Improving the Microbiological Safety of Candy Apples and Shredded Lettuce Through Applying Sequential Intervention Steps

by

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Abstract

Improving the Microbiological Safety of Candy Apples and Shredded Lettuce Through Applying Sequential Intervention Steps

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The following reports on the efficacy of sequential treatments to decontaminate fresh produce using candy apples and shredded lettuce as model systems. A forced air ozone treatment was developed to treat whole apples and lettuce heads. By using a combination of laboratory and pilot scale studies, the treatment time, air flow and ozone concentration was optimized to support a 1.90-2.40 log CFU of target bacteria. A further process based on advanced oxidation process (AOP) was developed using a combination of UV-C, hydrogen peroxide and ozone generated by a lamp emitting at a wavelength of 174nm. From optimization studies it was found that using a combination of UV-C and 6% hydrogen peroxide reduced *Listeria monocytogenes* by 0.86 log CFU. By using a combination of treatments it was possible to achieve a >5 log cfu reduction of target pathogens. The technologies developed during this study provides preventive controls that can be applied in post-harvest fresh produce processing.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Critical Control Points (CCP) – *specific points, procedures, or steps during the manufacturing of food products which reduce or prevent food safety hazards.*

Hazard Analysis Critical Control Points (HACCP) – *an approach to food safety that is systematic and preventive. It is recommended by the Codex Alimentarius Commission, the United Nations international standards organization for food safety. HACCP is used by most countries around the world and has been in use since the 1960s (CFIA).*

Good Agricultural Practices (GAP) – *specific methods which are designed to minimize the risk of food contamination, as well as documentation of clear records of production, handling and storage information.*

Good Manufacturing Practices (GMP) – *specific methods during production and testing which help ensure quality control of food products, testing is clearly defined, validated, reviewed, documented, as well included information regarding personnel, premises, and materials used for production.*

Colony Forming Units (CFU) – *a unit of measure to estimate the number of viable bacteria cells in a sample. Viable is defined as the ability to multiple under controlled conditions.*

1 Literature Review

1.1 Introduction

There is a general recognition that healthy diets should include a high proportion of fruit and vegetables. Through various campaigns by Provincial and Federal bodies the consumption of fresh produce has increased by 10% every year for the last decade. The growth in pre-prepared salads has been the main trend in the sector due to consumer acceptability and convenience. However, the growth in the fresh produce market has been mirrored by the rise in foodborne illness. Indeed, the number of outbreaks linked to fresh produce outnumber those linked to meat, dairy or seafood. There are several underlying reasons for fresh produce being a significant source of human pathogens with the main ones being that contamination can be introduced at any point in the chain with limited efficacy of post-harvest washes to remove acquired contaminants. Consequently, any human pathogens acquired in the field are likely to become distributed during processing as opposed to being inactivated. To date, the post-harvest wash has been recognized as the main intervention step in fresh produce processing. However, despite a diverse range of sanitizers being developed, none have been effective when assed within a commercial setting. Indeed, the best that can be achieved during washing is to avoid cross-contamination between batches. This has led to the consideration of seeking aqueous-free interventions.

In the following, the structure of the fresh produce sector will be described along with the different stages of growing and processing. Sources of contamination will be outlined along with potential interventions. The research was directed towards applying a sequential (hurdle) approach to achieve a greater degree of risk prevention and control compared to relying solely on post-harvest washes.

1.2 Produce Consumption and Market

Public Health officials have promoted the increased consumption of fresh produce to reduce obesity and chronic medical conditions (Perez 2002). Canada's Food Guide recommends consuming 7 – 10 servings a day, depending on age and gender (Health Canada 2016) with the World Health Organization recommending a minimum of 5 servings, or 400 grams per day (Riedieger et al. 2007).

In 2015, Ontario farmers grew 43.2% of the total value of vegetables grown in Canada, and Quebec grew 40.1% (Statistics Canada, 2016). Fruit sales were dominated by British Columbia (37.7%) followed by Ontario (27%) and Quebec (23.1%) (Statistics Canada 2016). In Canada, 7% of the land is used for agriculture, of which 70% is suitable for growing crops (arable land). On a global scale, Canada ranks 7th for the amount of available arable land (Statistics Canada 2016).

Because of the short growing season within Canada over 88% of fruit and vegetables requires to be imported (Denis et al. 2016; Statistics Canada 2002). In 2014 Canada imported $3.9 billion worth of processed produce, mainly from The United States, but also China, Mexico, and Brazil and exported $2.6 billion (AAFC 2016). From the USA, Canada imports mainly leafy greens, soft and citrus fruits, cauliflowers, broccoli, onions, beans and carrots, from Mexico, tomatoes, peppers, asparagus, avocados and cucumbers (AAFC 2014).

Fruit and vegetable processing is a significant industry in Canada and unlike growing, is an all year around activity (AAFC 2016). The key market trend in the sector is added value products (i.e. processed) that has seen a consistent 10% rise annually over the past decade (FAO 2005). Specifically, the growth in bagged leafy greens and processed fruit products have increased in recent years.

1.3 Microbial Contamination

The rise in demand for fresh produce has been mirrored by an increase in the incidence of foodborne illness outbreaks linked to the commodity. Indeed, fresh produce has been linked to more foodborne illness outbreaks than meat, seafood and dairy products. In principle, contamination by human pathogens can occur at any point during cultivation, processing and end user interface (Hour et al. 2013). Pre-harvest contamination points can occur via bio solids/manure, irrigation (water quality and method), run-off, soil, insects, animals (domestic and wild), equipment and field workers (Doering et al. 2009, Hou et al. 2013, Beuchat 1995). In the course of harvesting, human pathogens can be transfer via workers or disseminated by cross-contamination events derived from contact surfaces including equipment and crates. Yet, the greatest cross-contamination risk is during processing where the produce is sliced/cut thereby creating openings and releasing nutrients to facilitate pathogen entry, in addition to potential growth. Furthermore, significant dissemination of pathogens between product batches can occur during the post-harvest wash thereby representing a key food safety risk (Doering et al. 2009 Mercanoglu et al. 2011; Lynch et al. 2009).

1.3.1 Safety Interventions in the Fresh Produce Chain

Given that the fresh produce chain has no effective intervention steps there is a strong emphasis of preventative controls to reduce the risk of introducing pathogens. To this end, there have been several industry led initiatives to enhance food safety within the fresh produce chain. The most relevant example is the California Leafy Greens Agreement that formed the foundation of the Final Fresh Produce Rule under the Food Safety Modernization Act (FSMA). The main approaches taken under the guide relates to manure management, irrigation water quality, worker

hygiene, wildlife exclusion and pathogen screening. However, the open nature of farming, along with the inherent limitations of testing, has questioned if such approaches have, or will result in an improvement in the food safety status of fresh produce. Consequently, there has been a focus on post-harvest interventions with continuing reliance on the wash process to remove field acquired contamination. Yet, regardless of the sanitizer applied only a 0.5 – 1.0 log CFU reduction in pathogen levels on fresh produce is achieved with commercial wash processes (Barrera et al. 2012).

1.3.2 Foodborne Illness Outbreaks Linked to Fresh Produce

There have been many outbreaks associated with fruits and vegetables in the past decade. Produce, defined as: fruits/ nuts, fungi vegetables, leafy vegetables, root vegetables, sprout vegetables, and vine-stalk vegetables, had the highest amount of illness, with leafy vegetables being the highest within the category (CDC 2013). Although the produce category showed less deaths then meat, it did have more than double the illnesses (CDC 2013). There have been 400 known produce outbreaks in North America since 1990 over 50% of which could not be linked to a specific source of fresh produce (Table 1.1; CDC 2016). The lack of source attribution is due to a combination of factors with the short-shelf of product along with sporadic contamination events being most significant.

Table 1: Summary of fruit and vegetable outbreaks in North America (CDC 2016).

Produce Outbreaks In North America of the Last Six Years			
Year	**Product**	**Pathogen**	**Number of Recorded Infections**
2016	Frozen Vegetables	*Listeria monocytogenes*	9
2016	Alfalfa Sprouts	*E. coli* 0157:H7	11
2016	Packaged Salads	*Listeria monocytogenes*	19
2016	Alfalfa Sprouts	*Salmonella enterica* - Muenchen	13
2015	Cucumbers	*Salmonella enterica* - Poona	907
2014	Caramel Apples	*Listeria monocytogenes*	35
2014	Bean Sprouts	*Salmonella enterica* - Enteritidis	115
2014	Cucumbers	*Salmonella enterica* - Newport	275
2012	Cucumbers	*Salmonella enterica* – Saintpaul	84
2013	Shredded Lettuce	*E. coli* 0157:H7	30
2012	Organic Spinach/ Spring Mix Blend	*E. coli* 0157:H7	33
2012	Romaine Lettuce	*E. coli* 0157:H7	24
2012	Cantaloupes	*Salmonella enterica* - Typhimurium & Newport	261
2012	Mango	*Salmonella enterica* - Braenderup	127
2011	Cantaloupes	*Listeria monocytogenes*	147
2011	Romaine Lettuce	*E. coli* 0157:H7	58
2011	Cantaloupes	*Salmonella enterica*- Panama	20

1.3.3 *Escherichia coli*

The majority of *E. coli* are nonpathogenic and naturally carried within the gastrointestinal tract brining benefit such as producing vitamin K along with acting as a biological buffer.

(Donnenberg 2013). However, approximately 4.5 million years ago a group of E. coli followed a different evolutionary path whereby virulence factors where acquired leading to the immergence of a range of pathotypes. Specifically, 6 toxigenic E. coli groups emerged that included Enteropathogenic *E. coli* (EPEC), Shiga toxin producing *E. coli* (STEC) also known as Verocytotoxin-producing *E. coli* (VTEC) or enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) **(**Kaper et al. 2004). Sepsis and meningitis is caused by meningitis-associated *E. coli* (MNEC), and urinary tract infections (UTIs), of which 85% are associated with uropathogenic *E. coli* (UPEC) (Kaper et al. 2004). Of most concern are those E. coli that belong to the STEC group with E. coli O157:H7 being the most virulent although additional serotypes (termed non-O157 STEC) are becoming equally significant (CDC, 2015). STEC infection can range from the classic symptoms of gastroenteritis through to development of life threatening hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Erickson et al. 2010). It has been shown in previous studies that the infectious dose of *E. coli* may be as low at 10 cells (Tomás-Callejas et al. 2011).

On February of 2016, there was a press release warning consumers not to eat alfalfa sprouts made by Jack and the Green Sprouts. Later the product was recalled after a multistate (Minnesota and Wisconsin) outbreak of *E. coli* (STEC O157), infecting 11 people, with 2 needing hospitalization (CDC 2016). The celery and onion in the salad was implicated and voluntarily recalled by Taylor Farms Pacific Inc. Again in 2015 there was an outbreak of *E. coli* STEC O26 associated with Chipotle Mexican Grill restaurant across several states, infecting a total of at least 60 people with 22 being hospitalized, unfortunately the source of the pathogen was never identified (CDC 2015). In November 2013, there were 33 people, across five states,

which became infected with *E. coli* O157 after consuming Wegmans brand Spring Mix lettuce, and two of those people developed hemolytic uremic syndrome (CDC 2013). In January of 2013, 30 Canadians became sick from shredded lettuces distributed by FreshPoint Inc. to KFC and Taco Bell restaurants (PHAC 2013). In April 2012, 24 Canadians and several Americans became infected with *E. coli* O157:H7 after consuming romaine lettuce at a Jungle Jim's restaurant (CDC 2012). Most at risk are young children under 5 years old, immunocompromised individuals, pregnant women and the elderly (Ilic et al. 2012; Critzer et al. 2010).

1.3.4 *Salmonella*

Salmonella, a genus belonging to the family Enterobacteriaceae, currently has two species: *S. enterica and S. bongori. S. enterica* is further divided into 6 subspecies*, enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (Andino and Hanning 2015*)*. There are also 2,579 serotypes of *Salmonella*, which are usually found within the intestinal track and environment of birds, reptiles, animals and humans (PHAC 2015). These serotypes are classified into 50 groups based on their antigen composition (Ekperigin & Nagaraja, 1998). Salmonellosis causes four different clinical disease patterns: Gastroenteritis, enteric fever, bacteremia, and chronic carrier state, causing abdominal pain, diarrhea, chills, fever, and vomiting (Wong et al. 2011).

Gastroenteritis is caused by over 150 serotypes of *Salmonella*, Typhimurium and Enteritidis being most common, which is caused by the ingestion of food or water contaminated with animal waste (Wong et al. 2011). Enteric fever on the other hand occurs with the ingestion of food or water contaminated with human waste containing *Salmonella* Typhi (typhoid fever) or the milder Paratyphi A, B and C (paratyphoid fever) (Wong et al. 2011). Typhoid fever is an acute enteric infectious disease that is defined by prolonged fever, relative bradycardia, apthethic

facial expressions, reoseola, splenomegaly, hepatomegaly, leukopenia, and intestinal perforation and hemorrhaging (Wong et al. 2011). Bacteremia is caused by high invasive serotypes such as Cholearaesuis or Dublin, which cross the intestinal barrier and enter the blood stream (Wong et al. 2011). *Salmonella* can persist in certain individuals, making them chronic carriers, infecting many other people. Nontyphoidal serotypes are known to be able to persist within the intestinal tract from 6 weeks to 3 months (Wong et al. 2011).

There are 1.3 billion cases of gastroenteritis, 16 million cases of typhoid fever, and 3 million deaths worldwide due to *Salmonella* (Wong et al. 2011). The number of outbreaks associated with fresh produce has more than doubled between 1973 to 1987 and 1988 to 1991 in North America (Andino and Hanning 2015*)*. *Salmonella* causes the largest number of deaths and also causes the highest economical with respect to health care costs, in addition to lost productivity (Andino and Hanning 2015*)*. Salmonellosis associated costs were calculated in 2010 for America to be 2.7 billion for the 1.4 million reported cases, with the consumption of chicken, turkey and eggs being implicated in 70% of the cases (Andino and Hanning,2015*)*. Although poultry and eggs are known carriers of *Salmonella* (accounting for 145 and 117 outbreaks respectively between 1998 and 2008), fruits/nuts and sprouts also had quite a few outbreaks, 36 and 21 respectively (Wong 2011*)*. Especially of concern were apples, cantaloupe, alfalfa sprouts, mango, lettuce, cilantro, unpasteurized juice, tomato, melon, and parsley. Most frequently implicated in the outbreaks was serovar Enteritidis followed by Typhimurium, Newport, Heidelberg, and Montevideo (Andino and Hanning 2015*)*.

The ability for *Salmonella* to persist on foods depends on the serotype, but generally includes a number of variables such as pH (> 4 <5.5 pH) temperature (>5.3 < 45 °C), water activity (>0.9 aw), and chemical composition (Gomez Aldapa et al. 2012). In February of this

year, there was a *Salmonella* Muenchen outbreak involving Sweetwater Farms Alfalfa Sprouts, which infected 13 people from four (Kansas, Missoiuri, Oklahoma, and Pennsylvania) states, with 5 of the ill people needing hospitalization (CDC 2016). Samples later taken from the farm by investigators found irrigation water and sprouts contaminated (CDC 2016.)

In May to September of 2014, cucumbers were implicated in *Salmonella enterica* outbreak, serovar Newport infected 275 patience, in 29 states as well as the District of Columbia (CDC 2014). In 2015, cucumbers were found to be infected with *Salmonella* Poona, where a total of 907 people were infected from 40 states, 204 of them needed hospitalization and six passed away (CDC 2015). The cucumbers were imported from Mexico and distributed by Andrew and Williamson Fresh Produce and Custom Produce Sales (CDC 2015). Usually *Salmonella* Newport outbreaks are associated with tomatoes with cases from 2010 with (52 people), 2007 (65 people), 2006 (115 people), 2005 (72 people), and 2002 (333 people) (CDC 2011). In 2014, there was an organic chia spout powder (by Oriya Organics, LL) outbreak involving 31 people infected with either *Salmonella* Newport (20 people) or *Salmonella* Hartford (7 people) or *Salmonella* Oranienburg (4 people), across 16 states, with 5 people requiring hospitalization.

