INACTIVATION OF MURINE NOROVIRUS ON GREEN ONIONS AND IN SALSA BY HIGH PRESSURE PROCESSING, AND INACTIVATION OF SALMONELLA ON BLUEBERRIES BY A NOVEL ULTRAVIOLET LIGHT AND WASHING PROCESS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Food Science

Fall 2014

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ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Haiqiang Chen, for the opportunity and funding to conduct and present this research, and for his patient guidance and advice throughout this process.

I would also like to thank my committee members, Dr. Rolf Joerger and Dr. Changqing Wu for their encouragement throughout my graduate career, and for agreeing to be part of my thesis committee.

Furthermore, I would like to thank Dr. Xinhui Li for teaching me proper cell culture technique; Dr. Mu Ye for teaching me how to operate the pressure unit; Jonathan Huang for all of his help on my second project; Fiona Liu and Runze Huang for being flexible with their experiments and schedules; and Shuanguan Guo and Xinang Cao for assisting me in the completion of the last of my experiments.

Last, but by no means least, I would like to thank my friends and family for their unflagging support throughout all of my educational endeavors.

I dedicate this thesis to Stephanie Deal, whose love, patience, and encouragement have made all the difference.
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ABSTRACT

Two projects are presented in this thesis. In the first project, high pressure processing was tested as a method for inactivation of a surrogate for human norovirus (murine norovirus) in green onions and salsa. Conditions for high pressure treatment, inactivation kinetics at 300 and 350 MPa, and persistence testing in salsa at as-received and acidified pH were tested. Over the range of 1-20°C, 1°C and addition of water to the treatment package was found to improve effectiveness. The most effective treatment for green onions resulted in elimination of the virus below the detection limit (>5.5 log reduction; 350 MPa, 3 min, 1°C). D-values calculated at 300 and 350 MPa (66 s and 36 s, respectively) indicate increasing barosensitivity at higher pressures. A 300-MPa treatment in salsa for 2 minutes resulted in a 2.2-log reduction that was not dependent on pH over the range 3.8-4.0; storage trials lasting 24 and 72 h resulted in ~0.5-log reductions, demonstrating that murine norovirus is insensitive to acidic pH both during pressure treatment and during storage. These results demonstrate the effectiveness of high pressure processing as a useful method for decontamination of green onions and salsa.

In the second project, a novel combined washing and ultraviolet light process was tested to inactivate Salmonella on blueberries. This combined process was intended to both inactivate bacteria on blueberries and to prevent cross contamination by washing water. Existing static UV processes are limited by shadowing and are only effective on one surface at a time. In this study, simple water washing, UV-washing, and combined UV-sanitizer washing treatments lasting 1 and 2 minutes were tested on
blueberries inoculated with a 4-strain cocktail of *Salmonella*. The robustness of these treatments was tested with the addition of crushed berries and blueberry juice to the washing water on skin, calyx and dip inoculated blueberries. Effectiveness of the treatments ranged from $0.74\pm0.55$ log reduction (calyx inoculation, 1 minute, blueberry juice) to $4.91\pm1.56$ log reduction (skin inoculation, 2 minutes, UV and chlorine in deionized water). Use of hydrogen peroxide as sanitizer reduced the risk of cross contamination and increased *Salmonella* inactivation on the blueberries; addition of chlorine to washing water improved effectiveness except in the presence of blueberry juice. The results of these treatments indicate that ultraviolet light is a promising technology for decontamination of blueberries when coupled with a washing process.
Chapter 1

INTRODUCTION

This thesis is the culmination of two projects: Inactivation of Murine Norovirus on Green Onions and in Salsa by High Pressure Processing and Inactivation of *Salmonella* on Blueberries by Combined Washing and Ultraviolet Light Process. The chapters in this work will begin with an overall discussion of both projects followed by the specifics of each project. A review of the literature relevant to each project will be presented in Chapter 2, followed by each of the projects in Chapters 3 and 4. Overall conclusions and a discussion of future work will be presented in Chapter 5.

Fresh foods are becoming increasingly popular in the United States and outbreaks of foodborne disease associated with fresh produce are increasingly common. Consumers are demanding safe, minimally-processed, additive-free produce that maintains “fresh” and “uncooked” sensory qualities (Oey et al., 2008). Nonthermal technologies represent an important opportunity to reduce the spread of foodborne illness in green onions, salsa, and blueberries while maintaining the safety and sensory quality that consumers demand.

1.1 Inactivation of Murine Norovirus on Green Onions and in Salsa by High Pressure Processing

Green onions and fresh salsa made with them are a source of norovirus and other foodborne pathogens, but are frequently consumed raw as a garnish, and are valued for their fresh flavor and texture. Though thermal treatment for both green
onions and salsa would eliminate the threat of norovirus, texture and flavor would be altered. An outbreak of hepatitis A virus traced to contaminated green onions in 2003 sickened 600 people and killed three because the green onions were served raw and added to salsa offered to everyone who ate at the restaurant (Wheeler et al., 2005).

On average, one outbreak of norovirus was reported per day between 2001 and 2008 (Hall et al., 2012) in the United States, and the frequency of outbreaks has continued to rise since that time (Karst, 2010). Increases in the rate of norovirus transmission may be caused partly by increases in consumption of fresh produce (Dragoslava, 2012). High Pressure Processing (HPP; also High Hydrostatic Pressure Processing, or HHP) is a nonthermal food preservation method used commercially for cold pasteurization, or “pascalization”, of salsas, guacamole, and other products to inactivate pathogens without producing the “cooked” sensory qualities caused by thermal processing (Bermúdez-Aguirre and Barbosa-Cánovas, 2011).

1.2 Inactivation of *Salmonella* on Blueberries by Combined Washing and Ultraviolet Light Process

Fresh blueberries are an increasingly popular food in the United States and can be a source of foodborne disease. *Salmonella*, a pathogen typically associated with poultry and eggs, has caused an increasing number of outbreaks on fresh produce, including blueberries (Calder et al., 2003; CDC, 2014; CSPI, 2014). Washing is a frequently employed unit operation in the produce industry, and ultraviolet light is an emerging technology for decontamination of foods but has not yet been used with produce washing. Ultraviolet light has been shown to cause minimal changes to the sensory qualities of foods while inactivating pathogens and spoilage organisms.
Outbreaks of *Salmonella* Muenchen and *Salmonella* Newport, as well as other pathogens, have been traced to fresh blueberries (CDC, 2014; CSPI, 2014). Blueberries are currently picked and packed into clamshell containers in the field without any food safety intervention; and since consumers do not reliably handle and wash produce properly (Li-Cohen and Bruhn, 2002), pathogens on the surface of the blueberries could easily infect consumers. Use of a combined UV-washing process could reduce the likelihood of an outbreak and prevent cross-contamination while maintaining sensory quality.
Chapter 2

LITERATURE REVIEW

This literature review will introduce the organisms, foods and processing methods used in each project. Commercial practices and experimental studies will be discussed and analyzed to better contextualize the original work which is the topic of this thesis.

2.1 Inactivation Of Murine Norovirus On Green Onions And In Salsa By High Hydrostatic Pressure

Green onions are a widely consumed food in the United States and around the world; salsa has been the most widely consumed condiment in the US since 1992 (Seitz-Wald, 2014). Green onions are typically trimmed and assembled into bunches, then bagged and stored on ice for shipment at the farm. In restaurant and foodservice settings they are typically washed and chopped before service (or addition to salsa) and stored under refrigeration until needed (Wheeler et al., 2005). Salsa is prepared fresh at restaurants and some grocery stores and stored under refrigeration, typically for no more than a few days (Wheeler et al., 2005). Salsa is also available as a shelf-stable canned product or as a refrigerated pressure-treated product for home and foodservice use.

Green onions and salsa have been linked to multiple outbreaks of foodborne illness (see Table 1 below) featuring a wide variety of pathogens. It is probable that outbreaks of human norovirus (HuNoV) have occurred as well, though none have been recorded specifically.
2.1.1 Norovirus

Norovirus was first identified in 1972 as the causative agent of an outbreak of acute gastroenteritis traced to a school cafeteria in Norwalk, Ohio in 1968. The illness was previously described in 1929 as “winter vomiting disease”, since the rate of norovirus infections tends to peak during winter (Adler and Zickl, 1969). Symptoms of norovirus infection include nausea, vomiting, diarrhea, and abdominal pain. Children (patients under age 15) are more likely to suffer vomiting, nausea and abdominal pain than adults, who are more likely to suffer diarrhea (Sala et al., 2014). The elderly and persons with compromised immune systems are at greatest risk of complications from norovirus; for healthy individuals, the symptoms typically resolve themselves within 24-48 hours.

