorganisms, in addition to induced resistance of broccoli surface 24 h after treatment to reduce possible secondary contamination. Field application could be made possible through a carefully designed technology where UV-C lights move slowly over broccoli heads in the field and deliver a recommended dose of irradiation. The second option is as a postharvest application for stored broccoli. Broccoli may be stored at chilling temperatures for days or weeks after harvest and so, 24 h before processing, stored broccoli could be treated in the pack-house. This can be achieved by moving intact broccoli heads held in an up-right position through a chamber with UV-C lights on a conveyer belt and then processing the next day. Data from this research indicated that maximum suppression of *L. monocytogenes* could be achieved 24 h after UV-C irradiation. Further work is required to determine whether UV-C treated broccoli remains safer if inoculated beyond this time period.

6.4 Future work

6.4.1 Identifying volatile compounds in UV-C treated broccoli

GC-MS analysis of phytochemicals in broccoli extracts revealed presence of some volatile compounds such as dimethyldisulfide and dimethyltrisulfide. However, highly volatile compounds in broccoli such as hydrolysis products of GLSs may have been lost during extraction and heating of the samples. Therefore a headspace analysis of volatiles of broccoli treated with UV-C, would be a better alternative to identify volatile compounds that may have an immediate effect on suppressing *L. monocytogenes* on UV-C treated fresh cut broccoli in future studies.

6.4.2 Effect of UV-C on attachment and morphology of *L. monocytogenes*

Growth of *L. monocytogenes* was suppressed on UV-C treated fresh cut broccoli inoculated 24 h after treatment compared to non UV-C treated broccoli. Since one possible mechanism may involve alteration to the broccoli surface, CLSM or SEM imaging of *L. monocytogenes* cells growing on prior UV-C treated fresh cut broccoli may elucidate the nature of the changes occur that restrict growth of the pathogen.

6.4.3 Antimicrobial activity of putative compounds

In this study, six candidate compounds were tentatively identified by LC-HRMS analysis that may be associated with defence mechanisms of broccoli. Identification and screening of such candidate compounds that may have antimicrobial effect is important to determine the degree of inhibition, mode of action and effective doses against different foodborne pathogens through real time bio-efficacy assays. Such antimicrobial compounds may damage cellular structures of the pathogen or affect virulency of the pathogen by interrupting quorum sensing and preventing biofilm formation. Therefore, thorough knowledge on inducible bioactives and their effect on pathogens is important for designing appropriate postharvest technologies that target elimination of foodborne pathogens on fresh produce.

6.4.4 Gene expression associated with UV-C

An alternative approach for measuring phytochemicals that may be induced by UV-C irradiation would be to identify genetic markers that induce particular phytochemicals with antimicrobial activity against human pathogens including *L.*

monocytogenes. This approach may also contribute to the development of cultivars with increased resistance to pathogen attachment.

6.4.5 Commercial adaptive research

This technology has to be commercially tested and developed into a practical procedure to be used in the horticulture industry. The results obtained in this research were based on fresh broccoli stored at 15 °C after UV-C irradiation and inoculation with *L. monocytogenes.* However, in the real industrial situation broccoli may be stored for few weeks after harvest at about 4 °C. The defence mechanisms of stored broccoli may not react similarly to the freshly picked broccoli and UV-C induced desirable changes may not occur if broccoli is stored at an optimal temperature (0 – 4 °C) for a long period of time. Therefore, current results need to be validated to develop a robust treatment regime suitable for industrial situations.

6.5 Conclusion

Hormetic doses of UV-C irradiation significantly reduced growth and survival of *L. monocytogenes* inoculated onto fresh cut broccoli 24 h after treatment compared to non UV-C treated control. Similar reduction was observed in broccoli inoculated with *L. monocytogenes* 6 h after treatment, if treated with 5.2 kJm^{-2}. This is an important finding that may help to reduce risk of human pathogen contamination of fresh produce and foodborne diseases among people. The suggested mechanisms of observed suppression by UV-C irradiation are: 1. modification of surface properties in such a way that the surface becomes less supportive for the pathogen to grow and 2. induced production of phytochemicals that are harmful for the pathogen. Identification of three compounds that are associated with plant defence system by LC-HRMS analysis confirmed possible correlation between UV-C induced phytochemicals and growth suppression of *L. monocytogenes*. Their antimicrobial activity against *L. monocytogenes* should be determined through further research. In addition, presence of surface wax components such as linolenic acid in UV-C treated broccoli at a slightly higher level also indicate possible enhancement of surface waxes and modification of surface properties that do not support growth of *L. monocytogenes*.

GLSs are ranked the most important secondary metabolite in broccoli by many researchers due to their nutritional, antimicrobial, chemopreventive and many other

health benefits while their hydrolysis products are also involved in plant defence system. Therefore, it was suspected that GLSs and their hydrolysis products could be responsible for growth suppression of *L. monocytogenes*. The current study identified 10 GLSs but there was no significant treatment effect on GLSs content suggesting no effect on growth suppression of *L. monocytogenes*. In addition to these compounds, there could be volatile compounds such as hydrolysis products of GLSs that are responsible for the immediate suppression of *L. monocytogenes* and a headspace gas analysis of UV-C treated broccoli may be a better approach to identify those compounds.

UV-C irradiation is a technology studied for decades as a postharvest technology mostly for the purpose of surface sanitation due to its well-known germicidal effect. This is the first time that its potential in induced resistance of fresh produce against post inoculated human pathogens has been revealed. Therefore, it may be a useful novel hurdle technology to reduce human pathogen contamination during processing and provide consumers with clean, fresher, and safer products.

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