Bean sprouts (possibly produced by Wonton Foods Inc.) were associated with another outbreak in 2014, with strains of *Salmonella* Enteritidis infecting 115 people from 12 states (CDC 2014). In 2013, cucumbers were again involved in a *Salmonella* outbreak, where 84 people from 18 states were infected with serovar Saint Paul, which originated from cucumbers out of Mexico, supplied by Daniel Cardenas Izabal and Miracle Greenhouse (CDC 2014). In 2012 there was a cantaloupe outbreak (*Salmonella* Typhimurium and Newport, 261 people, 24 states) and a mango outbreak (*Salmonella* Braenderup, 127 people, 15 states) (CDC 2012).

1.3.5 *Listeria monocytogenes*

Species of the genus *Listeria* are usually classified into two typically non-hemolytic species (*L. innocua and L. welshimeri*) and three hemolytic species (*L. monocytogenes, L. ivanovii, and L. seeligeri*) (Saunders et al. 2011). *Listeria* can be isolated from a variety of sources including soil, water, vegetation, farms, food processing facilities, gastrointestinal tract and sewage (Sauders et al. 2011). In foods, *Listeria* is primarily linked to processed meats, fish, raw vegetables, fruit, milk and dairy products (Wing & Gregory 2002). *Listeria monocytogenes* causes listeriosis, an often-deadly infection that leads to meningitis sepsis, as well as acute gastroenteritis (Schuppler & Loessner 2010). People most at risk are immunocompromised individuals, older adults, and pregnant women, as *Listeria* can also cause infection and abortion of fetuses (Schuppler & Loessner 2010). *Listeria* has a low incidence rate, however because it high virulence (30% mortality rate) it is one of the deadliest of the foodborne pathogens (Schuppler & Loessner 2010).

In 2016, there was an outbreak of listeriosis linked to packaged salad products made at a Dole processing facility in Ohio, where 19 people were infected from 9 states (CDC 2016). In 2015 there was a recall on Granny Smith and Gala apples from several suppliers after 35 people became infected with listeriosis after eating candied apples across 12 states (CDC 2015). Unfortunately, ten of the cases were of pregnant individuals, in which one patient had a miscarriage (CDC 2015). In 2011, there was a listeriosis outbreak involving cantaloupes, where 147 people became infected across 28 states, resulting in 143 hospitalizations and 33 deaths, and a miscarriage (CSC 2011).

1.3.5.1 Surrogates

In the course of validating and verifying intervention process there is a need to establish operating parameters that assures a decrease in the pathogen levels by a designated level. With respect to pathogens, the ideal level of reduction is 5 log CFU that equates to a 99.99% decrease in numbers. Demonstrating a 5 log CFU reduction within a contained laboratory is relatively straightforward however it is frequently questioned how such results can be translated to commercial-scale processing (Waite-Cusic et al. 2011). Obviously, introducing pathogens into commercial processing to validate and verify processes is not possible. Consequently, a surrogate is required to exhibits the same or enhanced resistance compared to the pathogen target but itself is non-pathogenic (FDA 2000). *Listeria innocua* is often used as a surrogate for *Listeria monocytogenes* given that both are genetically and phenotypically related although the former is non-pathogenic (FDA 2010). However, because *Listeria* spp. is used as a hygiene indicator in environmental monitoring it is not possible to use *L. innocua* in commercial scale trials. Consequently, there is interest in alternative surrogate that can be used to predict the level of efficacy of a process to support pathogen introduction. For example, the lactic acid bacteria, *Enterococcus faecalis* NRRL C-2354 has been used as a surrogate for *Salmonella* in the pasteurization of almonds. *E. faeclais* is a suitable surrogate for this purpose given it is easy to detect and differentiate, along with being non-pathogenic and corresponding thermal resistance compared to Salmonella. There are currently no alternative surrogates for *L. innocua* although lactic acid bacteria could be suitable given the similar environments inhabited (Waite-Cusic 2011).

1.4 Food Safety of Leafy Greens and Caramel Apples

The main focus of the current thesis was to develop effective decontamination methods to enhance the food safety of leafy greens and caramel apples. The selection was based on the contrasting features of the fresh produce types and also current commercial significance. The following summarizes the research to date on leafy greens and candy apples.

1.4.1 Leafy Greens

Leafy greens such as lettuce and salad spinach have been implicated in the majority of produce related foodborne illness outbreaks and recalls. This can be partly attributed to volume of product consumed and intermit contact of plants with soil. It is also noted pathogens can colonize plants tissue especially through natural openings such as stomata, trichomes and cracks in the cuticle of the leaves (Li et al. 2001; Delaquis et al. 2002). Cutting or shredding lettuce results in the release of latex that results in foaming, sequestering of sanitizers and enhances adhesion of bacteria to the leaf surface (Brandl 2008). Consequently, washing lettuce to remove pathogens is very limited thereby requiring more effective decontamination treatment (Sessa et al. 2000).

1.4.2 Caramel Apples

In 2014, a multistate listeriosis outbreak was linked to the consumption of caramel-coated apples in the US. In total there were 35 cases in 12 states with 34 hospitalizations and 7 deaths. Although apple cider has been previously linked to foodborne illness outbreaks involving *E. coli* O157:H7, whole fruit has been considered a lower food safety risk. In the 2014 outbreak the specific source of *Listeria* was never reported only that the contaminated fruit was supplied by a single producer. A possible hypothesis provided was that the *Listeria* was derived from a

contaminated dunk tank and introduced into the inner apple core via insertion of the stick with the pathogen growing in the course of storage at room temperature. Two studies have been published with respect to the growth of *L. monocytogenes* on and within candied apples (Glass et al. 2015; Salazar et al. 2016).

Salazar et al (2016) employed three *L. monocytogenes* strains that were isolated from patients that had contracted listeriosis from candy apples. The *Listeria* were cultivated at overnight and cells harvested to form a cell suspension of 5-9 log CFU/ml. The apples were inoculated with 10 µl aliquots at the stem end and along the equatorial part of the fruit. The inoculated samples were held for 3h at 25°C for 2h or 24h at 5°C. Upon completion of the drying period the original population had decreased by 1-4 log CFU (99% - 99.99% of the original population). Upon completion of the drying period, a wooden stick was inserted into individual fruit. The apples were then dipped into a molten caramel (Aw 0.66) maintained at 76°C. For apples dried at 5°C were conditioned at room temperature prior to dipping. The apples were held for 2 h to allow the caramel to harden then the resultant candy apples placed in a clam shell then held at 4 or 25°C. Periodically, candy apples were removed then placed in a 3.5l stomacher bag along with 350 ml of enrichment broth and the sample homogenized by stomaching. A dilution series was prepared then plated onto PALCAM that was incubated 37°C for 48h. The lower detection limit was 2.5 log CFU/ml.

For candy apples stored at 5°C *Listeria* increased by 3 log CFU on the stem end after 15 days' storage. Those on the surface did not change in levels over the 15-day storage at 5°C. For candy apples stored at 25°C the levels of *Listeria* increased by 3-4 log CFU at the stem scar tissue. Again, *Listeria* on the surface of apples did not change in levels through the 15-day

storage period. The results within the paper by Salazar et al (2016) show that drying apples alone results in a significant log reduction of the *Listeria*. Although shorter lag times were observed with candy apples stored at 25°C the benefits derived from holding at 5°C were an addition 4-day lag period. With non-dried apples the extent of growth of *Listeria* on candy apples did not differ between samples stored at 5 or 25°C. The authors concluded there are many knowledge gaps that exist with respect to the growth of *Listeria* on candy apples but significantly, did not suggest that storing candy apples at 5°C could be used as a risk mitigation strategy and that interventions are required.

A further study on the fate of *Listeria* on apples was published by Glass et al (2016). Here, a 4 strain cocktail of *L. monocytogenes* was cultivated and a suspension prepared. The apples were spot inoculated onto the surface, stem and calyx areas of the apple to achieve a final cell density of 4.2 log CFU per apple. The inoculated apples were allowed to air dry for 5-10 minutes prior to inserting a stick then dipped in molten caramel held at 95°C. The candy apples were allowed to set then transferred to 7 or 25°C incubators. Candy apples were removed periodically with *Listeria* being recovered by submerging the sample in 100 ml of buffered peptone then physically massaged. The rinse samples were then plated onto Modified Oxford Agar. A 0.8 – 1.2 log reduction resulted from the coating caramel coating process. With candy apples stored at 25°C supported growth of *Listeria* without a lag period. Growth of *Listeria* on candy apples stored at 7°C after a 7-day lag period then increased by 2 log CFU over a 28-day storage period. Apples coated with caramel but without stick insertion supported *Listeria* growth stored at 25°C with no growth at 7°C. The conclusion of the study was that the growth of *Listeria* could be supported by the juice released from apple cells through insertion of the stick. The

authors suggested that the risk derived from *Listeria* could be reduced by refrigeration of candy apples. Although the Glass et al (2016) study did illustrate the influence of storage temperature on *Listeria* growth it should be noted that the inoculum was actively growing at the time of inoculation. Although this would represent a contamination event at the point of caramel application it could not mimic pathogen contamination at pre- or immediately following harvesting.

From reviewing previous studies performed on the interaction and growth of *L. monocytogenes* on candy apples it is apparent that the fate of the pathogen is dependent on several factors. Specifically, *Listeria* on the surface of apples is susceptible to inactivation if dried onto the surface and limited growth occurs regardless of storage temperature. However, survival and growth of *L. monocytogenes* can occur at the stem end where the stick is inserted thereby releasing exudate/apoplastic fluid from the apple. When actively growing *L. monocytogenes* are inoculated onto apples the pathogen increases in numbers within 2 days when stored at 25°C. However, if *L. monocytogenes* is inoculated onto the stem end then dried the effect of storage temperature is less apparent with an extended lag phase of the pathogen. The only benefit derived from refrigeration of candy apples in this case is a 4-day extension in lag phase before the pathogen initiates growth. It can be envisaged that under natural conditions, the *Listeria* on apples would be in a stressed state and hence would likely experience the growth patterns reported by Salazar et al (2016). In contrast, the scenario outlined by Glass et al (2016) would be less likely encountered although potentially could occur in dunk or wash tanks where actively growing *Listeria* could reside. Regardless of this fact, temperature control of *Listeria* is not effective and decontamination methods are required to ensure food safety.

1.5 Current Treatments

Currently chlorinated water (50-200 mg/L) is applied widely in industry to sanitize fresh produce (García-Gimeno and Zurera-Cosano 1997; Zhang et al. 2009). Although hypochlorite is low cost, and readily available, this treatment is far from satisfactory due to the low antibacterial effects within the processing environment. Washing apples or lettuce in aqueous sanitizers has found to have limited efficacy in removing contamination (<1 log CFU reduction) and potentially can lead to cross-contamination (Perez-Rodriguez et al. 2014). In addition, residual moisture on apples impedes the coating of caramel on apples thereby causing production problems. In the case of lettuce, washing results in hydration of the product that can promote spoilage. Therefore, there is demand for aqueous free approaches as an alternative to post-harvest washing.

As well as the limited efficiency of chlorinated water, it is also of concern due to the possible formation of carcinogenic chlorinated compounds in the water. Used at low concentrations chlorine still has the potential to alter taste and odor in treated products, further increasing the interest in finding alternative sanitizers (Singh et al. 2002). Organic load can reach a point where the chlorine is sequestered and not able to efficiently sanitize the produce (Van Haute et al. 2013). The harmful disinfection by-products (DBPs) this treatment generates have caused this practice to be banned in some countries (Van Haute et al. 2013). These DBPs include total trihalomethanes such as chloroform, bromoform, dibromochloromethan and dichlorobromomethane, and studies have shown that although they accumulate within the wash water, none is found on the produce afterwards (Van Haute et al. 2013). DBPs not only pose a problem to human heath, but also cause environmental concerns.

Quaternary ammonium compounds (QUATS) are also used in industry, as they have many

advantages including being odorless and colourless, they are stable at high temperatures, nonirritating to skim, non-corrosive to equipment, and they are able to penetrate food contact surfaces more readily than other sanitizers. QUATS are more effective on fungi and Gram-positive bacteria, although there is some concern for the potential for resistance to form upon exposure to sub-lethal concentrations of the sanitizer.

1.6 Alternative Post-Harvest Decontamination Treatments

1.6.1 Ultraviolet Radiation

Ultraviolet (UV) electromagnetic radiation spans from 100 to 400 nm wavelengths. The spectrum is divided into four main categories: vacuum UV is designated for wavelengths from 100 – 200 nm, short-wave UV-C is from 200 to 280 nm, middle-wave UV-B from 280 to 315 nm and long-wave UV-A is from 315 to 400 nm (Parish 1979). On the electromagnetic spectrum UV wavelengths are between X-rays (<100 nm) and visible light (>400 nm). Exposure to UV can be very harmful, especially to the eyes, which is why it is very important for personnel working in processes with UV to protect their eyes and skin from the harmful rays. Overexposure to UV radiation can cause sunburns, premature skin aging, skin cancer, eye damage, and can weaken the immune system (Health Canada 2014). As well there are many factors that can make some individuals more susceptible to these effects than others, such as a fair complexion, light hair, numerous and large moles, freckles, heredity disorders and family history of skin cancer (Health Canada 2014).

Low wavelength UV (<200 nm) also termed vacuum UV, is characterized as having low photon penetration in air although is sufficient energytic to split oxygen that recombines to form

ozone. Therefore, the antimicrobial action of vacuum UV is indirect through the generation of ozone.

UV-C (250-270 nm) inactivates microorganisms by damaging the genetic material thereby inducing lethal mutations in DNA (Dai et al. 2012). Low pressure vapor lamps are often used in small operations because they emit > 90% of their radiation at 254 nm along with no excessive generation of heat as observed with medium pressure types (Shama 2007). The effectiveness of UV radiation to kill microbes depends on may factors besides wavelength including; temperature, the type of organisms and the distance from UV, intensity, organic matter, suspension, and contact surface (CDC 2012). UV radiation is used in many industrial process and work environments such as labs and hospitals. Bacterial spores tend to be more resistant to UV radiation than vegetative cells and viruses (Dai et al. 2012).

The ability for cells to protect themselves, or to recover, from UV damage depends on factors such as the efficiency of DNA repair mechanisms of the organism and the production of UV absorbing pigments. Biofilms can also be more resistant to UV radiation due to both the polysaccharide matrix in which cells are embedded and the metabolic state the cells are in. The germicidal effects of UV can be increased with the addition of certain oxidants such as ozone and hydrogen peroxide. Although each is germicidal on their own, the combination of these sanitizers is synergistic in deactivation nature due to the increased hydroxyl radical production. For the majority of materials UV is very strongly absorbed, so the UV is limited to acting on microbes present on the surface of the object or food item. As well, if there are cervices in the object (like knife indents on a cutting board or the grooved surface of a cantaloupe) microbes may be shielded from the radiation (Shama 2007).

1.6.2 Ozone

The use of ozone (O_3) has a long history of use for decontaminating water, air and surfaces. Ozone has Generally Recognized as Safe (GRAS) status for direct contact of foods although actual levels are limited due to the potential to generate byproducts (Donnell and Cullen 2012; Graham et al. 1997). Ozone is also permitted in Canada for use as a food additive (up to 2 ppm in water), according to Health Canada Food and Drug Regulations Table VIII, section B.16.100. There are also other regulations in place in to ensure the safety of the personnel working with the additive. These regulations include defining how much ozone is safely permitted in the air from the generator, which is a limit is 0.05 ppm (Health Canada 2007). Although ozone is permitted when following the specific guidelines outlined by Health Canada, there are also regulations in place ensuring the consumers know that the product has had ozone added. For bottled water it needs to be specified both in the ingredient list as well as on the main display label (Canadian Food Inspection Agency 2015).