Norovirus is a nonenveloped, positive-sense RNA virus in the Caliciviridae family. The virion particles are approximately 37 nm across. The family is named for the cup-like (calyx) appearance of the virions in electron micrographs. Other genera in the Caliciviridae family are Lagovirus, Sapovirus and Vesivirus, with Recovirus proposed as an additional genus (Yu et al, 2013).

Many strains of norovirus exist. They have been collected into five genogroups (each with constituent genotypes) based on differences in VP1 (major capsid protein) coding sequences. Groups I, II, and IV affect humans; Group III affects bovines, and Group V affects mice (CDC Guidelines report). Outbreak strains typically arise from Group II, genotype 4 (GII.4), as a result of mutations in the VP1 structural protein that prevents recognition of the virus by the humoral immune system (Donaldson et al., 2010).
The 50% infectious dose (ID50) of norovirus appears to be somewhat controversial; an oft-repeated statistic in the literature is that 18 virions are sufficient for infection, but a recent study by Atmar et al. (2014) suggests that between 1300 and 2800 genome copies (corresponding, presumably, to the same number of virions) is needed. A challenge study by Teunis et al. (2008) found a dose-response relationship. Increasing numbers of virions increases the chance that a person will become ill; they estimated a 10% chance of illness with exposure to $10^3$ genome copies and up to a 70% chance of illness with exposure to $10^8$ genome copies, though some challenge subjects did not become ill, even at very high doses. In either case, the actual infectious dose on a volume basis is very small; reports exist of norovirus infection by aerosolized vomit at distances exceeding 10 meters (Marks et al., 2000).

2.1.1.1 Norovirus and Public Health

The CDC estimates that 20 million cases of acute gastroenteritis are caused by norovirus every year in the United States. Of these, nearly 2 million result in a visit to a doctor or emergency room, and several hundred people die (CDC Norovirus Trends and Outbreaks). Norovirus outbreaks occur worldwide and affect all age groups (Patel et al., 2009). The elderly and very young are most vulnerable to norovirus infection, and norovirus outbreaks commonly occur in nursing homes and day care centers (Hall, 2014; Kosa et al., 2014). Humans are the only known reservoirs of norovirus (CDC, n.d.). Sources of norovirus are therefore traceable to infected humans or to infectious human waste. In a case examined by Marks et al. (2000), an outbreak of norovirus traced to aerosolized vomit occurred in 1998 when a restaurant patron unexpectedly vomited in a hotel restaurant with a large number of other diners present. Those nearest the vomit were more likely to become ill (and to do so with shorter incubation
period) in the following days than those who were seated further away. The waiter who cleaned up the vomit fell ill shortly thereafter, though none of the other staff or diners in another restaurant within the hotel did (Marks et al., 2001).

Recreational water is known to be a source of norovirus once contaminated with infectious vomit or feces. Swimmers, boaters and others can both contaminate and be infected by water in lakes, swimming pools, and even water fountains. An outbreak of norovirus in schoolchildren in the Netherlands was traced to a fountain where the children had played on a school outing; of the 191 children who attended the outing, 90 (or an attack rate of 47%) fell ill with norovirus (Hoebe et al., 2004). Viral RNA extracted from stool samples matched the RNA from a sample of water from the fountain. Even children who showed no symptoms expressed detectable levels of viral RNA in their feces, which suggests that asymptomatic and apparently healthy individuals can spread norovirus. Furthermore, the fraction of children who became ill and the results of the challenge study by Teunis et al. (2008) suggest that the viral dose received by the children was on the order of $10^0$-$10^2$ particles, assuming that the children were of similar susceptibility as the challenge subjects.

Norovirus can spread rapidly in hospitals if not controlled effectively. Khanna et al. (2003) reported a new variant of a GII strain, named ‘Basel’ (Switzerland) for the city of the outbreak; the source of the outbreak was not identified and the authors conjectured that an infected patient may have brought the disease into the hospital. Kanerva et al. (2009) reported three new subvariants of GII.4 2006b, which together caused a six-month outbreak of norovirus across multiple wards in a Finnish hospital. Once again, it was conjectured that the source of the outbreak was individuals who brought the disease into the hospital from the local community. Complicating the issue
of norovirus in healthcare facilities is that healthcare workers frequently fall ill themselves, and must be either sent home while ill and remain there for 48 hours after the cessation of symptoms or be reassigned to care for other norovirus patients. The former is not always practical since it leaves hospitals short-staffed during an outbreak (Khanna et al., 2003; Kanerva et al., 2009). To compound this issue, a survey by Kosa et al. (2014) showed that most infection preventionists (healthcare professionals who develop procedures to prevent the spread of disease, typically employed by hospitals) did not recognize norovirus as one of the top three foodborne pathogens (it is the leading cause), and only 5% recognized the three most likely sources of a norovirus infection.

2.1.1.2 Norovirus and Food Safety

As an enteric virus, norovirus outbreaks are frequently associated with food. In the period from 2001 to 2008, as studied by Hall et al. (2012), 2922 confirmed or suspected foodborne outbreaks of norovirus were reported: an average of one foodborne outbreak attributable to norovirus every day. In the same period, 364 outbreaks of norovirus were attributed to a single food commodity; the leading categories were leafy vegetables, at 33%; fruits and nuts at 16%, and mollusks at 13% (Hall et al., 2012). These categories of foods are both water-intensive and frequently consumed raw.

Food handlers are the largest source of foodborne norovirus contamination. Of outbreaks with a traceable source, 53% were attributed to food handlers (Hall et al., 2012). The same study found that commercial and institutional food-handling settings were the source of 89% of foodborne norovirus outbreaks, with private homes, picnics and religious facilities contributing only 11%. This demonstrates that places where
large amounts of food are prepared and stored are particularly vulnerable to norovirus outbreaks, either from infected food handlers who can shed norovirus onto food or from cross-contamination of safe food by contaminated foods.

Contaminated water can contaminate produce as irrigation water (Hanning et al. 2009), infect people directly (as drinking or recreational water; see the fountain-related outbreak above), and contaminate shellfish such as oysters and clams (Kingsley et al., 2002). Irrigation water is known to be a source of many foodborne pathogens in fresh produce, including viruses, bacteria, protozoa, and helminthes (worms) (Steele and Odumeru, 2004). Viruses, including norovirus, are known to be stable in groundwater and surface water for very long periods of time (Bae and Schwab, 2008) and norovirus can spread long after a contamination event is thought to be over. Green onions in particular have been shown by Chancellor et al. (2006) to draw virus-sized particles into the inner tissues, with decreasing concentration of particles observed with increasing distance from the roots.

In January 2006, 362 restaurant patrons and 15 restaurant employees were infected with norovirus spread by an infected food handler (Bohm et al., 2007). A server became ill and infected a bartender who then infected a line cook. This line cook fell ill and vomited at home in the early morning, but came to work and eventually vomited into an open trash can near a station where salads, antipasti platters and pizzas were being prepared. This line cook left the cooking area and later went home, but reported back to work the next day despite suffering from diarrhea. Inadequate sanitizing procedures targeted mainly at bacteria (wipes and towels saturated with quaternary ammonium compounds) were ineffective against norovirus (see Gulati et al., 2001; discussed in 2.1.2.2). Food items besides those contaminated
directly by aerosolized vomit were thought to have been contaminated by these surfaces. The attack rate (number of illnesses divided by number of diners) peaked at 40% during dinner service on the day when the line cook vomited. This case study and the case study reported by Marks et al. (2001) show that food and people exposed to the vomit of an infected person are at risk. Furthermore, if the surrounding area is not adequately sanitized, norovirus can linger and contaminate food for days.

Shellfish are also a common source of norovirus. An outbreak reported by Iizuka et al. (2010) involved an asymptomatic food handler who became infected after being exposed to contaminated frozen shucked saltwater clams, though only restaurant patrons who consumed the contaminated seafood actually became ill. This suggests that asymptomatic food handlers who maintain good hygiene standards do not necessarily spread norovirus on their own. The ultimate source of the contamination was not discovered, but was almost certainly contaminated water.

Norovirus contamination of food can be prevented by good hygiene and improved sanitation. It may not be possible for food handlers to stay home when ill; if that is the case, ill workers should be assigned non-food-contact roles for up to three days after the cessation of symptoms (Bohm et al., 2007).

2.1.2 Norovirus Surrogates in Research

Human norovirus is not culturable and as a result, a variety of surrogates have been proposed (Karst, 2010). Murine norovirus (MNV), feline calicivirus (FCV), Tulane virus, and a variety of other surrogates have been used to simulate human norovirus inactivation during food safety and surface disinfection procedures (Richards, 2012; Gulati et al., 2001). The major difference between the surrogates and human norovirus is that the surrogates are detectable by plaque assay, which tests
infectivity, rather than only by RT-PCR, which tests for the presence of viral genomes (that may or may not be infectious). The different surrogates are sensitive to different environmental conditions and processing techniques. In general, surrogates should be slightly less sensitive to the tested treatment than the original pathogen. When such a surrogate is not available, multiple surrogates are tested in parallel. Treatments used in industry are then designed around persistence of the toughest surrogate.

2.1.2.1 Murine Norovirus

Wobus et al. (2004) found that murine norovirus replicates in mouse macrophage and dendritic cells and eventually causes lysis of those cells. Therefore, MNV can be detected by plaque assay. Further work by Baert et al. (2008) showed that cell-culture infectivity of MNV decreases more than the number of genome copies detected by RT-PCR. Work by Tang et al. (2010) showed that high pressure processing produces a similar effect. Both of these results demonstrate that plaque assays are a more precise method for determining the titer of infectious virions than RT-PCR, which detects the intact genomes of virions that have been rendered harmless by heat treatment, high pressure processing, or by other mechanisms.

Murine norovirus is thought to be the closest surrogate to the behavior of human norovirus for high pressure processing studies (Richards, 2012). A study of viral persistence in groundwater and surface water samples by Bae and Schwab (2008) found that persistence rate of genome copies of MNV and human norovirus were very similar.

Resistance of human norovirus and MNV to high pressure was tested head to head in a study by Sanchez et al. (2011). The authors found that inactivation of human norovirus was difficult to determine accurately because it is quantifiable only by RT-
PCR, but that reductions of murine norovirus genome copies were less than reductions of human norovirus genome copies. The pressure treatment conditions (450 MPa at 45°C for 15 minutes) are not well suited to the inactivation of murine norovirus, since murine norovirus is known to be more sensitive to pressure at lower temperatures (Huang et al., 2014).

Murine norovirus was tested as a surrogate for human norovirus in a study by Huang et al. (2014). Effectiveness of high pressure treatment on quartered strawberries and strawberry puree was examined. The authors found that decreased treatment temperature improved effectiveness and that addition of water to the treatment package also improved effectiveness. Frozen storage of strawberries and strawberry puree did very little to reduce MNV on its own; only a 1.2-log reduction was observed during 28 days of frozen storage.

2.1.2.2 Feline Calicivirus

Feline calicivirus, though a member of the vesivirus genus of *Caliciviridae* rather than a member of the norovirus genus, has been tested as a surrogate for norovirus in a variety of settings. FCV can be cultured in Crandell-Reese feline kidney (CRFK) cells and can be propagated using ordinary cell culturing techniques (Gulati et al., 2001; Lee et al., 2012) similar to those used for propagation of MNV in RAW cells (ATCC, 2014).

Lee et al. (2012) found that FCV was more sensitive than MNV to low-pH environments. In particular, the authors tested the survival of FCV in actively fermenting *dongchimi* pickles (which consisted of 40% sliced radishes seasoned with ginger, scallions and garlic and 60% brine at 2.5% salinity) and found ~3-log reduction over the course of 21 days of fermentation. This result shows that FCV is
less suitable for use a surrogate than MNV in strongly acidic foodstuffs such as salsa or pickles (initial pH of salsa was measured as part of this study; every jar measured 4.00 when opened, and the *dongchimi* prepared by Lee et al. did not approach pH 4.00 until 15 days of fermentation).

Bae and Schwab (2008) found that FCV deteriorated significantly faster in surface and groundwater samples than MNV or any of the other surrogates tested besides poliovirus. They mentioned that FCV was a respiratory virus and therefore likely to be more pH sensitive than enteric viruses which must survive passage through the acidic stomach before initiating infection in the intestinal tract.

Surface disinfection of FCV on strawberries, lettuce, and stainless steel was tested by Gulati et al. (2001) using a variety of sanitizers used in both foodservice operations and food processing facilities, including 1.75% iodine and 6.5% phosphoric acid, quaternary ammonium compounds, sodium hypochlorite solution, and Microbac II (4.75% *o*-benzyl *p*-chlorophenol and 4.75% *o*-phenylphenol). The authors saw minimal effectiveness at a contact time of 1 minute, and minimal effectiveness gains beyond 10 minutes. Therefore, they only reported data for 10 minutes of contact time. FCV was unaffected by iodine and phosphoric acid at concentrations below 150 ppm iodine (double the recommended dilution), mildly reduced (1.1-log reduction) by sodium hypochlorite (at 800 ppm free chlorine), moderately reduced by quaternary ammonium compounds (2.3-log reduction at 1800 ppm), and all but eliminated by Microbac II (7.0-log reduction at 1:64 dilution, though this is four times the recommended concentration). In all cases, none of the sanitizers tested produced a satisfactory effect (defined by the authors as >3-log reduction) at the recommended concentration. This finding and food handler-related outbreaks described above
reinforce the need for intensive decontamination measures to eliminate norovirus on environmental and food contact surfaces.

### 2.1.2.3 Tulane Virus

Tulane virus (TV or TuV) is a calicivirus (genus recovirus) isolated from rhesus macaques and propagated in LLC cells (Farkas et al., 2008). It causes respiratory infections, gastroenteritis, and hemorrhaging in its native host. The virion is of similar structure to human norovirus and other *Caliciviridae* (Yu et al., 2013).

Tulane virus was found to be more sensitive than MNV in blueberries, oyster meat, and cell culture medium (Li et al., 2013); both viruses were found to be more sensitive to pressure treatment when water was included in the treatment package. Aichi virus and TV were both found by Cromeans et al. (2014) to be essentially unaffected by alcohol-based surface sanitizers; they also noted that TV and MNV were the best surrogates overall for sanitizer testing.

Hirneisen and Kniel (2013b) found that MNV and TV were of similar stability when exposed to chlorine at levels ranging from 0.2 to 2000 ppm and heat treatments at 50 and 75°C. However, MNV was more stable than TV at extreme pH values and at refrigerated (4°C) storage over five days; MNV showed no loss of titer, but TV showed a significant decrease. These conditions are of particular relevance to green onions and salsa, which are both stored for at refrigerated temperatures, and, in the case of salsa, have a low pH.
2.1.2.4 Other Surrogates

Poliovirus has been suggested as an alternative surrogate for murine norovirus; however, its structural differences make it unattractive as a surrogate when other options are available. Like the members of the *Caliciviridae* family, poliovirus is a single-stranded RNA virus; however, its capsid proteins and structure are different (Hogle et al., 1985; Hansman et al., 2011). Bae and Schwab (2008) found that genome copies of poliovirus deteriorated much more quickly in groundwater and surface water samples than human norovirus or murine norovirus.

As a subject in its own right, inactivation and persistence of poliovirus has been examined on green onions. Kurdziel et al. (2001) found no measurable decline in poliovirus during refrigerated storage of green onions over the course of a 14-day testing period (which exceeds some recommendations for green onion shelf-life [Smith et al., n.d.]). This finding suggests that human norovirus and murine norovirus would not decline measurably during refrigerated storage on green onions since poliovirus is less stable than norovirus.

MS2, a bacteriophage which attacks *E. coli* bacteria with a specific fertility factor, is also a positive sense single-stranded RNA virus; however, it varies significantly from the *Caliciviridae* in terms of structure and genome size, and cannot be detected by infectivity assay as easily as MNV (Bae and Schwab, 2008). MS2 was tested by D’Souza and Su (2010) as a surrogate for surface disinfection; it was inactivated at a lower rate than FCV and MNV by 2% glutaraldehyde solution, but was more sensitive than MNV to 2% trisodium phosphate solution.

Outbreaks of hepatitis A virus (HAV) are frequently traced to the same foodstuffs that cause outbreaks of human norovirus, which suggest that it has similar persistence and distribution characteristics. Like norovirus, it is also a non-enveloped
virus with a single-strand RNA genome (Wheeler et al., 2005; Kingsley et al., 2005). However, it is a poor choice as a surrogate for the purposes of this study because it becomes more sensitive to pressure at high temperatures and less sensitive at low temperatures (Kingsley et al., 2005). Norovirus, however, has the opposite response to temperature: it becomes more sensitive at lower temperatures (Huang et al, 2014; Kingsley et al., 2007; Li et al, 2014).

2.1.3 Green Onions

Green onions, also referred to as “bunching onions”, “scallions”, and other regional names, are the partially matured bulblets and stems of Allium cepa, or cultivated onions. Many varieties have been developed to accommodate different growing season weather and day lengths, since onions begin to form bulbs as a response to changes in temperature and day length (Smith et al., n.d.). Green onions are consumed worldwide and are frequently used raw as a garnish.