The use of ozone has assisted with the physical, chemical, and biological cleaning of food processing units within the industry. Ozone use is not new, as it has been used in water treatment facilities since 1906 (Donnell and Cullen 2012), however has recently come into the spotlight due to the demand for minimally processed foods and its potential for reducing foodborne outbreaks. Ozone has the benefit of not leaving harmful residues as it spontaneously degrades to O_2, a nontoxic product (Kim et al. 1999). It also has the benefit of being able diffuse through the biological cell membrane, making it an ideal treatment for leafy greens. Ozone is produced by many different methods including: chemical, thermal, electrolytic, photochemical, and by electric discharge (Kim et al. 1999).

Ozone is able to react with organic material up to 3000 times faster than chlorine, and has

been reported to reduce pathogens on lettuce by nearly 2- $\log_{10}$ CFU/g (Singh et al. 2002). There are several factors that influence the antimicrobial efficacy of ozone, including: concentration, temperature, pH, humidity, and organic substances present (Karaca & Velioglu 2014). The use of both ozonated water and ozone gas have been shown successful for reducing the bacterial load on other fresh fruits and vegetables. Najafi and Khodaparast (2009) showed that dates treated with ozonated water were reduced by up to 4-$\log_{10}$ CFU/g in coliform bacteria. Selma et al. (2008) showed that the effects of ozone treatments actually increase in efficiency throughout the self-life of non-ripe melons, showing a 5.4-$\log_{10}$ CFU/rind-disk reduction of *Salmonella* after 7 days of storage. The study concluded that gaseous ozone is ideal as a commercial sanitizing agent, especially for products which cannot be packaged when wet due to mold growth during shipping (Selma et al. 2008). Ölmez and Akbas (2009) reported a 2-$\log_{10}$ CFU/g reduction of *L. monocytogenes* on lettuce treated with ozone, however they also noted significant quality loss of the product.

Just as ozone is able to alter organic material meant for disinfection, if not used properly you can also have negative effects on human health. Studies have shown that 80 ppm of ozone exposure is considered the Lowest Observed Adverse Effect Level (LOAEL) for an exposure period up to 6.6 hours, in young healthy adults (Health Canada 2010). The symptoms of the exposure included many aspects of decreased lung function including: coughing, shortness of breath, wheezing, as well as chest pain, throat irritation, headache, upset stomach, and vomiting (Health Canada 2010). At 40 ppm of ozone, exposure for up to 6.6 hours showed no effects on the subjects and is therefore considered the No Observed Adverse Effect Level (NOAEL). Employers usually consider exposure by a Time Weighted Average (TWA), in which workers are not to not to be exposed to more than 0.1 ppm of ozone averaged over an eight hour work

shift, or 0.2 mg of ozone per cubic meter of air (mg/m3) as recommended by the CDC (2014). When considering Short Term Exposure Limits (STEL), a maximum concentration of 0.3 ppm is permitted (Health Canada 2010).

It is also worth nothing that there are other factors that also need to be taken into consideration when thinking about ozone damage. Plants are very susceptible to damage by ozone in indoor environments, and exposure can cause chlorosis and necrosis among other conditions. Ozone can also do a lot of damage to many building materials such as carpet, foam, rubber, electrical coatings, and much more. This is why 304 or 316 stainless steel is often used in ozone generators and air ducts (Health Canada 2010).

1.6.3 Hydroxyl Radicals

An alternative to ozone, which can be difficult to handle due to its highly corrosive nature, are hydroxyl radical generators. In solution, ozone decomposes into several radicals, hydroperoxyl ($^{\bullet}HO_2$), hydroxyl ($^{\bullet}OH$), and superoxide ($^{\bullet}O2^-$) (Kim et al. 1999). Like ozone, free hydroxyl radicals can be produced by a number of different means, or naturally by solar radiation of wavelengths less than 310 nm causing photolysis of ozone, which after reacting with water forms the hydroxyl radical. The hydroxyl radical is known as 'the detergent of the atmosphere' due to it being extremely reactive and capable of oxidizing most of the chemicals found in the troposphere. In biological systems, $^{\bullet}OH$ is generated from H_2O_2 by the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (Chen and Schopfer 1999). A variation of the original Fenton equation combines ultraviolet and visible light, Fe(III) and hydrogen peroxide to produce hydroxyl radicals photochemically $Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + {}^{\bullet}OH$ (Bolton 1999). Ozone absorbs UV radiation at 254 nm wavelength which then produces an intermediate, H_2O_2, which decomposes

to •OH (Munter 2001). Currently hydroxyl generating machines are used commercially for odour control, typically following a fire or flood, however preliminary testing shows antimicrobial activity after extended treatment times (Barrera et al. 2012).

1.6.4 Hydrogen Peroxide

Hydrogen peroxide is used within the food industry for many different food processes for different purposes that include as a bleaching agent and as a sanitizer Although treating food with hydrogen peroxide can cause destruction of methionine, cysteine, and ascorbic acid, with proper usage the loss of nutrition is insignificant (FDA 2015). Hydrogen peroxide can be applied to food directly, although it is limited due to the bleaching effects that can be detrimental to product quality. Residual hydrogen peroxide would not normally represent a hazard given that it decomposes to water and oxygen when acted upon by catalase. Yet, high concentrations of hydrogen peroxide can result in detrimental health effects and as a consequence, permitted residual levels are restricted to < 100 ppm (FAO 2015).

1.6.5 Chlorine Dioxide

Chlorine dioxide is generated by acidification of chlorite with the gas being used directly or infused into water (Zhang 1996). The main benefits derived from chlorine dioxide over chlorine, includes higher oxidation power, low disinfection byproduct generation, insensitive to organic matter and antimicrobial at lower concentrations (Gómez-López et al. 2009; Lee et al. 2004). The chlorine dioxide gas exists nearly all of monomeric free radicals, and thus when it is concentrated can potentially be explosive (WHO 2003). Chlorine dioxide lies between chlorite (+3) and chlorate (+5) in oxidation state and therefore decomposes to chlorite when not

oxidizing substances (WHO 2003). This explains why ClO_2 is much stronger than chlorine in HOCl, it accepts 5 electrons when reduced to chloride ion, which allows much more available chlorine, increasing the oxidation capacity, however also causing much slower reactions (Gómez-López et al. 2009). Although ClO_2 is now mainly used as a bleaching agent in paper production, numerous studies have shown it to be an efficient sanitizer for fresh produce. (Lee et al. 2004) showed that the ClO_2 gas works better after 3 hour treatment on lettuce leaves, compared to 30 minutes and 1 hour, achieving maximums of 4.4-$\log_{10}$ CFU/g reduction in *E. coli*, a 5.3- $\log_{10}$ CFU/g reduction in *Salmonella* Typhimurium and a 5.2-$\log_{10}$ CFU/g reduction in *L. monocytogenes* when compared with the control. Singh et al. 2002, showed reductions of shredded lettuce and carrots with maximum reductions of 2.2 and 3-$\log_{10}$ CFU/g using ClO_2.

1.7 Research Rationale

The cost of foodborne illness outbreaks can potentially result in bankruptcy of suppliers and processors with the cost of precautionary product recalls being quite significant. Given the limitation of post-harvest washing the food safety practices aim to prevent contamination at the farm level and attempt to detect pathogens through testing. By using different AOP processes pathogens contamination can be controlled on leafy green products and apples with no significant effects to the products. Although the risk of listeriosis from candy apples can still be regarded as low there is a need to apply interventions as a preventative control. Washing apples in aqueous sanitizers is one option although this has found to have limited efficacy in removing contamination (<1 log CFU reduction) and potentially can lead to cross-contamination (Perez-Rodriguez et al. 2014). In addition, residual moisture on apples impedes the coating of caramel on apples thereby causing production problems. Consequently, aqueous free approaches are

more compatible with candy apple production and moreover, have proven to be effective in decontaminating produce compared to traditional post-harvest washing (Back et al. 2014).

In the current study the efficacy of gaseous ozone introduced into a forced air flow was evaluated for decontaminating whole apples inoculated with *Listeria*. Ozone was selected based on high oxidation capacity, low byproduct formation and historical use as an antimicrobial gas (Khadre et al. 2001). There have been previous studies that have demonstrated that ozone introduced into the atmosphere of storage rooms can reduce microbial loading on fruit (Yaseen et al. 2015). However, ozone in storage rooms is applied at low levels to prevent excessive corrosion of fittings and reduce potential hazards to workers. Consequently, an extended exposure time is required to achieve microbial reductions although contacting each individual apple (within a large contained bushel) represents a challenge. An alternative approach is to introduce ozone into the air stream used to raise the temperature of apples to prevent condensation forming on the fruit surface. By using the forced air approach, it is possible to use higher ozone concentrations and facilitating even (as opposed passive) air flow within the apple bed. An added advantage if introducing the ozone at the early stages of drying is that the relative humidity surrounding the fruit is high (due to the fact that they have just been removed from refrigerated temperatures and therefore have developed condensation around the skin due to the temperature change) that in theory will enhance the susceptibility of microbial cells to the lethal effects of ozone (Miller et al. 2013; de Candia et al. 2015).

Research Hypothesis and Objectives

The research hypothesis was that sequential interventions have an additive effect on inactivating human pathogens on apples destined for candy-apple production or shredded lettuce used to prepare bagged salad.

The objectives of the study were:

1) Evaluate a range of sequential gas-phase treatment to reduce human pathogens on apples or heads of lettuce.
2) Develop a produce decontamination method based on the Advanced Oxidative Process.
3) Assess the efficacy of sequential interventions on reducing the human pathogens on apples and lettuce.
4) Determine the fate of human pathogens during post-treatment storage of candy apples and shredded lettuce.

2 Materials and Methods

2.1 Bacterial Cultures and Growth Conditions

The pathogens used in this study included Shiga toxin-producing *E. coli*- STEC, serotypes O157:H7 (two strains) and one strain of O111, O45, O26. As well as *Listeria monocytogenes* (serotypes 4a, 4b, 1/2b, 1/2a, and 3a). These isolates are of particular relevance as they are associated with past outbreaks, and were obtained from the University of Guelph's Food Science culture collection. *Lactobacillus fructivorans* ATCC 8288 was also applied in the study as a surrogate for *L. monocytogenes* and obtained from American Type Culture Collection (Atlanta US).

The concentration of each bacteria was determined by both optical density (OD) and serial dilution. After each bacterial was diluted to the same concentration (8-log $_{10}$ CFU/ml) the *E. coli* strains were mixed together to make a final inoculum, as well as the *L. monoctyogenes* strains. The inoculums were stored at 4°C for up to 12 hours before use and vortexed (IKA™ Vortex 3 Shaker) for 1 minute once removed. Each bacteria stain was streak plated onto selective agar to allow for isolation of single colonies, which were then removed and grown in tryptic soy broth (TSB) for 24 hours at 37°C or 30°C in MRS broth for 48h in the case of Lactobacillus. Cells were harvested by centrifugation (Sorvall™ ST 8) at 5000 x g for ten minutes. The supernatant was discarded and the pellet of bacteria suspended in saline, vortexed (IKA™ Vortex 3 Shaker) for one minute and stored at 4°C for 48 hours, to allow for stress adaptation.

2.2 Produce Samples and Inoculation

Non-waxed apples and whole head of iceberg lettuce were provided from industrial partners and stored at 4°C until required. It was important that the produce used was intact

without obvious signs of mechanical damage such as bruising and abrasions. Therefore, apples with any visible signs of damage (bruises, cuts, missing stems) or any spoilage were not used. The lettuce heads were prepared for treatment by removing the outermost layers of leaves which have had mechanical damage during processing. This helped to ensure consistency between samples, and to not add further factors in which may change how the bacteria will react between trials.

Apples were spot inoculated on the skin, around the top of the fruit, with 100 µl of the test bacterium at a concentration of 8-log $_{10}$ CFU/ml. The apples were then dried in a biosafety cabinet for 20 minutes to 4 hours, before being transferred to 4°C storage for a maximum of 24 hours. To internalize the bacteria, 1 ml of the suspension was added to the stem crevice and put under a vacuum for 1 minute, removed from the vacuum and left for 1 minute, before being vacuumed once more for another minute.

2.3 Treatment

2.3.1 Laboratory Scale Forced Air Ozone Reactor

The reactor consisted of an ozone generator (Netech™, ozone output of 6g/hour, flow rate 10 l/minute, power – 120 W, 50/60 Hz.) that was positioned at the based or top of a 3.5’ x 3.5’ x 3.5’ - ½” plywood box lined with 0.157” corrugated plastic. Within the chamber apples were placed in a perforated box (30 cm depth). The ozone was pulled up or down through the apple pile via a fan at a velocity of 9.5 m/s (measured with a CFM/CMM Thermo-Anemometer - Extech™ - Model # AN100 - 20 point average for air flow and 3% velocity accuracy). The reactor was an enclosed system with the humidity being poised at 85 - 90% relative humidity via a humidifier (Honeywell # 3043-5974-0, 1-gallon capacity, 36 hour run time, low – high

settings). The temperature, humidity and ozone concentration was measured using a Aeroqual series 940 monitoring unit (Auckland, NZ) calibrated by Aeroqual to a certified accuracy of <±0.008 ppm 0-0.1 ppm, <±10% 0.1-0.5 ppm. The unit included a real time digital display of ozone concentration and an alarm to alert of high/low concentrations and diagnostic issues. The air was exhausted from the chamber via a fan and passed over 4 UV-C lamps (254nm) to decompose residual ozone after treatment. The temperature of the apples themselves was also recorded using a thermometer probe (Fisher Scientific™ Traceable™ - accuracy ±0.05°C - range -50° to +150°C) which was placed 0.5 or 1.0 cm into an apple (positioned in the middle of the pile). The treatment time was set for 20 minutes after which the apples were removed then sub-divided into those at the top, middle or bottom of the pile. The surviving *L. monocytogenes* was recovered as described above and enumerated on MOx with the initial loading being determined using non-treated fruit.

After the produce was inoculated and the pathogens allowed time to adhere, as described above, they were then placed in a chamber and exposed to ozone at a rate of 6g/h. The parameters were controlled within the chamber as mentioned, with the ozone concentration starting from 30 ppm at five minutes of generation up to 80 ppm after 20 minutes. Humidity was stable from 85 – 90%, as kept constant by the humidifier, and temperature from 24.4°C to 26.8°C. Upon completion of the treatment, residual ozone was broken down by 4 UV lights (254 nm wavelength – peak ozone destruction) and removed from the chamber with fans. The survivors of ozone treatment were recovered (described below) and log count reduction relative to controls determined.

2.3.1.1 Effect of Air Flow on Efficacy of Ozone Decontamination of Apples

To determine if the point of introduction of ozone impacted the process, efficacy trials were undertaken where the gas was introduced into the chamber at different locations. In the one arrangement, the ozone generator was placed on the bottom of a perforated container with 30 cm depth of apples (Figure 1). Three inoculated apples were placed at the base, middle or top. The ozone was drawn through the apple bed via a blower then passed the exhaust air over UV lamps to degrade residual ozone. In the other arrangement the ozone was placed above the apples (outside of the chamber) with the air flow being forced downwards rather than upwards as before (Figure 2). Humidity, temperature and ozone concentrations were kept at constant rates as described above.