2.1.3.1 Green Onion Production and Storage

Green onions for the North American market are primarily grown and harvested year-round in California; Ohio, North Carolina and Mexico also produce green onions in significant quantities (Smith et al., n.d.). Green onions are typically trimmed and assembled into bunches, then bagged and chilled. Green onions can be maintained up to two weeks under modified atmosphere storage (Hong et al., 2000) at 5°C; mild heat treatment at 55 °C for 2 minutes to improve shelf-life was found to slow the rate of “telescoping” (growth of inner leaf layers) but did not affect chemical composition (total dissolved solids, pH, thiosulfinate content).
2.1.3.2 Green Onions and Food Safety

Outbreaks of disease have been traced to green onions; the details of some recent outbreaks are provided in Table 1.

Table 1: Outbreaks of disease traced or attributed to green onions. CSPI = Center for Science in the Public Interest Outbreak Alert Database; CDC = CDC FOOD Database (full citations in References section)

<table>
<thead>
<tr>
<th>Year</th>
<th>Pathogen</th>
<th>Location</th>
<th>Setting</th>
<th>Reported illnesses</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994</td>
<td><em>Shigella flexneri</em></td>
<td>Multistate</td>
<td>Multiple locations</td>
<td>97</td>
<td>CSPI</td>
</tr>
<tr>
<td>1996</td>
<td>Hepatitis A virus</td>
<td>California</td>
<td>Multiple Locations</td>
<td>60</td>
<td>CSPI</td>
</tr>
<tr>
<td>1997</td>
<td><em>Cryptosporidium parvum</em></td>
<td>Washington</td>
<td>Restaurant</td>
<td>54</td>
<td>CSPI</td>
</tr>
<tr>
<td>1998</td>
<td>HAV</td>
<td>Ohio</td>
<td>Picnic</td>
<td>42</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2000</td>
<td>HAV</td>
<td>Multistate</td>
<td>Restaurant</td>
<td>32</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2003</td>
<td>HAV</td>
<td>Pennsylvania</td>
<td>Restaurant</td>
<td>601</td>
<td>CDC, CSPI, Wheeler et al., 2005</td>
</tr>
<tr>
<td>2003</td>
<td>HAV</td>
<td>Multistate</td>
<td>Homes, Restaurants</td>
<td>935</td>
<td>CDC</td>
</tr>
<tr>
<td>2009</td>
<td><em>Salmonella Javiana</em></td>
<td>Multistate</td>
<td>Unknown</td>
<td>9</td>
<td>CDC</td>
</tr>
</tbody>
</table>

The most prolific pathogen in these eight outbreaks is hepatitis A virus, plus an outbreak each from *Shigella, Salmonella* and *Cryptosporidium*. It is possible that hepatitis A is so well represented over other pathogens because its symptoms are relatively unique: jaundice, amber colored urine, and pale feces are unusual symptoms not normally associated with foodborne illness (Acheson and Fiore, 2004).

The 2003 Pennsylvania outbreak of hepatitis A was investigated by Wheeler et al. (2005), who found that restaurant patrons who had consumed green onions were 33
times more likely to contract hepatitis A than those who did not. Mild salsa, offered to all restaurant patrons upon seating, was determined to be the main food vehicle. The source of the contamination was green onions (used in the salsa and as a garnish for other dishes) imported from specific farms in Mexico that were contaminated before arrival at the restaurant. Surprisingly, green onions were a relatively small component in the salsa: each 38-L batch of salsa contained only 170 g of green onions, or 0.45% w/v. The investigators suggest that the practice of washing the green onions while still bundled and not drying them carefully before cutting, rather than removing them from their bundles and washing and drying them individually or in a single layer, may have helped spread the virus.

A study in Malaysia by Noor Hidayah et al. (2011) found that 4 of 30 green onion samples acquired from local markets tested positive for human norovirus; the rate was lower for other types of onions tested. However, as the authors point out, green onions are the least likely to be cooked and are, therefore, even more likely to cause disease than other types of onions. A study in the United States by Allwood et al. (2004) found no detectable human norovirus on 40 green onion samples acquired from local markets and a restaurant undergoing investigation for a norovirus outbreak, though F-specific coliphages, an indicator of fecal contamination, were found on 33% of retail samples and all of the restaurant samples. Comparison of these studies suggests a lower incidence of norovirus contamination in North America (the country of origin of the green onions tested by Allwood et al. was not specified) than in Malaysia; this is probably due to improved food safety and sewage management practices.

Green onions are a water-intensive crop (Smith et al., n.d.) and are susceptible
to contamination through irrigation water or soil. A study focusing on the spread of hepatitis A virus in green onions found that fluorescent virus-sized particles mixed into soil and hydroponic medium were drawn into the tissues of growing green onions; the authors note that once there they are not easily removed (Chancellor et al., 2006). A study by Hirneisen and Kniel (2013a) found that no detectable HAV or MNV was drawn into spinach and green onions from contaminated soil over 20 days of growth, but that up to 4 log of MNV and HAV could be drawn up from contaminated hydroponic medium, thus demonstrating the importance of clean irrigation water.

2.1.4 Salsa

2.1.4.1 Salsa Formulation and Production

Fresh salsa is made from a mixture of vegetables: according to Merriam Webster, salsa is “a spicy sauce made with tomatoes, onions, and hot peppers that is commonly served with Mexican food”. No standard of identity has been published by the FDA (Association for Dressings and Sauces, 2014); this is probably because salsas can be made with a wide variety of ingredients as a base. A survey of amateur and professional literature suggests that salsa consists of a base of tomatoes, tomatillos or fruits seasoned with onions (red, yellow, white, green, or a combination), chili peppers, garlic, herbs, and lemon or lime juice (Waters, 2007; America’s Test Kitchen, 2012; Association for Dressings and Sauces, 2014). Fresh salsa is typically consumed the same day it is made, though it can be stored for a few days at refrigerated temperature (America’s Test Kitchen, 2012). In the case of the hepatitis A outbreak associated with green onions and salsa, 38 L of salsa were prepared each day and stored up to three days at refrigerated temperature (Wheeler et al., 2005).
Commercially processed jarred salsa and home-made cooked salsas are not known to be sources of foodborne illness other than botulism, which is easily prevented by acidification or by retort processing at the commercial level (Schneider et al., n.d.).

2.1.4.2 Salsa and Food Safety

Fresh salsa has been traced as the source of many (>97) disease outbreaks (CDC FOOD Database). Confirmed outbreaks of norovirus specifically are collected below in Table 2. An extra column has been added because many of these outbreaks were linked to salsa and one or more additional food products. For the sake of brevity, unconfirmed or suspected outbreaks were omitted from this summary; an additional 20 unconfirmed or suspected outbreaks of norovirus in salsa occurred in this time period.
Table 2: Confirmed outbreaks of norovirus traced or attributed to salsa and related foods. CSPI = Center for Science in the Public Interest Outbreak Database; CDC = CDC FOOD Database (full citations in References section)

<table>
<thead>
<tr>
<th>Year</th>
<th>Foods</th>
<th>Norovirus Genogroup</th>
<th>Location</th>
<th>Setting</th>
<th>Reported Illnesses</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>ground beef; ice; salsa</td>
<td>GI</td>
<td>Georgia</td>
<td>Restaurant</td>
<td>41</td>
<td>CDC</td>
</tr>
<tr>
<td>2003</td>
<td>chips; salsa</td>
<td>Unknown</td>
<td>Kansas</td>
<td>Private home; Restaurant</td>
<td>25</td>
<td>CDC</td>
</tr>
<tr>
<td>2004</td>
<td>lettuce-based salads; salsa</td>
<td>GI</td>
<td>Ohio</td>
<td>Restaurant</td>
<td>7</td>
<td>CDC</td>
</tr>
<tr>
<td>2004</td>
<td>rice; salsa</td>
<td>GI</td>
<td>California</td>
<td>Restaurant</td>
<td>22</td>
<td>CDC</td>
</tr>
<tr>
<td>2004</td>
<td>salsa</td>
<td>GI</td>
<td>Ohio</td>
<td>Restaurant</td>
<td>9</td>
<td>CDC</td>
</tr>
<tr>
<td>2005</td>
<td>chips; salsa; guacamole</td>
<td>GI</td>
<td>Idaho</td>
<td>Restaurant</td>
<td>44</td>
<td>CDC</td>
</tr>
<tr>
<td>2006</td>
<td>salsa</td>
<td>Unknown</td>
<td>Oregon</td>
<td>Restaurant</td>
<td>17</td>
<td>CDC</td>
</tr>
<tr>
<td>2007</td>
<td>chips; tortilla; salsa</td>
<td>GI</td>
<td>Georgia</td>
<td>Restaurant</td>
<td>6</td>
<td>CDC</td>
</tr>
<tr>
<td>2008</td>
<td>chips; salsa</td>
<td>GI</td>
<td>Minnesota</td>
<td>School</td>
<td>17</td>
<td>CDC</td>
</tr>
<tr>
<td>2008</td>
<td>salsa</td>
<td>Unknown</td>
<td>New York</td>
<td>Restaurant</td>
<td>10</td>
<td>CDC</td>
</tr>
<tr>
<td>2009</td>
<td>guacamole; salsa</td>
<td>Unspecified</td>
<td>Washington</td>
<td>Unspecified</td>
<td>13</td>
<td>CDC</td>
</tr>
<tr>
<td>2009</td>
<td>salsa</td>
<td>Unknown</td>
<td>California</td>
<td>Workplace, not cafeteria</td>
<td>14</td>
<td>CDC</td>
</tr>
<tr>
<td>2010</td>
<td>multiple items; salsa</td>
<td>GII</td>
<td>Wyoming</td>
<td>Banquet Hall; Private Home</td>
<td>33</td>
<td>CDC</td>
</tr>
<tr>
<td>2010</td>
<td>chicken; salsa</td>
<td>GII</td>
<td>Oregon</td>
<td>Restaurant</td>
<td>10</td>
<td>CDC</td>
</tr>
<tr>
<td>2011</td>
<td>chips; salsa</td>
<td>GII</td>
<td>Alabama</td>
<td>Restaurant</td>
<td>18</td>
<td>CDC</td>
</tr>
<tr>
<td>2011</td>
<td>cilantro; salsa</td>
<td>Unknown</td>
<td>California</td>
<td>Workplace, NC</td>
<td>27</td>
<td>CDC</td>
</tr>
<tr>
<td>2011</td>
<td>carnitas; salsa</td>
<td>Unknown</td>
<td>California</td>
<td>Workplace, NC</td>
<td>136</td>
<td>CSPI</td>
</tr>
</tbody>
</table>
Table 2 summarizes details from 17 confirmed outbreaks from 1999 to 2011; a simple calculation reveals that a confirmed outbreak of norovirus attributed at least partly to salsa occurred approximately every nine months during this time period. Attribution of an outbreak to salsa alone is rare (2/17); this could occur for one or more of three reasons. First, it is likely that in restaurants which serve fresh salsa with only some menu items, e.g., chips or nachos, few, if any, of the patrons would eat the salsa without its accompanying chips, and in the absence of food samples, investigators would be unable to definitively determine either of the two items as the source of the outbreak. Second, it is possible for cross contamination to occur between salsa made from contaminated ingredients and other food items prepared at the same time, using the same implements, or with ingredients in common, e.g., guacamole. Third, it is possible that an infected food handler contaminated several food items that were prepared and served using different implements, e.g., ground beef, ice and salsa.

A *Salmonella* outbreak in 2008 was linked to tomatoes, jalapeños, and serrano peppers. A study by Li et al. (2010) found that though *Salmonella* did not grow in salsa made from inoculated versions of those ingredients, it was not inactivated unless fresh garlic or lime juice was present. Fresh, uncooked salsa contains a variety of ingredients that have been linked to foodborne outbreaks, such as tomatoes, jalapeños and serranos (Kendall et al., 2013), and represents an increasing source of foodborne disease.

**2.1.5 High Pressure Processing**

High Pressure Processing is a nonthermal food preservation method in which foods are exposed to pressures exceeding 200 MPa either in bulk or in flexible containers. It is used commercially for cold pasteurization, or “pascalization”, of a
variety of products such as juice, jams, salad dressings, sliced lunch meat, and other products to inactivate spoilage microorganisms, pathogens and enzymes with minimal changes in sensory quality (Bermúdez-Aguirre and Barbosa-Cánovas, 2011).

2.1.5.1 Usage and Effects of High Pressure Processing on Food

High pressure does less damage to the sensory quality of food than comparable thermal treatments (Kingsley et al., 2005). Flavor and aroma of foods are typically less affected than texture and color (Oey et al., 2008; Kovač et al., 2010), and the effects are dependent upon the structure of the food. High pressure processing is used on a variety of foods with a variety of objectives, including shelf-life extension, quality improvement/value addition, inactivation of pathogens, and denaturation of enzymes. Oysters, for instance, are processed with high pressure to extend shelf life and separate the oyster meat from the shells, though the pressure treatments are generally not sufficient to eliminate pathogens (Leon et al., 2011). Salsa and guacamole are processed to extend shelf life and inactivate pathogens (Kendall et al., 2013). Fruit juice and smoothie blends are processed to preserve the “raw” flavor and texture while denaturing enzymes and inactivating pathogens.

2.1.5.2 Effects on Microorganisms and Viruses

High pressure processing inactivates microorganisms and viruses by altering the quaternary, tertiary and secondary structures of proteins and other macromolecules (Kovač et al., 2010). Properties of the food being processed affect the susceptibility of microbes to pressure; water activity, salt concentration and the processing temperature strongly affect effectiveness (Cheftel, 1995).
High pressure treatment is effective against viruses because viral infection cannot occur unless the virus capsid and envelop proteins (if present) are intact. Work by Tang et al. (2010) demonstrates that the capsid protein is damaged by HPP treatment of MNV-1 (and, presumably, other noroviruses). The authors found that MNV subjected to HPP treatment at 400 MPa for 5 minutes at 0°C and then treated with a protease and RNase resulted in far fewer genome copies as detected by RT-PCR, even though binding to antibodies was not affected. Without an intact capsid, the virion simply decomposes and become harmless.

Bacteria are also sensitive to high pressure; early work with high pressure by Hite, Buchner and Bridgman focused on bacteria rather than viruses. One early use of high pressure was by Larson et al (1918), who used high pressure as a method for inactivating bacteria to produce vaccines. Most of the bacteria tested (referred to in the manuscript as “B. coli, B. tuberculosis, B. proteus, B. subtilis, staphylococci, streptococci and pneumococci”) were inactivated by the treatment, except the spores of B. subtilis. Even now, nearly a century later, techniques for inactivating bacterial spores are the subject of ongoing research (Ahn et al., 2006). Like viruses, bacteria seem to be inactivated by disruption of their inner and outer surfaces (Smelt, 1998), though these surfaces are plasma membranes that can be repaired rather than a protein capsid that cannot.

2.1.5.3 Existing Equipment

High pressure processing units are capital-intensive, but have low operating costs due to their relatively low energy consumption as compared to thermal processing units (Pereira and Vincente, 2010; Bermúdez-Aguirre and Barbosa-Cánovas, 2011). Liquid foods can be pressure-treated in bulk, but solid foods are
typically treated in vacuum packages that are surrounded by water. Some liquid foods are also processed in-container so that the containers do not have to be sanitized separately.

High pressure processing machines for solid foods consist of a sealable pressure chamber that is charged with the food packages and filled with heated or cooled water at low pressure using a centrifugal pump or other high-volume, low-head pump. Once filled, a pressure intensifier compresses the treatment water to the specified pressure. A pressure intensifier is a mechanical device which converts low pressure exerted over a large area into high pressure exerted over a small area (Torres and Velazquez, 2004). The pressure is held for the specified time and then released. The water is drained and the food packages are removed.

Machines designed for bulk processing of liquid foods are fairly similar in design, except that the food itself is pumped into the chamber and compressed. Of some concern is the potential for accumulation of pressure-tolerant mutant organisms, but this is only an issue for bulk treatment of liquid foods, where some of the previously treated food could remain in the chamber (San Martin et al., 2002).

2.1.6 Experimental Methods for Nonthermal Decontamination of Green Onions and Salsa

Experimental nonthermal interventions for viruses on green onions and in salsa can be divided into two groups: chemical methods, involving sanitizers such as ozone or calcium hypochlorite, and physical methods, involving processes such as high pressure and UV light.
2.1.6.1 Chemical Methods

Chemical methods for inactivation of pathogens on green onions are limited by the ability of sanitizers to reach internalized bacteria and viruses (Lynch et al., 2009). Pathogens on the smooth and waxy surface, however, can be readily inactivated or removed from the surface with impressive efficacy.