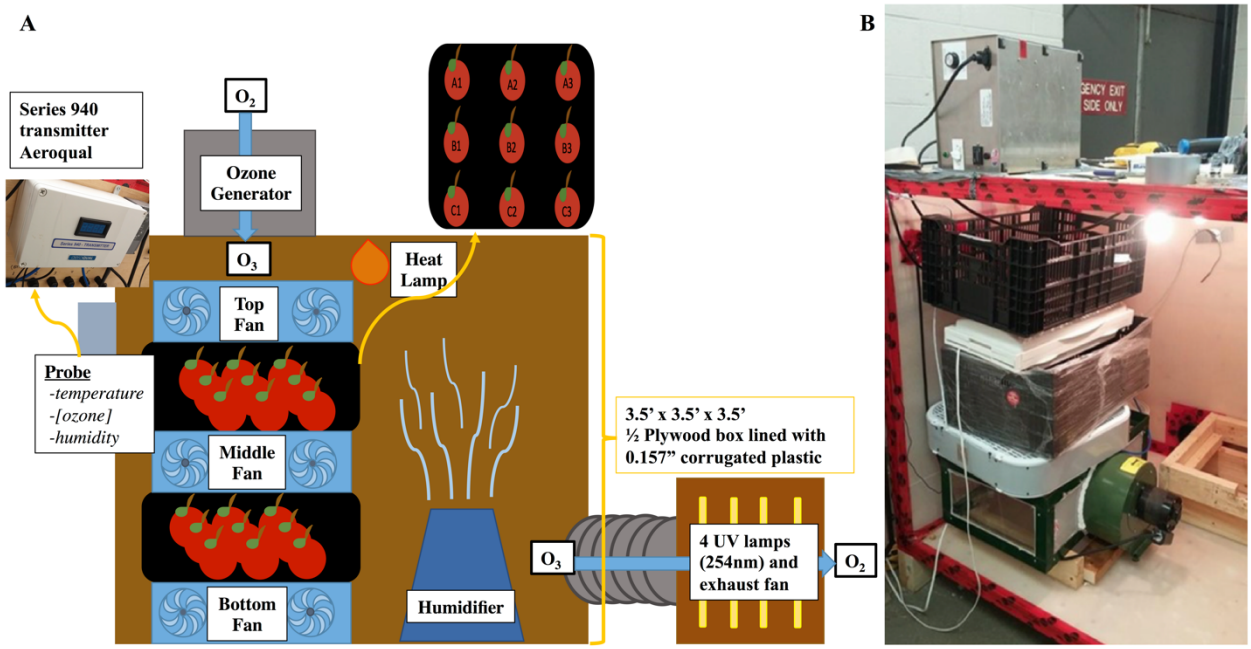

Figure 1: Forced air reactor with ozone air stream being introduced at the top of the apple batch containing *Listeria* inoculated fruit.

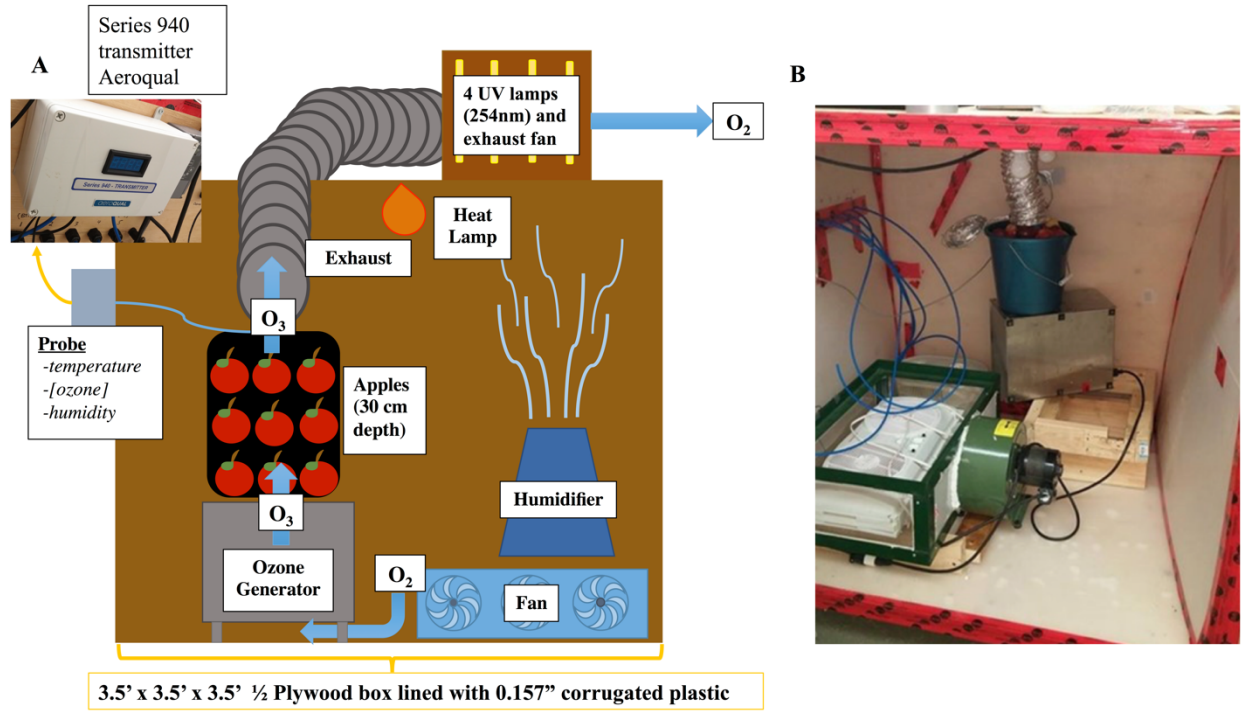

Figure 2: Forced air reactor with ozone air stream being introduced at the base of the apple batch containing *Listeria* inoculated fruit.

2.3.1.2 Efficacy of Ozone to Inactivate *L. monocytogenes* on Apples in Multiple Layers

Apples were inoculated with *L. monocytogenes* and left to attach for 2 hours at room temperature (21°C). Inoculated apples (n=3) were then placed in the center row (B) in the tray (bottom) and the layer completed with non-inoculated apples. A further layer of inoculated apples (n=3) were place on the bed of apples (middle) and again layer completed with non-inoculated fruit. Finally, 3 inoculated apples were placed on the dual layer and surrounded by non-inoculated apples (top). Therefore, the tray had 3 layers of apples in total. The apples were placed in the chamber then treated with ozone for 20 minutes under high relative humidity for 20 minutes.

2.3.1.3 Ozone Treatment of Conditioned Apples

Trials were performed to deterred the efficacy of ozone on apples with and without condensate. The apples were inoculated with *Listeria* with one set being placed at 4°C for 12 hours with the other being held at 20°C. The apples were removed from 4°C then placed directly in the treatment chamber and ozone (6 ppm) applied for 20 minutes.

2.3.2 Commercial Scale Forced Air Ozone Generator Reactor

Apples were inoculated with 7 log CFU *Lactobacillus* suspension and transported in a cooler to facility. The reactor consisted of an ozone generator (Medallion Indoor Environmental, model 03-20-24 UV Ultra High Output –twenty 24" AT987 ozone lamps, 224/ 240-volt AC 50/60HZ 8 amp, max ozone output 161.2 g/h, maximum air capacity 1200 CFM) placed at the top of a 4.0' X 3.5' X 10.0' – stainless steel unit (assembled by the apple factory owner) which introduced ozone at a rate of 60 g/h (37 ppm) into the chamber. The apples within bins were held in a cooler prior to use and transferred to the treatment chamber directly to ensure condensate formed on the fruit surface. Two bins (3.9' x 3.3' x 2.5') of apples were used for each trial that were stacked on top of each other and wrapped with plastic film to contain the ozone within the apple stack. A seal was formed on the top of the bin by the lid of the ozone delivery nozzle with the air velocity being controlled by an exhaust fan positioned at the base of the reactor (Figure 4). The ozone concentration was measured at close proximity to the ozone exhaust port using an ozone monitor (2B Tech^{TM}, model 106-L, range 0-100 ppm ozone, accuracy 1.5 ppb). The concentration of ozone within the chamber ranged from 50 ppm -100 ppm. The treatment time and fan speed was set electronically along with an evacuation step upon completion of the process –a fan drawing the ozone through four 25W lamps (measured at 254nm at 100 hours and 80°F, 24" long and 15mm diameter - Standard UV lamps (serial # 05-1348). The inoculated apples were arranged at the top, middle or bottom of the chamber. Upon completion of the process the apples were removed and surviving *Lactobacillus* enumerated.

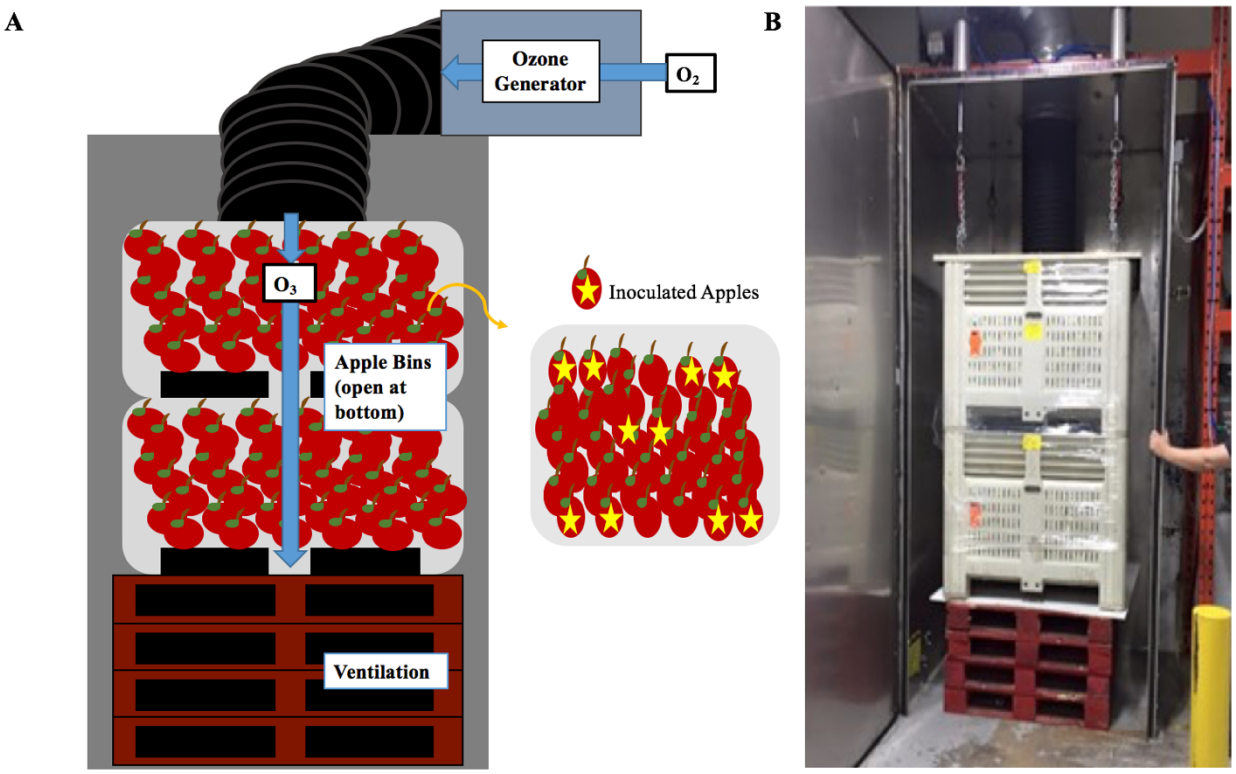

Figure 3: Commercial ozone treatment chamber and position of inoculated apples.

2.3.4 Hydrogen Peroxide: UV Reactor

The UV reactor consisted of an ultraviolet fixture (Sani-Ray™- stainless steel, 24" x 9" x 5", 120v 50/60Hz) containing 4 x 25W lamps (measured at 254nm at 100 hours and 80°F), 24" long 15mm in diameter, UV output of 8.5 when held a distance of 16 cm from the conveyer surface. Standard UV lamps (serial # 05-1348) and ozone lamps (# 05-1349, ozone output of 2.3) were both used (2 of each). A UVX radiometer (UVP™ calibrated to +/- 5% according to manufacturer instructions) was used to monitor the lamps intensity to ensure consistency. The hydrogen peroxide (obtained from Sigma-Aldrich™, 30% solution) was prepared at varying concentrations (2-4% v/v) and pre-warmed in a hot water bath to 22 or 48°C then placed in the reservoir of an atomizer/vaporizer. The treatment chamber was pre-warmed with a fan heater that was switched off just prior to placing the inoculated apples into the unit. The apples were held in the center of the unit for the allotted time period removed at the opposite end.

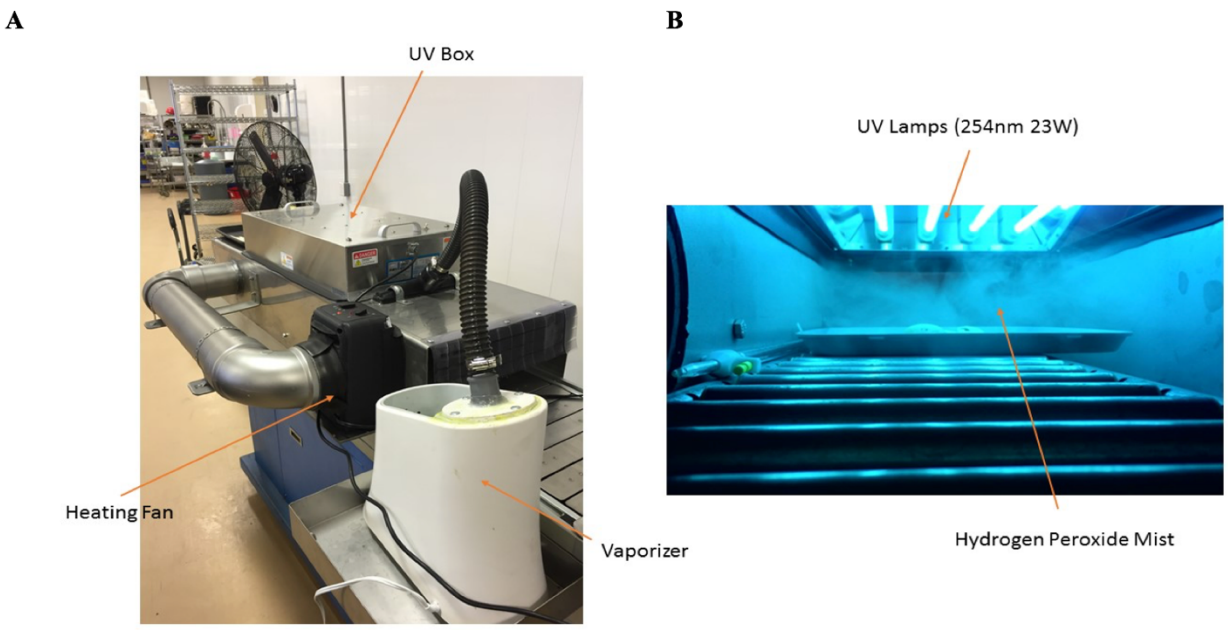

Figure 4: UV: hydrogen peroxide reactor. Controlled temperature, circulation and humidity (A), with UV lamps inside (B).

2.3.4 Peracetic acid

Peracetic Acid (obtained from Sigma-Aldrich™, 40% in acetic acid: water) was diluted to various concentrations from 20-70 ppm was used either as a wash or a spray.

2.3.5 Verification of Combined Ozone and AOP Process to Control *L. monocytogenes*

Apples were spot inoculated (5 $\log_{10}$ CFU/apple) at the calyx of apples with a five strain cocktail of *L. monocytogenes* as described above. The apples were transferred to a cold room and held overnight prior to treatment. Batches of 13 apples were placed in the ozone reactor and treated for 40 minutes. The apples were then dried for a further 50 minutes without ozone then directly transferred to the AOP unit. The AOP treatment was performed placing the apples within the chamber with the calyx facing the UV-C: Ozone lamp. Hydrogen peroxide vapor was generated from a 6% v/v solution pre-warmed to 48°C. Treatment was performed for 30 seconds after which a wooden stick was inserted into the calyx before coating with caramel maintained at 80°C. For the red apples, an additional chocolate layer was added. The apples were then stored on trays within a room maintained at 22°C. Control groups of apples were prepared at the same time as apples to be treated, and stored in the same manner. However instead of being treated with ozone and hydrogen peroxide, they were only exposed to air and water (at otherwise same flow rates and temperatures to mimic the process).

Periodically, candy apples (n=3) were transferred for microbiological analysis. Here, the core was removed using a sterile corer and placed in a sterile bag then re-suspended in One-step enrichment broth to a 1:10 dilution. The core was homogenate in a stomacher for 60 seconds. The part of the stick that was embedded in the apple was manually massaged in the homogenate

to release any attached *Listeria*. The remainder of the apple was submerged in 100 ml of One step enrichment broth and manually massaged to release the candy: caramel layers.
The samples were plated onto Modified Oxford Formula media that was incubated at 30°C for 48 hours. In parallel, the homogenates were enriched at 37°C for 24 hours then streaked onto MOx agar that was incubated at 30°C for 48 hours. A presumptive positive colony from each plate was subjected to confirmation using API test strips.

2.4 Bacteria Recovery and Enumeration

2.4.1 Lettuce

After treatment, lettuce heads were chopped, suspended in 500 ml of saline and stomached for 1 minute, a dilution series was prepared in saline. To enumerate STEC, the samples were then spread plated onto MacConkey Sorbitol agar (CT-SMAC) and chromogenic culture media (CHROMagar) incubated at 37°C for 24 hours. *L. monocytogenes* was plated onto Modified Oxford Agar (MOX) incubated at 35°C for 24 – 48 hours.

2.4.2 Apples

After having challenges recovering pathogens from apples in the same manner as lettuce, baseline studies were performed in order to determine the optimal method for recovering *Listeria* from the surface of apples. The apples were spot inoculated with 100 µl of *Listeria* [8-log CFU] then allowed to attach for 4 hours. The *Listeria* was then recovered by one of three methods to evaluate the efficiency of each method. The methods were as follows: method (1) whole apples were placed in sterile plastic pouches and suspended in 100 ml of saline and manually rubbed for 1 minute. For method (2) a peeler was used to remove the apple peel which was then placed in 50 ml of saline and vigorously shaken for 1 minute. Lastly, method (3) was the same as

described for (2) except the peel was homogenized using a lab top blender. Regardless, of the method of recovery, a dilution series was prepared in saline then spread plated onto Modified Oxford Agar (MOX) incubated at 35°C for 24 – 48 hours. Presumptive positive colonies were counts being reported a log CFU.

2.4.2.1 Effect of *Listeria* Incubation Temperature on Attachment

To determine if the incubation temperature of *Listeria* is important for its attachment to apples, the bacteria was cultivated at both 25°C (were *Listeria* express flagella) and at 37°C (i.e. no flagella expressed). The bacteria were allowed time to adhere to the apple before being removed (method 1) as described above.

2.5 Statistical Analysis

Each experiment was repeated at least three times with triplicate samples being analyzed. The bacterial counts transformed into $\log_{10}$ values with differences between means performed using ANOVA in combination with the Tukey test.

3 Results

3. 1 Suitability of *Lactobacillus fructivorans* as a Surrogate for *Listeria monocytogenes*

The relative resistance of *Lactobacillus* to ozone compared to *L. monocytogenes* was assessed using inoculated apples placed inside a biobubble in which the antimicrobial gas was introduced. It was found that the extent of inactivation of *Lactobacillus* and *L. monocytogenes* by ozone treatment was dependent on the applied time (ozone concentration). In relative terms there was no significant difference (P>0.05) in the log reductions of *L. monocytogenes* compared to *Lactobacillus* receiving the same ozone exposure (Figure 5). Therefore, the *Lactobacillus* strain is a suitable surrogate for *L. monocytogenes* that can be applied in commercial trials for accessing the efficacy of ozone treatment.

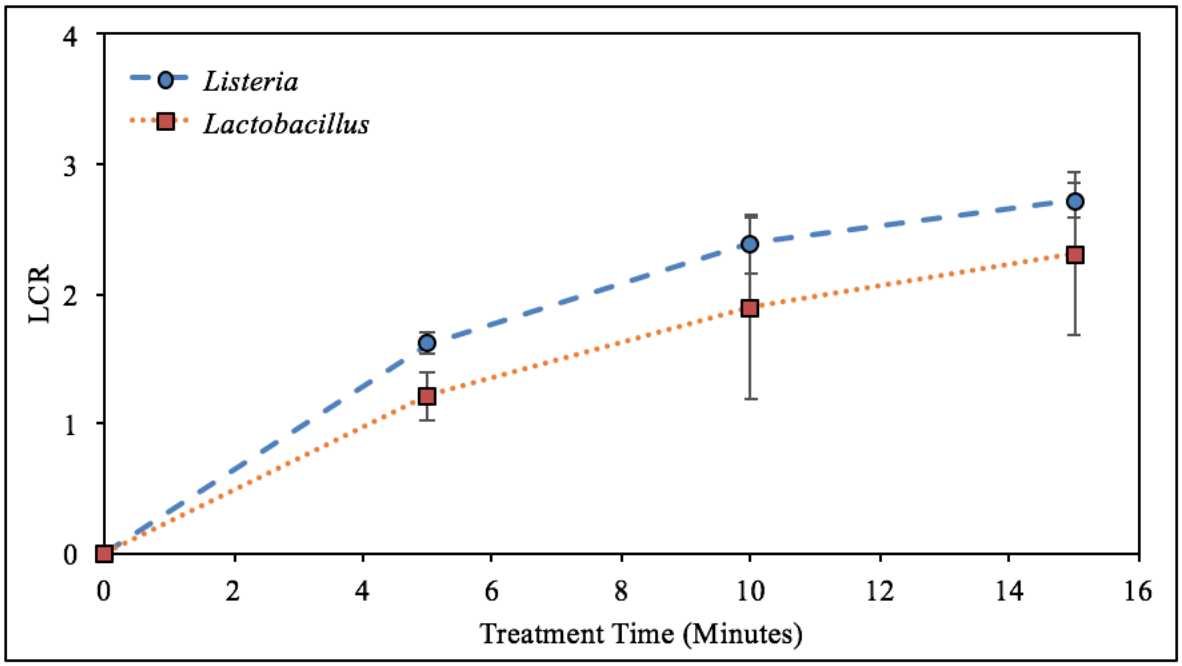

Figure 5: Log reduction of *Listeria monocytogenes* and *Lactobacillus* inoculated onto apples then treated with ozone introduced at a rate of 6g/h for different time periods. At five minutes of exposure ozone concentration measured 30 ppm ± 2, at ten minutes 55 ppm ± 2, and at fifteen minutes 77 ppm ± 2.

3.2 Effect of Air Flow Direction on the Efficacy of Ozone to Inactivate *Listeria monocytogenes* Inoculated into Apples

Inoculated apples were placed in the laboratory scale reactor then treated with ozone either by the gas being introduced at the top or bottom of container. The relative humidity was held between 65-85% relative humidity and treatment time set for 20 minutes (Table 2).

It was found that the log count reductions of *Listeria* was independent on the position of the apple within the pile and also if the ozone was introduced at the top or base of the bed (Table 2). The results indicate that ozone can successfully infuse through the apple bed thereby enabling uniform contact with the fruit regardless of the air flow direction.

Table 2: Average log count reductions of *Listeria* inoculated onto apples then treated in the Top or Bottom reactor as described in Figure 3. Treatment was performed for 20 min with apple fruit initially stored at 4°C prior to loading into the reactor. Here the mean of the samples is reported followed by the standard error (the standard deviation divided by the square root of the sample size n, where n is ≥ to 3).

Location of inoculated apple within the batch	Upward Ozone Flow	Downward Ozone Flow
	Listeria Log Count Reduction	
Bottom	2.12±0.94[a]	2.54±0.37[a]
Middle	2.62±0.80[a]	2.63±0.96[a]
Top	2.55±0.25[a]	3.07±0.45[a]

Means followed by the same letter are not significantly different.

Although the contact of ozone with apples was independent of the location of fruit within the pile there were differences with respect to the temperature profiles of fruit within the bed. Specifically, apples that received the incoming ozone stream equilibrated with room temperature quicker than those at the base (Figure 5). As maybe expected, the temperature at 0.5 cm depth of

apples increased more rapidly compared to 1 cm into the fruit (measured as described previously). The significance of the result is that the surface of the apple would retain moisture (condensation) provided a temperature differential with room temperature exists. The rapid temperature increase of apples at the top of the pile would cause a decrease in condensation compared to those at the base that in turn could reduce the efficacy of ozone. However, this was not the case according to comparable log count reductions of *Listeria* that was obtained irrespective of the position of the apple within the bed. It is possible that the surface apples would be exposed to a higher concentration of ozone that would compensate for the decrease in surface moisture.

The apples at the bottom of the column took longer to reach ambient and still had moisture on the surface at the end of the treatment period (Figure 6). Those apples on the top closest to the exhaust reached ambient temperature more rapidly with no visible residual moisture on the surface. Therefore, for conditioning apples, a 60 minute treatment would be required that is well over the 20 minute required for ozone treatment required to inactivate *Listeria*.

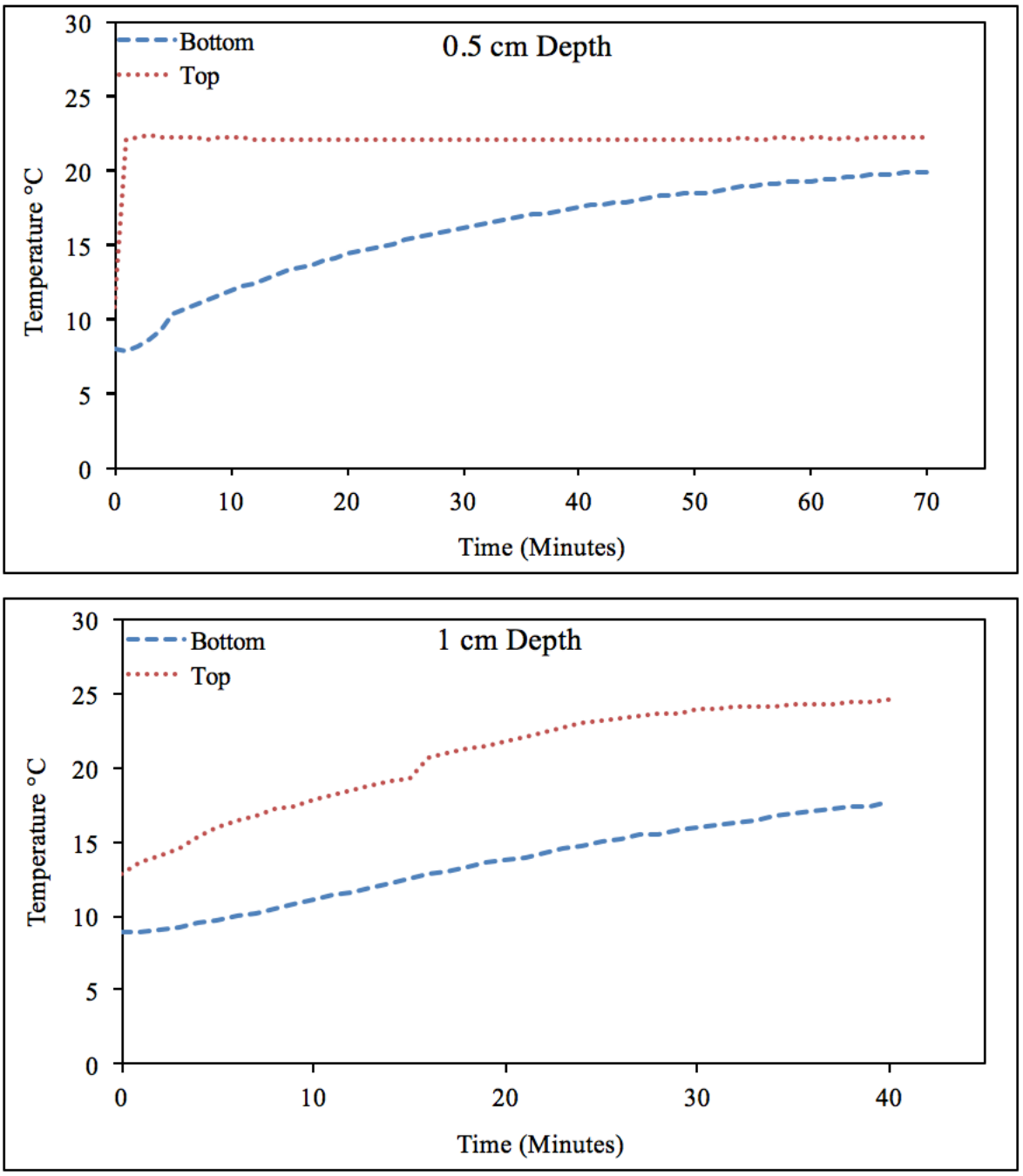

Figure 6: Temperature profiles of the subsurface of apples at the top or bottom of the apple column in the laboratory scale reactor. The ozone was introduced at the top and drawn through the apple pile. The ambient temperature within the reactor was 23°C.

3.3 Commercial Scale Forced Air Ozone Reactor

A commercial scale reactor was constructed as described earlier by the apple producer, based on the findings of the laboratory trials. From an engineering prospective it was easier to introduce the ozone at the top of the unit then draw it down through the apple pile and exhaust at the bottom of the chamber. In validation trials, the inoculated apples were place in different positions in the apple pile to determine if the ozone treatment was being applied uniformly onto the apples. The ozone concentration was determined by ozone monitors as described above and the airflow monitored with an anemometer (as described above) which measures a combination of air velocity and volumetric measurements over a set period of time, in Cubic Feet per Minute (cfm), in which 1 cubic foot equals approximately 28 liters.

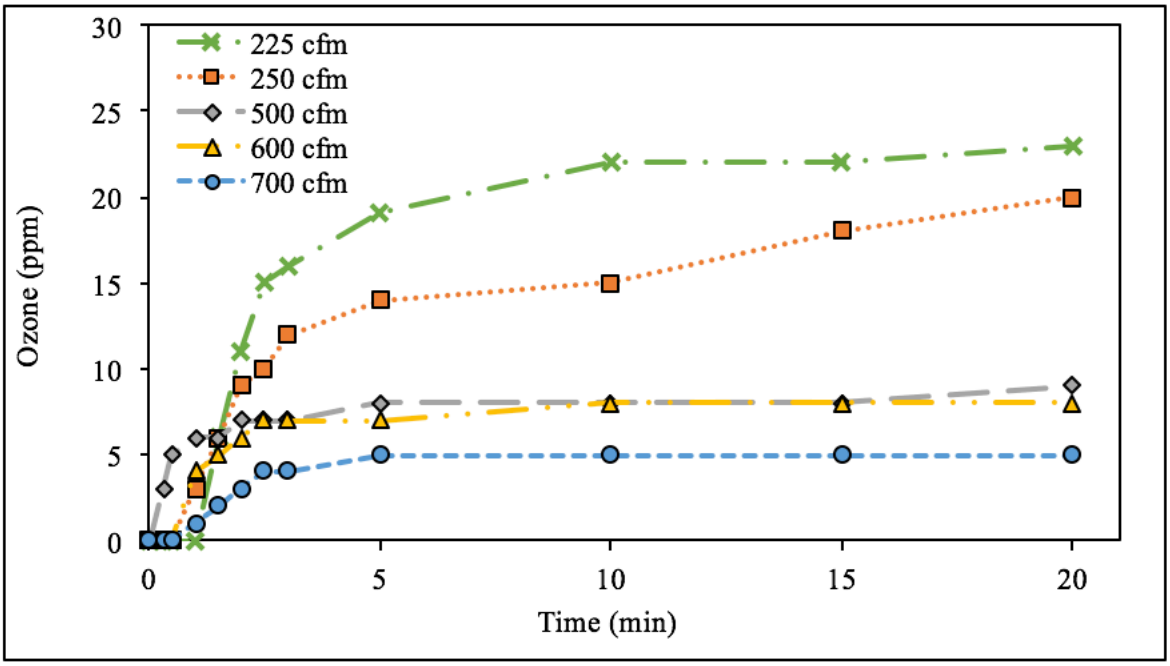

Figure 7: Effect of exhaust air velocity (cubic feet per minute) on the ozone concentration within the forced air ozone reactor. Two bins of apples were placed in the reactor and speed of the exhaust air fan set to give different air velocities. The ozone was introduced at the top of the reactor and measured after passing through the apple bed. The treatment was performed for a 20 minute period with the ozone concentration being logged every 30 seconds.