Table 3: Chemical Methods for Decontamination of Green Onions

<table>
<thead>
<tr>
<th>Pathogen/Surrogate</th>
<th>Sanitizer</th>
<th>Process</th>
<th>Time</th>
<th>Maximum Effectiveness (log₁₀ reduction)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNV</td>
<td>Lactic acid</td>
<td>Fermentation</td>
<td>20 days</td>
<td>1.0</td>
<td>Lee et al., 2008</td>
</tr>
<tr>
<td>FCV</td>
<td>Lactic acid</td>
<td>Fermentation</td>
<td>20 days</td>
<td>2.5</td>
<td>Lee et al., 2008</td>
</tr>
<tr>
<td>MNV</td>
<td>Aqueous ozone, 6.25 ppm</td>
<td>Gas sparged into water</td>
<td>10 min</td>
<td>2.9 (external) 1.5 (internal)</td>
<td>Hirneisen and Kniel, 2013b</td>
</tr>
<tr>
<td>MNV</td>
<td>Calcium hypochlorite 200 ppm</td>
<td>Surface spraying</td>
<td>2 min</td>
<td>2.6 (external) 0.4 (internal)</td>
<td>Hirneisen and Kniel, 2013b</td>
</tr>
<tr>
<td>MNV</td>
<td>Aqueous ozone, 6.25 ppm</td>
<td>Gas sparged into water</td>
<td>10 min</td>
<td>3.78</td>
<td>Hirneisen et al., 2011</td>
</tr>
<tr>
<td>Salmonella enterica Typhimurium</td>
<td>Chlorine, 4 ppm</td>
<td>Chlorine wash</td>
<td>1 min</td>
<td>3.2</td>
<td>Xu and Wu, 2014</td>
</tr>
<tr>
<td>Salmonella enterica Typhimurium</td>
<td>Thymol, 0.4 mg/ml</td>
<td>Sanitizer wash</td>
<td>1 min</td>
<td>4.8</td>
<td>Xu and Wu, 2014</td>
</tr>
<tr>
<td>Salmonella enterica Typhimurium</td>
<td>Citric acid (2 mg/mL) + SDS (4%)</td>
<td>Sanitizer wash</td>
<td>1 min</td>
<td>&gt;5.3</td>
<td>Xu and Wu, 2014</td>
</tr>
</tbody>
</table>

Lactic-acid fermentation in a traditional Korean kimchi (dongchimi) by Lee et al. (2008) only resulted in a reduction of 1.0 log of MNV. This and the very slow inactivation of norovirus in water samples (Bae and Schwab, 2008) suggest that norovirus will not be inactivated over the shelf-life of fresh salsa. The previously mentioned persistence of poliovirus on green onions suggests that norovirus can
persist on green onions without inactivation. Aqueous ozone soaking and calcium hypochlorite sprays, as conducted by Hirneisen and Kniel (2013b) and Hirneisen et al., (2011), were moderately effective against surface viruses but not as effective against viruses internalized by the green onions. The increase in effectiveness seen with ozone might be due to the longer exposure time, which allowed diffusion of the ozone into the layers of the green onion that was not possible with the calcium hypochlorite sprayed onto the surface and then permitted to stand for 2 minutes. The washing processes tested by Xu and Wu (2014) are not directly comparable to the results achieved by Hirneisen and Kniel (2013b), but demonstrate that sanitizers can be effective against surface pathogens on green onions.

2.1.6.2 Physical Methods

Physical methods for decontamination of green onions and salsa include pulsed light and high pressure processing.
Table 4: Physical Methods for Decontamination of Green Onions and Salsa

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Item</th>
<th>Process</th>
<th>Conditions</th>
<th>Maximum Effectiveness (log_{10} reduction)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCV</td>
<td>Green onions</td>
<td>UV light</td>
<td>240 mJ/cm²</td>
<td>5.6 TCID50</td>
<td>Fino and Kniel, 2008</td>
</tr>
<tr>
<td>MNV</td>
<td>Green onions</td>
<td>UV light</td>
<td>240 mJ/cm²</td>
<td>1.2 (external) 0.2 (internal)</td>
<td>Hirneisen and Kniel, 2013b</td>
</tr>
<tr>
<td>MNV</td>
<td>Green onions</td>
<td>High Pressure</td>
<td>500 MPa, 2 min, 20°C</td>
<td>&gt;6.4 (external) &gt;4.7 (internal)</td>
<td>Hirneisen and Kniel, 2013b</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Green onions</td>
<td>High pressure</td>
<td>375 MPa, 5 min, 21°C</td>
<td>4.75</td>
<td>Kingsley et al., 2005</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> Typhimurium</td>
<td>Green onions</td>
<td>Dry pulsed light</td>
<td>5, 15 s</td>
<td>4.6 (15 s)</td>
<td>Xu and Wu, 2014</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> Typhimurium</td>
<td>Green onions</td>
<td>Wet Pulsed Light</td>
<td>5-60s</td>
<td>3.6 (60 s)</td>
<td>Xu and Wu, 2014</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Green onions</td>
<td>Dry Pulsed Light</td>
<td>5, 15 s</td>
<td>5.2 (15 s)</td>
<td>Xu et al., 2014</td>
</tr>
<tr>
<td>MNV</td>
<td>Salsa</td>
<td>High Pressure</td>
<td>400 MPa, 1-10 min, 9°C</td>
<td>&gt;4.0 (5, 10 min)</td>
<td>Hirneisen et al., 2014</td>
</tr>
</tbody>
</table>

High pressure processing has been shown to inactivate hepatitis A virus (Kingsley et al., 2005), *Salmonella, E. coli* O157:H7, and other pathogens in green onions and in salsa containing green onions (Neetoo and Chen, 2011). Inactivation of murine norovirus by high pressure processing was tested on strawberries and strawberry puree (Huang et al., 2014).
Work by Hirneisen and Kniel (2013b) and Hirneisen et al. (2014) showed that norovirus can be reduced in green onions and salsa; however, the work presented here focuses on lower pressures and process optimization. The results show that complete elimination of murine norovirus is possible in green onions and salsa using high pressure, thus demonstrating the effectiveness of high pressure against norovirus in these food matrices.

Pulsed light combined with or without sanitizer washing was tested as a method to inactivate *E. coli* O157:H7 and *Salmonella enterica* Typhimurium (Xu et al., 2013; Xu and Wu, 2014) on green onions. Pulsed light alone inactivated $>4 \log_{10}$ of *E. coli* on the surface in 5 seconds, but damaged the sensory quality. A longer treatment with agitation (60 seconds) preserved the sensory quality while providing a comparable reduction, though complete elimination did not occur.

### 2.2 Inactivation of *Salmonella* on Blueberries by Combined Washing and Ultraviolet Light Process

Blueberries are an increasingly popular food in the United States, and are frequently eaten raw as a topping for breakfasts, desserts, or as a component in fruit salads (US Highbush Blueberry Council, 2014). Blueberries and other berries have been linked to outbreaks of a variety of pathogens, including *Salmonella*; likewise, *Salmonella* has caused outbreaks on many fresh produce items. These food safety issues will be discussed, along with the state of the art of produce washing and ultraviolet light processing. Experimental nonthermal methods for the inactivation of *Salmonella* on produce will be examined as well.
2.2.1 *Salmonella enterica*

*Salmonella* is a genus of rod-shaped, gram-negative bacteria in the *Enterobacteriaceae* family. Taxonomy within the genus has been controversial (Tindall et al, 2005), but it is now accepted that there are two species in the genus *Salmonella*: *S. bongori*, typically associated with reptiles; and *S. enterica*, which causes disease in humans and other mammals. Over 2500 serovars of *S. enterica* exist, spread across 6 subspecies; frequently, they are identified by the city of isolation (such as “Newport”, “Montevideo”, “Saintpaul”, “Muenchen”, “Dublin”, “Heidelberg” and others) or by names descriptive of the symptoms they cause in certain hosts (“Typhimurium”, “Pullorum”, “Bovismorbificans”, and others) (Coburn et al, 2006).

In humans, *Salmonella enterica* serovars can cause typhoid fever, or enterocolitis and diarrhea. The latter syndrome is referred to as *Salmonellosis*, and is the primary illness caused by *Salmonella* in the developed world (Coburn et al., 2006). Estimates of worldwide incidence of non-typhoidal *Salmonella* infections range from 200 million to 1.3 billion cases per year, resulting in approximately 3 million deaths. Typhoid fever, interestingly, was one of the first outbreaks of foodborne illness traced to an asymptomatic food handler; that food handler was named Mary Mallon, but is better known as “Typhoid Mary” (Soper, 1907). Relatively low numbers of bacteria are needed to cause disease; Blaser and Newman (1982) estimated that <10^3 bacteria were required for infection. This means that a single contamination event can sicken many people.

Salmonellosis, like norovirus and many other enteric diseases, is spread by the fecal-oral route. It can be prevented by good sanitation, personal hygiene, (as was the case with Typhoid Mary, who refused to wash her hands), and proper management of animal waste (Hanning et al., 2009), which can otherwise contaminate drinking and
irrigation water. The latter can contaminate produce (Steele and Odumeru, 2004), which can sicken consumers in the absence of food-safety interventions.

2.2.1.1 Salmonella and Produce Safety

*Salmonella* is typically associated with poultry, eggs, and other animal products, but has caused many outbreaks traceable to fresh produce (Hanning et al., 2009). *Salmonella* is capable of growth on a variety of fresh produce items, including tomatoes (Greene et al., 2008), mangoes, salad greens, sprouts, and other commodities (Hanning et al., 2009). A summary of *Salmonella enterica* outbreaks associated with produce items is presented in Table 5.
Table 5: Confirmed outbreaks of *Salmonella enterica* associated with fresh produce other than berries.

<table>
<thead>
<tr>
<th>Year</th>
<th>Foods</th>
<th>Serotype</th>
<th>Location</th>
<th>Setting</th>
<th>Reported illnesses</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>deviled eggs; green beans</td>
<td>Enteritidis</td>
<td>Virginia</td>
<td>Private home</td>
<td>29</td>
<td>CDC</td>
</tr>
<tr>
<td>2001</td>
<td>grapes, green</td>
<td>Senftenberg</td>
<td>Multistate</td>
<td>Private home</td>
<td>40</td>
<td>CDC</td>
</tr>
<tr>
<td>2001</td>
<td>ground beef, macaroni and cheese; peppers, green</td>
<td>Heidelberg</td>
<td>Louisiana</td>
<td>Restaurant</td>
<td>93</td>
<td>CDC</td>
</tr>
<tr>
<td>2002</td>
<td>mustard greens, potato salad</td>
<td>Johannesburg</td>
<td>Florida</td>
<td>Picnic</td>
<td>56</td>
<td>CDC</td>
</tr>
<tr>
<td>2004</td>
<td>green salad</td>
<td>Javiana</td>
<td>Florida</td>
<td>Restaurant</td>
<td>24</td>
<td>CDC</td>
</tr>
<tr>
<td>2005</td>
<td>green salad; sandwich, chicken</td>
<td>Muenchen</td>
<td>Pennsylvania</td>
<td>Restaurant</td>
<td>9</td>
<td>CDC</td>
</tr>
<tr>
<td>2007</td>
<td>cantaloupe; fruit salad; grapes, green salad; honeydew melon</td>
<td>Litchfield</td>
<td>New Jersey</td>
<td>Restaurant</td>
<td>30</td>
<td>CDC</td>
</tr>
<tr>
<td>2007</td>
<td>chicken; green salad</td>
<td>Newport</td>
<td>New Jersey</td>
<td>Other</td>
<td>16</td>
<td>CDC</td>
</tr>
<tr>
<td>2007</td>
<td>green salsa; pig head; red salsa</td>
<td>Montevideo</td>
<td>Illinois</td>
<td>Grocery store</td>
<td>9</td>
<td>CDC</td>
</tr>
<tr>
<td>2008</td>
<td>green salad; tomato</td>
<td>Braenderup</td>
<td>Iowa</td>
<td>Restaurant</td>
<td>12</td>
<td>CDC</td>
</tr>
<tr>
<td>2009</td>
<td>green chile</td>
<td>Newport</td>
<td>Colorado</td>
<td>Religious location</td>
<td>43</td>
<td>CDC</td>
</tr>
<tr>
<td>2009</td>
<td>green onion/scallion</td>
<td>Javiana</td>
<td>Multistate</td>
<td>Unknown</td>
<td>9</td>
<td>CDC</td>
</tr>
</tbody>
</table>

Some of the outbreaks (3/12) listed in Table 5 also involve animal products. It is possible that the implicated items were eaten together with contaminated produce; perhaps the 2007 Illinois *S. Montevideo* outbreak and the 2005 Pennsylvania *S.*
Muenchen outbreak fit this pattern. As discussed above in section 2.1.4.2, it is also possible that an infected food handler contaminated multiple items (ground beef, macaroni and cheese; green peppers), or that cross contamination occurred at a specific station at a restaurant (cantaloupe; fruit salad; grapes, green salad; honeydew melon). A search of the CSPI Outbreak Alert! Database yields even more outbreaks, but without a clear link to produce. The strains used in the present study make multiple appearances. S. Montevideo appears six times; S. Newport appears 32 times; S. Saintpaul appears eight times; and S. Stanley appears twice, for a total of 38 outbreaks.

2.2.1.2 Sources of Salmonella Contamination

Salmonella contamination of food can arise from a variety of sources, such as uncomposted manure applied to fields, runoff from sewage and grazing operations, and wild birds (Hanning et al., 2009; Simpson et al., 2002). Animal production in particular is a worrisome source of Salmonella since strains of Salmonella that flourish in such environments tend to be resistant to antibiotics (Ekperigin and Nagaraja, 1998). In the case of blueberries, contamination by wild birds is likely an important source of Salmonella. According to Pritts (n.d.), birds eat or damage up to 30% of blueberries grown in the Northeast. It stands to reason that birds eating blueberries will leave droppings on the bushes, and that these droppings could be a source of Salmonella contamination. Other fruits and vegetables are known to be contaminated in a similar manner (Matthews et al., 2014). For small operations, installation of fences might be sufficient to prevent intrusion of deer, sheep and cattle, which are also known to be reservoirs of Salmonella and other bacterial pathogens (Branham et al., 2005).
2.2.2 Blueberries

2.2.2.1 Blueberry Production and Storage

Blueberries are the climacteric fruits of *Vaccinium corymbosum*, a woody perennial shrub (Vander Kloet, 1980) grown commercially in the United States, Canada, South America and Australia. The fresh blueberries sold in supermarkets are typically harvested from dozens of highbush varieties, such as “Bluehaven”, “Bluejay”, “Blueray”, “Collins”, “Duke”, “Herbert”, “Patriot”, “Jersey”, and “Lateblue”, to name a few (University of Maryland Extension, n.d.; Penn State Extension, n.d.). Because blueberries are so perishable, different varieties have been developed to mature and ripen at different times of year. Though blueberries are climacteric fruits and can ripen after harvest, they do not develop full flavor unless allowed to ripen before harvesting (Mitcham et al., 2014). Blueberries are stored at 0°C, under 90-95% relative humidity; the shelf life of “Duke” can be extended to 6 weeks under controlled atmosphere storage (Harb and Streif, 2014).

Blueberries are an increasingly popular food in the United States; as part of a larger demand for fresh, minimally processed foods, consumers and foodservice operations are buying increasing numbers of fresh blueberries (US Highbush Blueberry Council n.d.). Blueberries are available year-round in many supermarkets. Blueberry harvesting in North America begins in April in Florida and continues northward along the East Coast until the last harvest of September in Maine (US Highbush Blueberry Council n.d.). When berries from North America are not available, berries are imported from South America. During off-season, consumers in Europe and Asia receive berries from Australia, where blueberries are harvested from July to April (Australian Blueberry Growers Association, 2014). Blueberries are
typically picked by hand and packed into PET clamshell containers without further processing (Strike, 2007).

### 2.2.2.2 Blueberries and Food Safety

A number of disease outbreaks have been traced to berries, including blueberries. Data from selected outbreaks have been summarized in Table 6.

**Table 6: Disease Outbreaks Involving Berries**

<table>
<thead>
<tr>
<th>Year</th>
<th>Foods</th>
<th>Pathogen</th>
<th>Location</th>
<th>Setting</th>
<th>Reported illnesses</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>strawberries (frozen)</td>
<td>Hepatitis A Virus</td>
<td>Multistate</td>
<td>Multiple Settings</td>
<td>51</td>
<td>CSPI</td>
</tr>
<tr>
<td>1997</td>
<td>strawberries (frozen)</td>
<td>Hepatitis A Virus</td>
<td>Multistate</td>
<td>School</td>
<td>256</td>
<td>CSPI</td>
</tr>
<tr>
<td>1998</td>
<td>strawberries (frozen)</td>
<td>Hepatitis A Virus</td>
<td>Texas</td>
<td>Multiple Settings</td>
<td>29</td>
<td>CSPI</td>
</tr>
<tr>
<td>2003</td>
<td>strawberries</td>
<td><em>Salmonella</em> Group B</td>
<td>California</td>
<td>Restaurant; daycare; school</td>
<td>13</td>
<td>CSPI</td>
</tr>
<tr>
<td>2006</td>
<td>blueberries; strawberries</td>
<td><em>E.coli</em>, O26 (STEC)</td>
<td>Massachusetts</td>
<td>Other</td>
<td>5</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2008</td>
<td>berries</td>
<td><em>Cryptosporidium cayatenensis</em></td>
<td>Tennessee</td>
<td>Banquet facility</td>
<td>3</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2008</td>
<td>mixed berries</td>
<td><em>C. cayatenensis</em></td>
<td>California</td>
<td>Workplace cafeteria; banquet facility</td>
<td>59</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2009</td>
<td>blueberries</td>
<td><em>Salmonella</em> Muenchen</td>
<td>Multistate</td>
<td>Private Home</td>
<td>14</td>
<td>CDC, CSPI</td>
</tr>
<tr>
<td>2010</td>
<td>blueberries</td>
<td><em>Salmonella</em> Newport</td>
<td>Minnesota</td>
<td>Private Home</td>
<td>6</td>
<td>CDC, CSPI</td>
</tr>
</tbody>
</table>

Between the CDC and CSPI, 46 unique outbreaks have been recorded involving berries. The outbreaks listed in Table 6 are relevant to the work discussed here because they involve blueberries, involve *Salmonella*, or could have been prevented by a combined UV-washing process as detailed in Chapter 4. Frozen foods, especially frozen fruit, represent a unique opportunity for food safety; they are
traceable and are of sufficiently high value that the extra costs associated with a safety intervention would almost certainly be economically beneficial for processors. The presence of bacterial pathogens such as *Salmonella* and *E. coli* indicate fecal or animal contamination at some stage in the chain from farm to fork; the presence of *Cryptosporidium* indicates water-related contamination (Ramirez et al., 2004). All of these pathogens are known to be sensitive to UV light (Bialka and Demirci, 2007; Kim and Hung, 2012; Hofman-Caris and Beerendonk, 2011).