The air velocity at different parts of the reactor were measured using an air flow meter placed at different positions. By using a set air flow setting at 500 cfm the intake at the ozone inlet was 0.08 m^3/s that decreased to 6.6 x 10^{-3} m^3/s at the bottom of the apple pile and 0.27 m^3/s at the air exit. The change in air velocity at different parts of the reactor is reflective of the diameter/area of the inlet, bed and outlet. The ozone concentration measured near the air exhaust port was dependent on the air velocity (Figure 7). At low exhaust air velocity, the ozone concentration stabilized 10 minutes into the run and attained the highest gas concentration. As the air velocity increased the level of ozone within the chamber decreased as did the time to achieve stable concentrations of the antimicrobial gas. At the highest exhaust air velocity (700 cfm) the ozone concentration recorded was 4 ppm that was significantly lower compared to when slower fan speed was applied.

At low fan exhaust air velocity, the log count reduction of *Lactobacillus* inoculated onto apples was dependent on the position of the apple within the pile (Figure 8). Specifically, a significantly higher log count reduction was obtained for those apples at the top of the pile compared those at the base. However, as the air velocity increased beyond 500 cfm there were no significant differences in terms of log count reduction of *Lactobacillus* at the top compared to the bottom of the apple pile. At the highest fan speed tested (700 cfm) the log reductions of *Lactobacillus* were significantly lower at the top of the apple pile compared to those positioned in the middle or at the bottom of the pile. Hence, the optimal exhaust air velocity is within the 500 – 600 cfm range.

The effect of air exhaust velocity is likely due to a combination of ozone concentration and the dynamics of flow around the apple pile. At low exhaust fan speed the ozone would primarily accumulate at the top of the bed then slowly pulled through. As the fan speed increases the air

being pulled through the generator dilutes the concentration of ozone but the flow through the bed is more homogenous. At the highest fan speed (700 cfm) the air being pulled through the ozone unit causes high dilution to the point that the concentration reduces in biocidal activity although can accumulate in the main body of the apple pile. Regardless of this fact, the optimal air exhaust speed lies between 500 – 600 cfm (Figure 8).

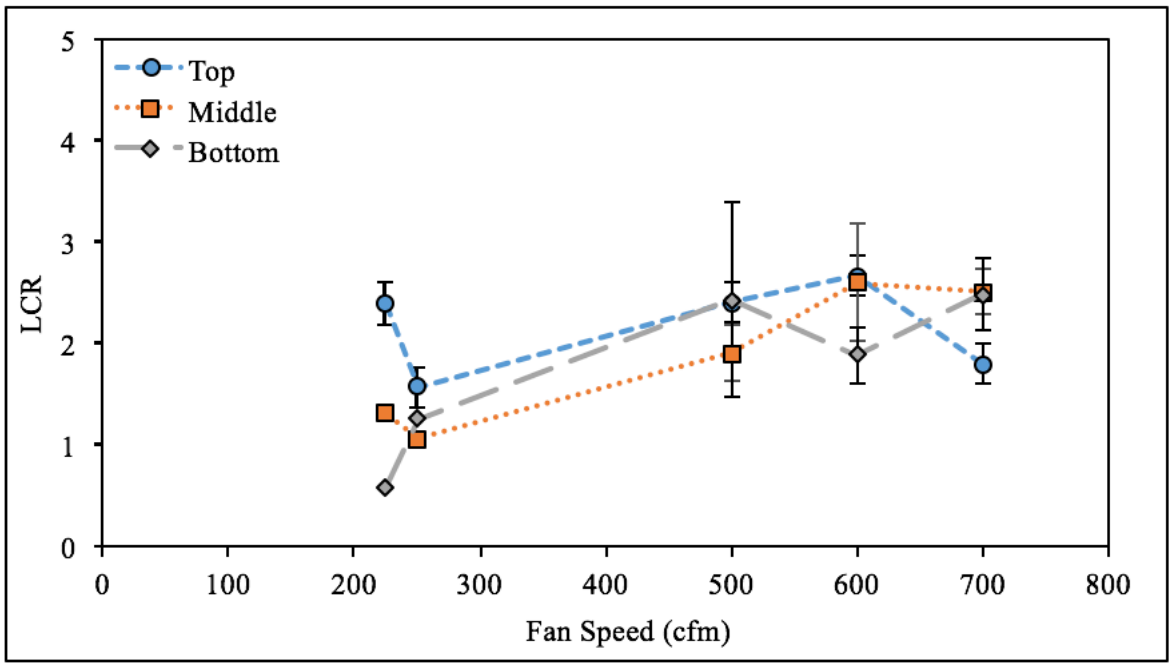

Figure 8: The Log count reduction of *Lactobacillus* on apples placed at the Top, Middle or Bottom within a forced air ozone reactor operating under different air exhaust velocities. Apples were spot inoculated with *Lactobacillus* around the stem end the 5 fruit placed on top level of the apple pile, 5 in the middle and 5 under the bottom bin. Ozone was introduced at the top and drawn through the apple pile (2 bins) at different rates set by the exhaust fan. After 20 min treatment the apples were removed and *Lactobacillus* recovered.

Trials were performed using an exhaust air velocity of 500 cfm to assess the effect of treatment time on the efficacy of the ozone mediated inactivation of *Lactobacillus* inoculated onto apples (Figure 9). It was found that 6 or 10 min treatment times were not significantly different compared to controls, where air was pulled through the apple pile without ozone

(0.19±0.29 log CFU reduction). However, treatment times >20 minutes supported a log reduction that was significantly greater compared to lower times. Increasing the treatment time to 40 minutes did not significantly increase the recorded log count reduction.

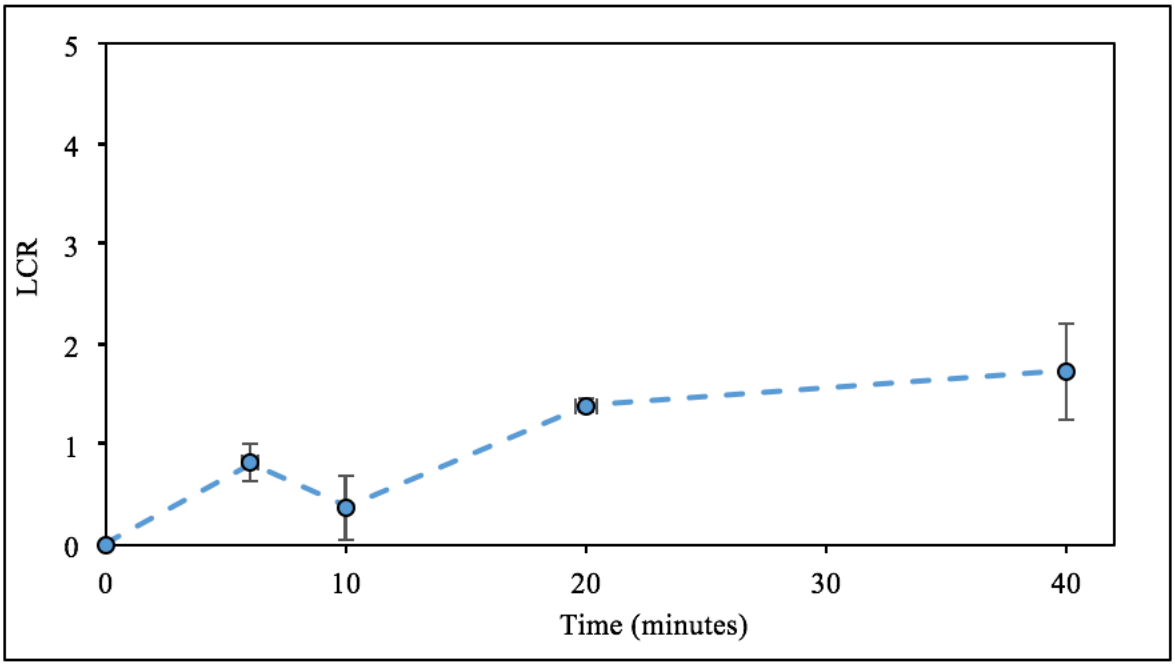

Figure 9: Effect of treatment time on the log reduction of *Lactobacillus* inoculated onto apples then treated within a forced air ozone reactor. The apple fruit were position at the top, middle and bottom of the apple pile (2 bins) then treated with ozone using an air exhaust fan speed of 500 cfm.

3.4 Optimization of UV: Hydrogen Peroxide Treatment

Baseline studies determined the effect of time, hydrogen peroxide concentration (1-1.5%) (22°C to 48°C) and temperature on the decontamination of apples inoculated with *L. monocytogenes*. To increase the sensitivity of the assay and avoid geometric effects, apple halves were used as opposed to whole apples. From the results it was found that UV alone supported a high log reduction of *Listeria* compared to when used in low concentrations of hydrogen peroxide (Table 3). The results can be attributed to the direct inactivation of *Listeria* in the absence of shading effects.

With hydrogen peroxide alone, negligible log reductions of *Listeria* were observed although the efficacy could be enhanced by operating the unit at 48°C compared to 22°C. A more distinct effect of temperature was observed when UV was combined with hydrogen peroxide (Table 3). Here, there were significantly higher log reductions when the unit was run at 48°C compared to 22°C. The highest log reduction was obtained with using a hydrogen peroxide concentration of 1.5% v/v introduced into the unit at 48°C with a 30 seconds residence time. The results can be explained by the AOP proceeding to a greater extent at 48°C compared to 22°C. At lower concentrations of hydrogen peroxide the lower observed lethality was due to insufficient radicles being formed from the UV decomposition of H_2O_2. Yet, the presence of hydrogen peroxide was sufficient to absorb the UV photons thereby providing a protective effect to the *Listeria* on the surface of apple.

Table 3: Inactivation of *Listeria monocytogenes* on inoculated apple halves by using different UV: hydrogen peroxide (1 – 1.5% v/v) combinations. Here the mean of the samples log count reduction is reported followed by the standard error.

		Initial Loading Log CFU	Log Count Reduction	
Treatment	**Time**		**22°C**	**48°C**
None		7.34±0.12		
UV	15s		>3.04	
	30s		>3.04	
H_2O_2 1%	15s		-0.95±0.19	0.97±0.70
	30s		-0.44±0.13	0.38±0.11
H_2O_2 1.5%	15s		0.14±0.18	-0.21±0.01
	30s		-0.02±0.29	2.78±1.01
UV:H_2O_2 1%	15s		1.29±0.20	2.75±0.38
	30s		0.92±0.06	>3.08
UV:H_2O_2 1.5%	15s		1.71±0.71	3.64±0.67
	30s		2.25±0.83	>4.40

3.5 Inactivation of *Listeria monocytogenes* Introduced on and Within Whole Apples

In practical terms, the treatment of whole apples is challenging due to the shading caused by the physical shape of the apple, in addition to *Listeria* being potentially present within the sub-surface of core. Because of the mentioned shading effects, the use of UV alone would be limited. The was indeed found in the current case where UV applied to inoculated whole apples resulted in < 1 log CFU reduction of *Listeria* on the surface of apples and no average reduction in levels of the pathogen within the internal core (-0.21±0.69). When UV was used in combination with hydrogen peroxide the LCR of *Listeria* was increased up to 4% v/v H_2O_2 beyond which did not support higher reductions of the pathogen (Figure 10). However, there was a correlation between hydrogen peroxide concentration and reduction in *Listeria* introduced into core tissue of apples (Figure 10). Here it was found that 6% hydrogen peroxide used in combination with UV could support a 0.86 log CFU reduction in *Listeria* levels that represents a reduction of 84% of the original population.

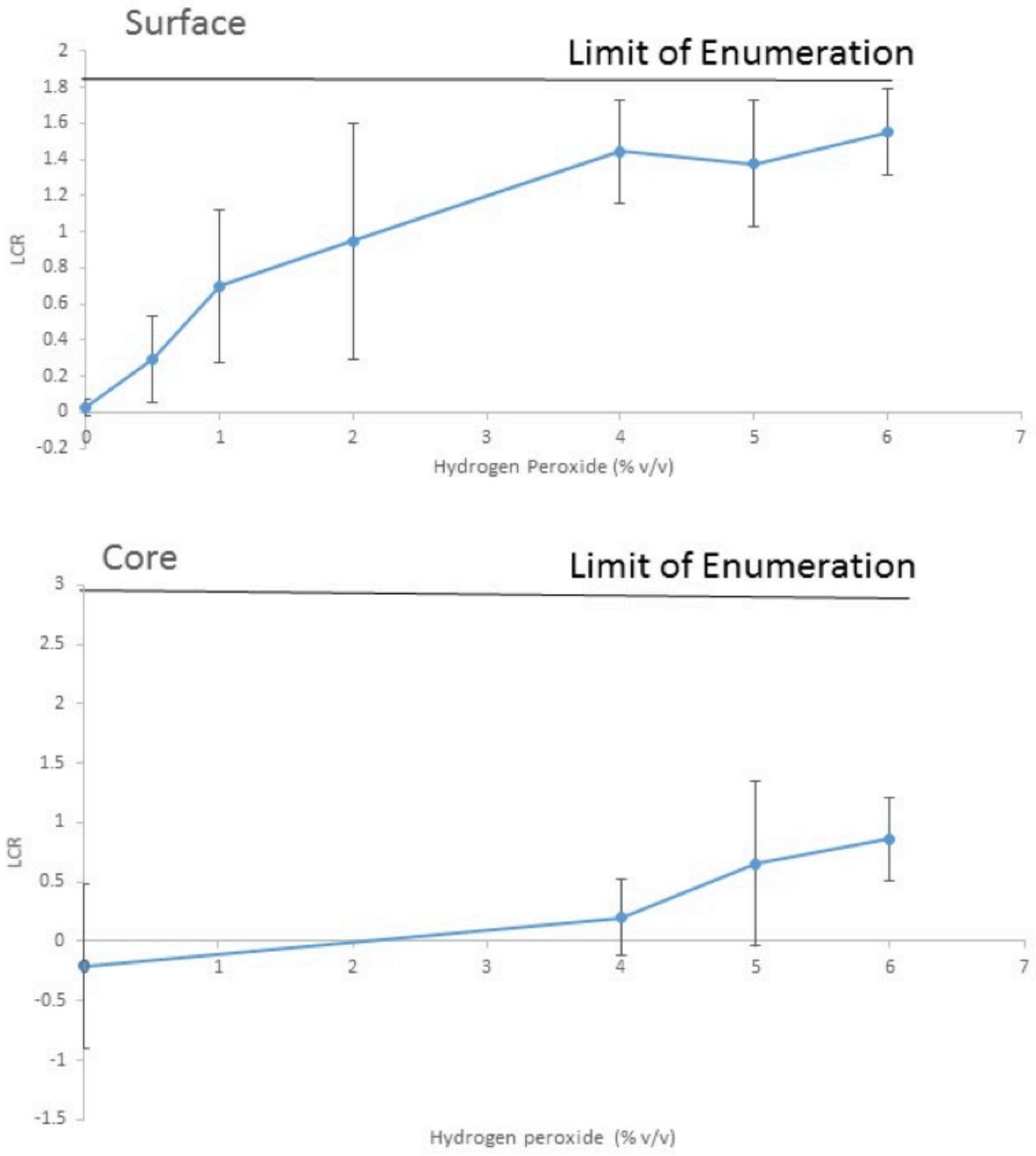

Figure 10: Inactivation of *Listeria monocytogenes* on and within apples by UV: hydrogen peroxide from 1 – 6%. Inoculated apples were placed in the chamber and different concentrations of hydrogen peroxide delivered. All treatments were performed for 60 seconds at 48°C.

3.6 Efficacy of a Combination of UV, Hydrogen Peroxide and Ozone to Decontaminate Apples

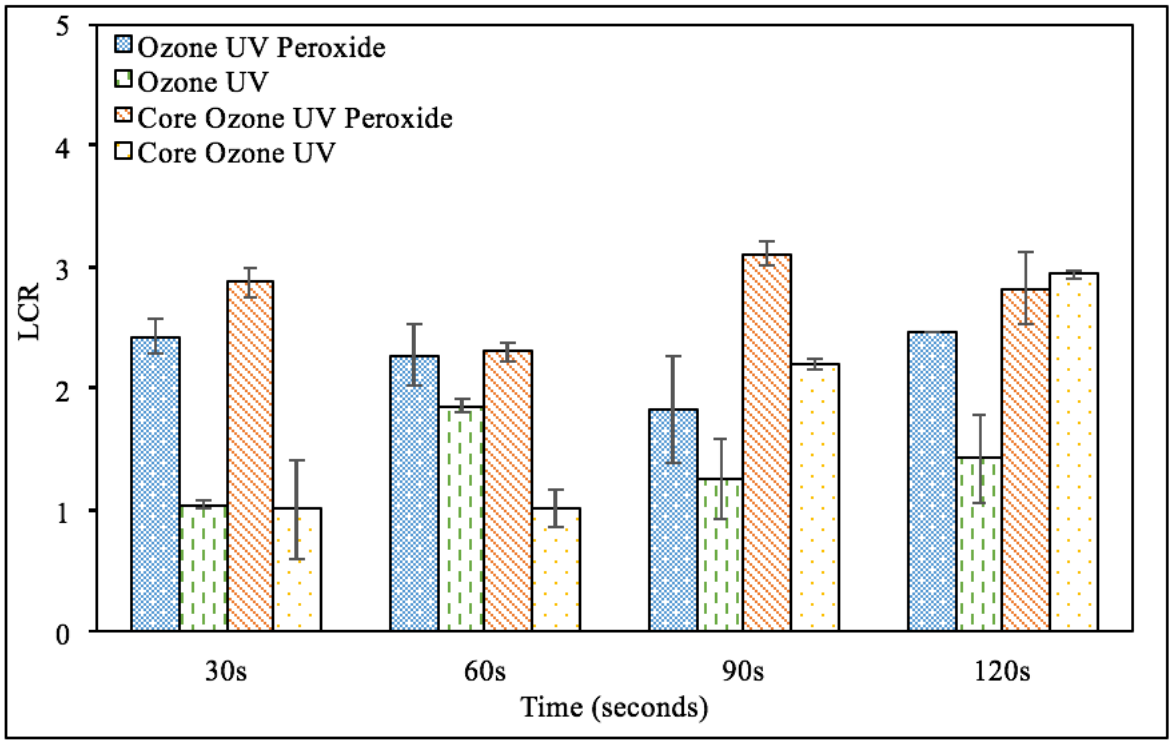

Figure 11: Inactivation of *Listeria monocytogenes* on the surface and core of apples using UV: ozone with or without 6% hydrogen peroxide at 48°C from 30 to 120 seconds.

Ozone was generated by replacing one of the UV-C lamps with one that emits at 174 nm with hydrogen peroxide being introduced via a vapor at 48°C. It was found that for a 30 second treatment time the reduction of *Listeria* on the surface of apples was significantly higher when UV: ozone: peroxide was applied compared to UV: ozone treatment. When longer treatment times were applied the extent of inactivation of *Listeria* on the surface of apples was not significantly different between UV: Ozone: peroxide and UV: ozone (Figure 11). The log reduction of *Listeria* on the surface of apples was independent of the treatment time suggesting that the residual survivors were in protective niches.

Similar to the trend of log reductions on the surface of apples, the inactivation of the pathogen within the core was significantly higher for UV: ozone: peroxide compared to UV: ozone at 30 second treatments (Figure 11). However, the extent of *Listeria* inactivation by UV: ozone: peroxide did not increase with extended treatment times. In contrast, the log reduction of *Listeria* within the core by UV: ozone did increase with time and was not significantly different from UV: ozone: peroxide at 120 seconds. The results would suggest that the action of UV: ozone: peroxide results in rapid inactivation of *Listeria* compared to when UV: ozone are applied without H_2O_2.

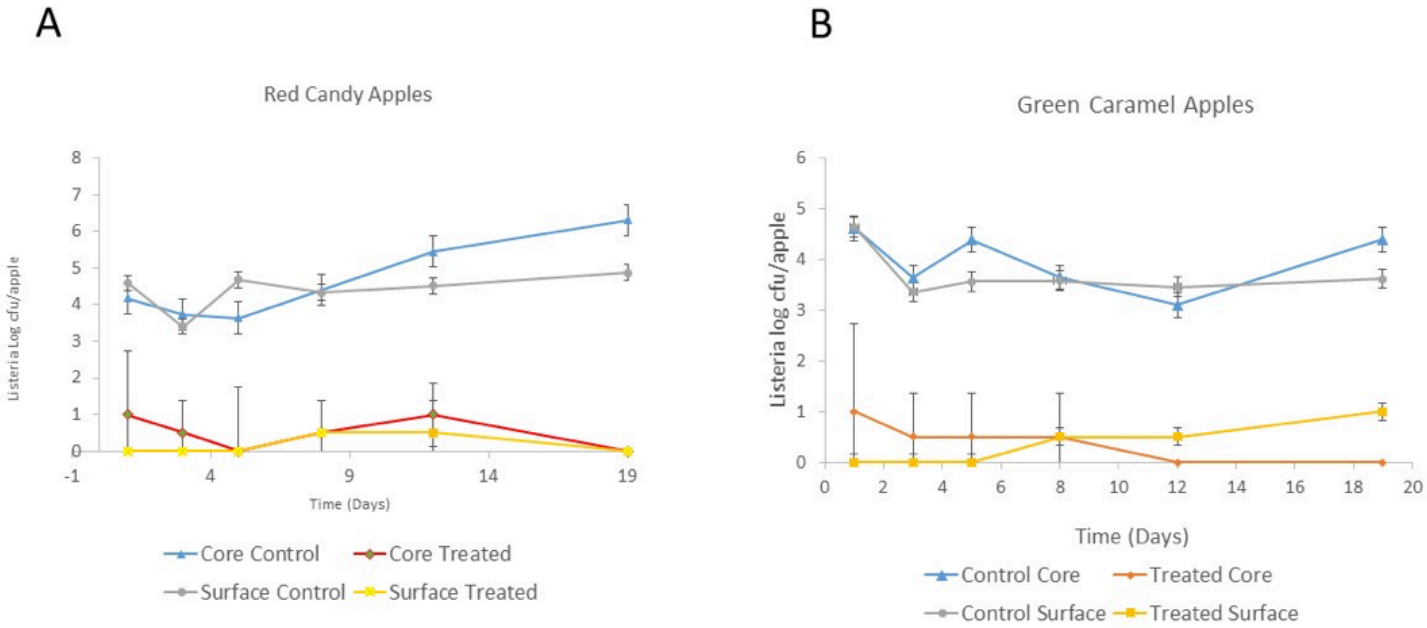

Figure 12: *Listeria monocytogenes* counts on the surface and core of candy apples stored at 22°C. The apples were inoculated with *Listeria* the treated using a combination of ozone (up to 50 ppm) followed by AOP. The apples were then coated with caramel-chocolate – red apple (A) or caramel- green apple (B) with 3 units of each being removed at the different sampling points.

Table 4: *Listeria monocytogenes* recovered from Candy Apples over a 19 Day Shelf-life at 22°C. Data from Figure 13.

A) Caramel Chocolate – red apples

Storage Day at 22°C	Log CFU/Apple (Positive by enrichment/Total tested)			
	Candy Apples from Non-treated (control) Red Apples		Candy Apples from Ozone & AOP Treated Red Apples	
	Surface	**Core**	**Surface**	**Core**
1	4.60±0.01	4.17±0.60	0 (0/3)	1.00±1.73 (1/3)
3	3.40±0	3.73±0.92	0 (0/3)	0.50±0.87 (1/3)
5	4.68±0.12	3.64±0.38	0 (0/3)	0 (0/3)
8	4.33±0.06	4.39±0.16	0.5 (1/3)	0.50±0.87 (1/3)
13	4.51±0.31	5.45±0.73	0.5 (1/3)	1.00±0.87 (2/3)
19	4.88±0.09	6.31±0.09	0 (0/3)	0 (0/3)

B) Caramel – green apples

Storage Day at 22°C	Log CFU/Apple (Positive by enrichment/Total tested)			
	Candy Apples from Non-treated (control) Green Apples		Candy Apples from Ozone & AOP Treated Green Apples	
	Surface	**Core**	**Surface**	**Core**
1	4.64±0.15	4.63±0.48	0 (0/3)	1.0±1.73 (1/3)
3	3.36±0.12	3.64±0.38	0 (0/3)	0.5±0.87 (1/3)
5	3.57±0	4.39±0.52	0 (0/3)	0.5±0.87 (1/3)
8	3.58±0.21	3.65±0.16	0.5±0.87 (1/3)	0.5±0.87 (1/3)
13	3.46±0.12	3.10±0.21	0.5±0.87 (1/3)	0 (0/3)
19	3.63±0.16	4.40±0.19	1.00±0.87 (2/3)	0 (0/3)

The *Listeria* levels on control non-treated apples (green and red) was 5 log CFU and numbers decreased by 0.4- 0.9 log CFU by the caramel coating process. In candy apples prepared from non-treated green apples, *Listeria* counts at the end of the 19-day shelf-life did not significantly ($P>0.05$) compared to Day 1 (Figure 12A; Table 4A). However, *Listeria* with the core of non-treated apples did increase in levels following a 3-day lag period and was significantly ($P<0.05$) higher at the end of the 19-day shelf-life (Figure 12A; Table 4A). *Listeria* counts on the surface of candy apples prepared from untreated green apples decreased by approximately 1 log CFU over the initial 3-day shelf-life but then remained constant for the remaining 16-day storage period (Figure 12B; Table 4B). The *L. monocytogenes* counts within the core of candy apples stored at 22°C fluctuated over the 19-day shelf-life with no overall significant change in counts at the end, compared to Day 1 (Figure 12B; Table 4B).
No *Listeria* was recovered from candy apples prepared from green or red fruit treated with Ozone then AOP (Figure 12; Table 4). With regards to the core samples, for both red and green apples, two of the three replicates tested negative for *Listeria* by enrichment following ozone and AOP treated (Figure 12; Table 4). Therefore, the overall log reduction of *Listeria* was 4-5 log CFU/apple in the case of both candy apple varieties.

In the course of storage, *Listeria* was sporadically recovered from the surface of candy apples prepared from treated red apples but levels of the pathogen did not increase in numbers. *Listeria* within the core of red apples was sporadically recovered over the 19 Day storage period with no overall increase in numbers being observed (Figure 12A; Table 3A). With treated green apples, surface counts on candy apples increased following after storage Day 5 attaining 1 log CFU/apple at the end of the 19-day storage period (Figure 12B; Table 4B). In contrast, surviving

Listeria within the core of green apples decreased over the shelf-life with none of the samples taken passed Day 8 testing positive for the pathogen (Figure 12B; Table 4B).

3.7 Decontamination of Lettuce Heads Using Gas Phase Interventions

The rational of the research approach was to inactivate pathogens on lettuce heads with the assumption that contamination would be restricted to the surface. This in effect would reduce the reliance of post-harvest washing and also minimize the risk of disseminating pathogens through the processing line. The two treatments evaluated were ozone and a treatment based on using a combination of hydrogen peroxide and UV (Advanced Oxidative Process).

3.7.1 Hydrogen Peroxide: UV

Lettuce heads were placed in the UV reactor chamber and sprayed with hydrogen peroxide (2 or 4%) with and without illumination with UV-C. As a control group inoculated apples were treated with H_2O vapor in place of the H_2O_2. The exact amount of vapor that comes in contact with the product is impossible to pin point exactly due to the nature of the produce conveyer type system where vapor enters a chamber from above as the produce passes, however after the process is finished the produce has been dried from the circulation fans. was Although 4% H_2O_2 is more effective in reducing *E. coli* on whole lettuce heads for shorter term treatments, 2% achieved higher reductions after 2 minutes (Figure 13). When replacing H_2O_2 with water, as a control, the treatment is just as effective. Illumination with UV alone supported the lowest log reductions with no increase in efficacy with treatment times > 60 seconds. Testing the treated apples with a catalase assay, there was no hydrogen peroxide residues detectable (level of detection >10ppm).

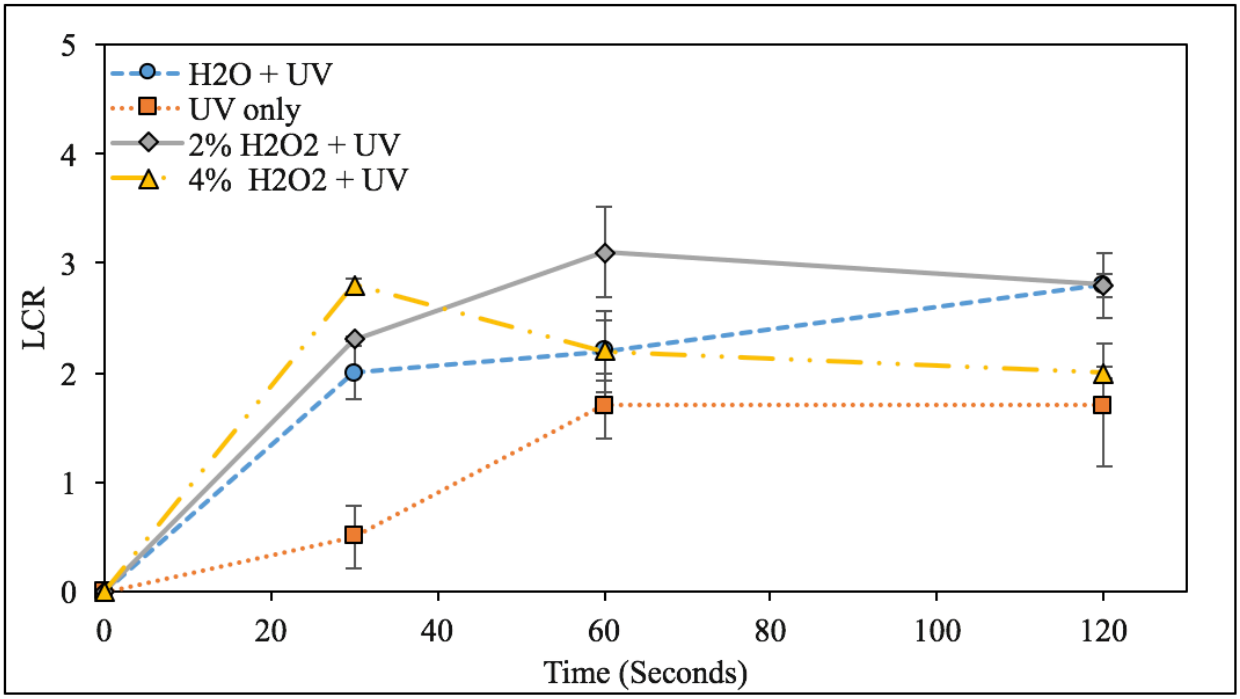

Figure 13: Log count reduction of *E. coli* on whole lettuce heads treated with hydrogen peroxide vapor (2 and 4%) for 30, 60, and 120 seconds. The log count reduction (LCR) was calculated by subtracting the average log survivors (3 units of each being removed at the different sampling points) from those recovered on non-treated fruit.

3.7.2 Ozone

Ozone applied under high humidity conditions resulted in the highest log reductions (just over 1 log CFU/g), slightly more than AOP process under the same humidity conditions (Figure 14). Overall the treatment achieved minimal log reductions.

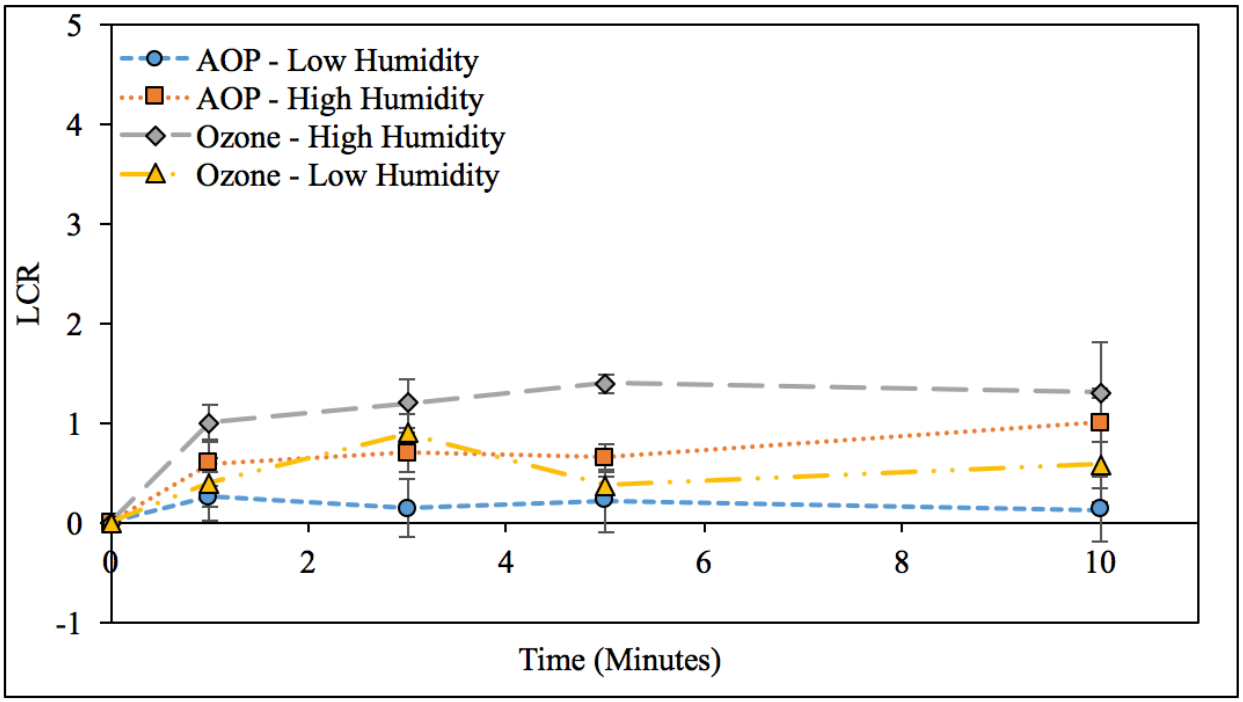

Figure 14: Log count reduction of *E. coli* on whole lettuce heads treated with ozone and AOP at high and low humidity. The log count reduction (LCR) was calculated by subtracting the average log survivors (3 units of each being removed at the different sampling points) from those recovered from non-treated fruit.

3.7.3 Efficacy of a Combination of UV, Hydrogen Peroxide, Ozone and Chlorine dioxide to Decontaminate Lettuce

A combined sequential treatment of ozone, 6 % H_2O_2 and 50 ppm chlorine dioxide achieved nearly 4 log CFU/g reduction (Figure 15). The combined treatment process (Figure 16) resulted in lettuce with very low *E. coli* levels, which was maintained throughout the 10-day shelf life, when compared to the untreated group which maintained a steady level of the pathogen (Figure 16).

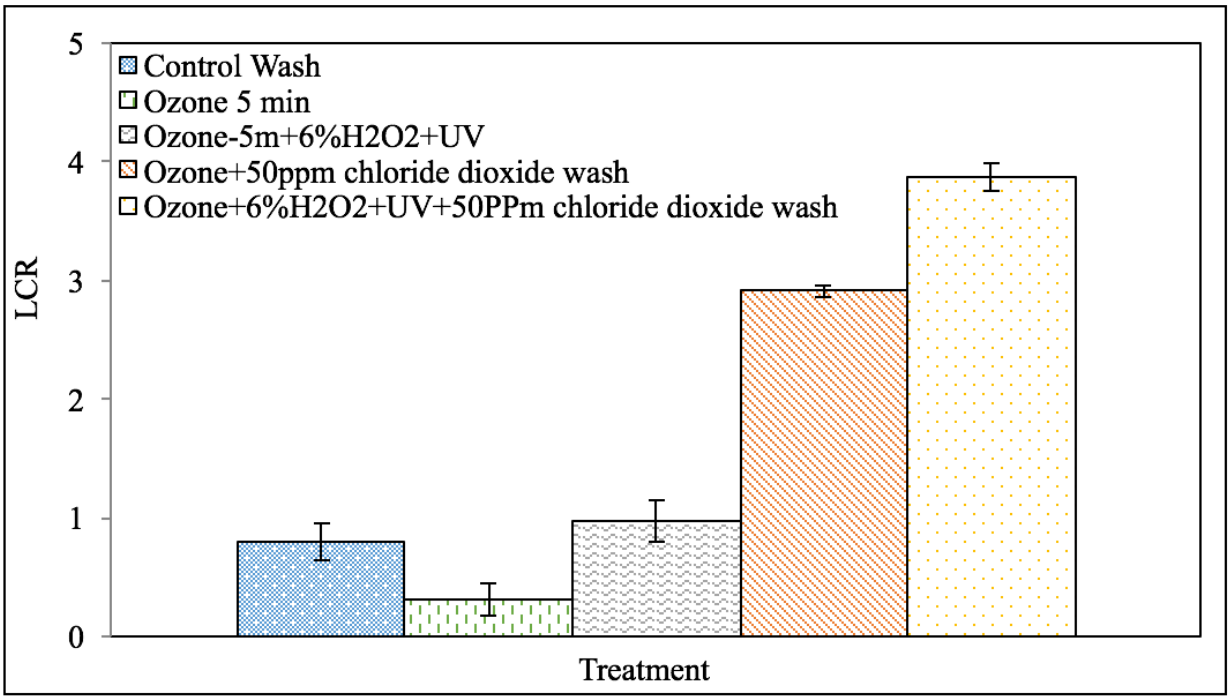

Figure 15: Average log count reductions of *E. coli* on whole lettuce heads treated with ozone gas for 5 minutes alone, and in combination with H_2O_2, UV, and chlorine dioxide. The log count reduction (LCR) was calculated by subtracting the average log survivors (3 units of each being removed at the different sampling points) from those recovered on non-treated fruit.

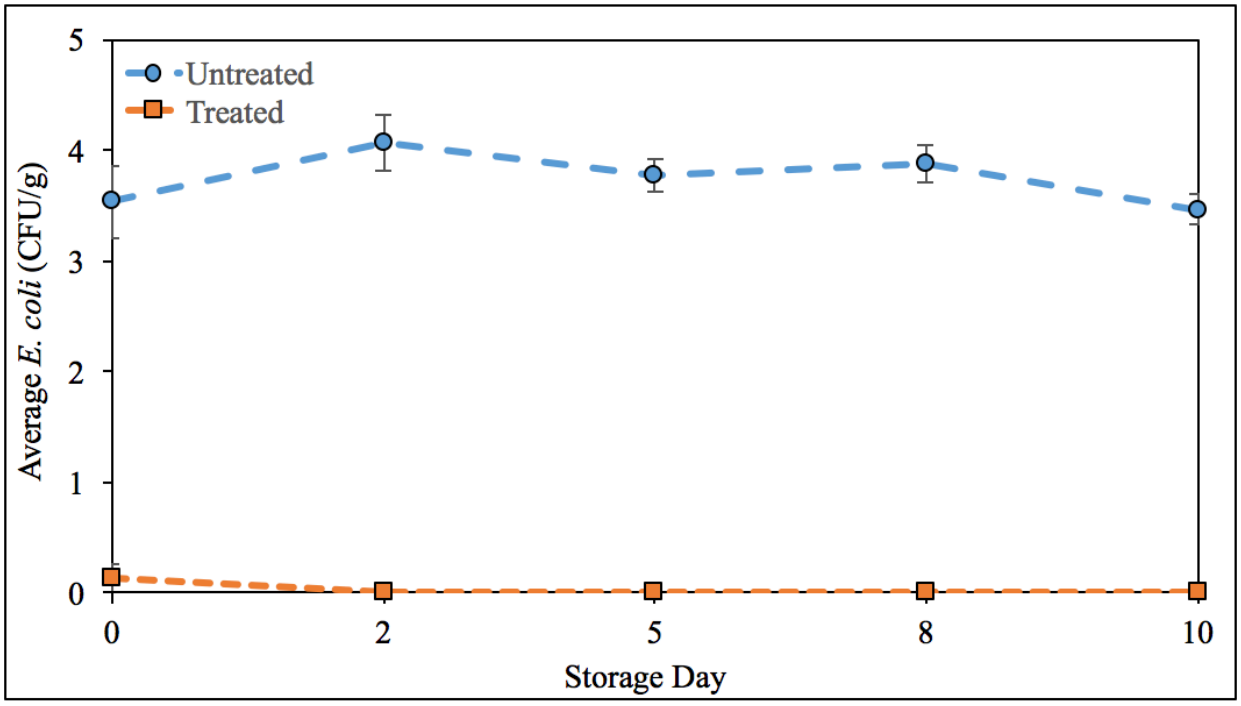

Figure 16: Shelf-life of whole heads of lettuce inoculated with *E. coli*, treated with ozone gas for 5 minutes alone, and in combination with H_2O_2, UV, and chlorine dioxide (Figure 15) as well as an untreated control group at storage days 0, 2, 5, 8, and 10.

4 Discussion

The research evaluated how a combination of intervention methods could collectively reduce the carriage of human pathogens of apples destined for candy apple production and lettuce used to produce bagged salad. An initial ozone treatment increased the susceptibility of *Listeria* and *E. coli* to biocidal washing followed by an AOP based process. Ozone has been previously applied for decontaminating fresh produce but more commonly in aqueous form (Alothman et al. 2010; Klockow and Keener 2010; Miller et al. 2013; Akata et al. 2015; de Candia et al. 2015). Gas phase treatments have also been applied but typically required extended contact times along with high concentrations (Horvitz and Cantalejo 2014). The limitation of applying ozone gas is the boundary layer that restricts the diffusion of the antimicrobial gas to the target bacteria on the produce surface. The novelty of the current approach was the introduction of ozone into a flowing stream of air that effectively disrupted the boundary layer thereby enhancing the contact of ozone with the target bacteria (Kim et al. 2003). This hypothesis was supported by the effect of air flow velocity on the extent and homogeneity of the decontamination efficacy in the packed apple bed. At low air velocities the majority of *Listeria* inactivation occurred on the top layer of apples but as the air flow became turbulent the ozone was drawn through the bed. It is also possible that the pressure differential caused by the air velocity also contributed to drawing the ozone through the packed apple bed although further studies are required to confirm this theory. Regardless of this fact it was noteworthy that treating apples within a packed bed was more effective compared to a monolayer of the fruit.

In agreement with other workers, the efficacy of ozone treatment was found to be dependent on the relative humidity with RH> 85% being more effective than lower concentrations (Selma et al. 2008; Rosenblum et al. 2012). In the current reactor design the

relative humidity was provided from the condensate of the apple entering the chamber. From trials performed it was found that under test conditions the temperature of apples equilibrated with the environment within 40 minutes. In this respect it was interesting to note that the log reductions of *Listeria* on apples did not increase beyond 30 minutes that could be attributed to a lowering of relative humidity required to inactivate Listeria.

The combination of UV and hydrogen peroxide has been previously applied for decontaminating fresh produce in a process referred to as an Advanced Oxidative Process (AOP). In agreement with other studies the efficacy of the process was dependent on treatment time, temperature and hydrogen peroxide concentration (Alaton et al. 2002; Hadjok et al. 2008; Assalin et al. 2010). The aforementioned parameters are optimal for the generation of free radicles. Although the log reductions of *Listeria* achieved using AOP were a fraction of that supported by ozone gas treatment it is noteworthy that populations of the bacteria present within the stem scar tissue were reduced. *Listeria* on and within the stem scar tissue can be considered significant given the potential of become further internalized when the stick is inserted during the candy apple making process (Salazar et al. 2016). Hence, inactivation of *Listeria* within the subsurface of apples can be considered as an advantage of the AOP process over that of ozone. The efficacy to inactivate internalized *Listeria* was enhanced by using an AOP based on using a combination of UV: ozone: hydrogen peroxide. The results can be explained by the generation of trioxane that has greater antimicrobial oxidative power compared to hydroxyl radicals produced from the breakdown of hydrogen peroxide alone (Alaton et al. 2002; Artes and Allende 2005; Rosenfeldt et al. 2006). Although the combination of UV: ozone: hydrogen peroxide has not been previously evaluated for fresh produce decontamination the treatment has been used to treat wastewater contaminated with toxic chemical agents (Alaton et al. 2002). From studies

performed it was found that the decomposition of pesticides was greater using the UV: ozone: hydrogen peroxide combination compared to UV: peroxide or UV: ozone (Alaton et al. 2002).

In agreement with other published reports, *Listeria* could grow within the core of candy apples when held at room temperature (Alegre et al. 2010; Graca et al. 2011; Salazar et al. 2016). The growth of *Listeria* on the internal tissue of apples can be attributed to hydration and access to nutrients. In contrast, *Listeria* on the surface of apples (i.e. located between the apple and candy layer) were inhibited by a combination of low water activity and lack of nutrients to support growth. The thermal shock by the addition of the candy layer also likely contributed to reducing the growth of *Listeria* at the apple: candy layer. By using a combination of ozone and AOP it was possible to support a 5-6 log CFU reduction of *Listeria* by an additive effect. Interestingly the low residual populations of *Listeria* did not undergo outgrowth even when the candy apples were stored at room temperature. The lack of *Listeria* growth could be attributed to the cells being subjected to oxidative stress in combination with the low pH of the internal apple core. However, further studies are required to support this hypothesis.

When the same combination of treatments was tested on lettuce the same additive antimicrobial effect was observed. Heads of lettuce were found to be more sensitive to ozone air flow compared to apples. This can be attributed to the weaker cell structure of lettuce that would lose turgor pressure leading to severe wilting. In a similar manner, the UV: hydrogen peroxide treatment was more detrimental to the quality of lettuce over prolonged treatments (Hadjok et al. 2008). Yet, a 3 log CFU reduction in *E. coli* was attained that is in agreement with values reported by others. The combination of ozone, UV: peroxide along with a chlorine dioxide dip resulted in an overall decrease in *E. coli* levels that did not recover during post-treatment storage.

The results clearly indicated by using a combination of treatments was significantly higher compared to relying on post-harvest washes alone.

4.1 Conclusions and Recommendations

From the studies performed along with those published by others, the fate of *L. monocytogenes* on candy apples depends on several factors. Specifically, the greatest risk posed by *L. monocytogenes* is introduced into the core as opposed to the surface of apples.
The extent to which *L. monocytogenes* grows on/within candy apples is more dependent on if the pathogen has been pre-stressed more so than the storage temperature. In this respect, the application of ozone gas and then AOP would lead to increased stress of *L. monocytogenes* that could explain the restricted growth of the pathogen with candy apples held at room temperature. In conclusion, by implementing the ozone gas treatment and AOP it is possible to reduce levels of *L. monocytogenes* on and within apples destined for candy apply production. Based on the fact that both interventions are aqueous free there is little risk of actively growing *L. monocytogenes* contaminating the product. Therefore, collectively the evidence presented indicates that storing apples at 4°C as opposed to room temperature does not bring significant benefits to *L. monocytogenes* control. Consequently, candy apples prepared as described can be stored at room temperature without any additional risk of being contaminated or supporting the growth of *L. monocytogenes*.

5 References

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