2.2.3 Washing

2.2.3.1 Theoretical Underpinnings

Washing is essentially a mass-transfer process (Atiemo-Obeng, 2003). Microbes, soil and other contaminants are dissolved from the surface of the berry into the surrounding medium when the liquid concentration is low and the surface concentration is high; however, the opposite occurs when the liquid concentration is high and the surface concentration is low. Therefore, correlations for mass transfer of solids dissolving into an agitated liquid could be used to conceptually model the transfer of microbes from the surface of the berry to the washing medium. Mass transfer of solids into agitated liquids occurs in two regimes, depending on the motion of the solids. At low agitation rates (i.e., low impeller speed, energy dissipation rate, or bulk flow velocity), the solids settle at the bottom or top of the liquid and mass transfer is slow (Atiemo-Obeng, 2003). At a critical impeller speed, the solids are lifted from the bottom of the vessel and do not return for more than 2 seconds at a time (Atiemo-Obeng, 2003); at and above this speed, the rate of mass transfer is only weakly dependent on the energy dissipation rate and impeller rotational speed.
Therefore, as long as the particles (in this case, the berries) do not remain on the top or bottom surface of the liquid, similar rates of mass transfer (and bacterial shedding) should be expected regardless of the washing configuration.

### 2.2.3.2 Washing of Produce

Sanitizer-washing processes are frequently used in the produce industry to prepare products for market while preventing cross-contamination. Chlorine is the most widely used sanitizer in the food industry because it is inexpensive and relatively simple to use (FDA, 2009). Other sanitizers, such as hydrogen peroxide, trisodium phosphate, and peroxycetic acid, are also used but on a smaller scale.

A variety of configurations for washers exist, depending on the product being washed. In some systems, produce fresh from the field is first washed in a “dump tank” to remove soil, leaves, and other debris before sanitizer spray washing, followed by a rinsing step in fresh water (Hoss, 2013). Potatoes for chip production are washed in a dump tank and then peeled and brush-washed in a single step using coarse brushes that remove soil and the skin of the potato simultaneously (Hoss, 2007). Such aggressive brush-washing is of course unsuitable for all but the sturdiest of fruits and vegetables, and spray-washing is therefore used for berries.

#### 2.2.3.2.1 Chlorine Treatments

Chlorinated water washing is a widely used processing step in the fresh produce industry (FDA, 2009). Chlorine treatments are typically followed by a potable water washing step to remove chlorine residue.

Chlorine levels in washing processes must be continuously monitored and controlled. Work by Luo et al. (2011), showed that although chlorine washing with 10
ppm free chlorine can prevent cross-contamination of *E. coli* O157:H7, it is not sufficient to eliminate *E. coli* O157:H7 on the surface of improperly washed, cross-contaminated lettuce, even at concentrations as high as 50 ppm free chlorine. This demonstrates that preventing cross-contamination is of critical economic and public health importance, since produce that has been cross-contaminated likely cannot be saved and must be destroyed.

### 2.2.3.2.2 Other Sanitizer Treatments

Hydrogen peroxide, trisodium phosphate, peroxyacetic acid, and a variety of other sanitizers have been tested experimentally and used commercially for washing of fresh produce.

Hydrogen peroxide has a long history of commercial use for sanitization of surfaces and containers (FDA, 1979), and has been tested for use in produce washing with inconsistent results (FDA, 2009). Hydrogen peroxide can be activated by ultraviolet light, and is used for decontamination of drinking water (Hofman-Caris and Beerendonk, 2011). Trisodium phosphate (TSP) is sometimes used for produce washing, and is very effective against pathogens with short contact times (Li and Wu, 2014); however, the alkaline pH of TSP solutions and the creation of phosphate-laden wastewater makes commercial use of TSP as a sanitizer a difficult proposition (FDA, 2009). Peroxyacetic acid has been used to prevent cross-contamination of produce (Peroxyacetic Acid as Disinfectant, 2011), though it is of limited effectiveness for surface decontamination (FDA, 2009). Other sanitizers, such as ozone, iodine and bromine have seen limited use; of these, ozone seems to be the most promising (FDA, 2009).
2.2.4 Ultraviolet Light

Ultraviolet light ranges in wavelength from 400 to 10 nm, though the most important range of wavelengths for food safety is 200-280 nm, referred to as the “germicidal” range for the damage it does to viruses, bacteria, fungi and protozoa (Guerrero and Beltran, 2004; Hofman-Caris and Beerendonk, 2011). Ultraviolet light has been used for many years to disinfect air, water and surfaces in food processing environments, movie theaters, cruise ships, hospitals and other large structures (Bintsis et al., 2000).

2.2.4.1 Existing Equipment

Ultraviolet light has been used for many years to purify drinking water (Hofman-Caris and Beerendonk, 2011), but the application of ultraviolet light to foods has been more recent (Guerrero and Beltran, 2004). The most widely used UV source is the low-pressure mercury vapor lamp which produces monochromatic light at 253.7 nm, within the germicidal range (Bintsis et al., 2000). These lamps operate at relatively cool temperatures and can produce radiation at intensities suitable for food processing. Alternative technologies include medium- and high-pressure mercury vapor lamps, which operate at much higher temperatures and produce significant visible and infrared radiation as well (Heraeus Noblelight, n.d.)

Processing of liquid foods by ultraviolet light is relatively straightforward. Equipment designs tend to be similar to those used for shell-and-tube heat exchangers, except that the heat transfer tubes are replaced with UV lamps in protective quartz sleeves. Food to be processed is pumped into the treatment chamber at a controlled rate to ensure that the proper dose and therefore level of inactivation is achieved (Murakami et al., 2005).
Processing of solid foods is more complicated since UV light can only act on one surface of solid foods at a time (Bialka and Demirci, 2007). Typical setups involve stationary UV lamps suspended over a conveyor belt which carries food to be processed (Heraeus Noblelight, n.d.). The problems caused by limited penetration depth can be fixed by flipping or otherwise rotating the food; the nature of the food determines the best method. Berries can be rotated using a washing process, which improves effectiveness of pulsed-light treatments and prevents thermal damage to blueberries (Huang and Chen, 2013).

### 2.2.4.2 Effects on Food

UV-C light (at approximately the same intensities as used in this study) has been shown to increase oxygen radical absorbance content (ORAC) of blueberries by up to 50% immediately after treatment (Wang et al., 2009; Perkins-Veazie et al., 2008). This is likely a response by the blueberry tissues to undo damage caused by the radiation. This production of phenolic compounds also slows the growth of bacteria and mold on the surface of the fruit, even if the bacteria are added after exposure (Guerrero and Beltran, 2004). Such hermetic effects are observed in a wide variety of produce items, from sweet potatoes to peaches and citrus fruits (Guerrero and Beltran, 2004).

Sensory effects on food are generally minimal, but some foods can be damaged by UV-C light. A study of apple slices showed that the application of UV-C light caused darkening and softening, with these effects increasing with dose; optical microscopy revealed that this was caused by damage to the cell walls of the apple slices (Gomez et al., 2010).
2.2.4.3 Effects on Microorganisms

Ultraviolet light causes DNA mutations as well as thymine-thymine dimerization; this effectively “jams” DNA replication and transcription to RNA (Witkin, 1976). Repair of DNA requires blue light as well as a sequence of enzymatic steps to replace the damaged sections.

Sommer et al. (1998) found that bacteria can be inactivated in drinking water by ultraviolet light across a 100-fold range of intensities and that the dose required for reduction decreased with increasing intensity. They also found that reduction of viruses and bacterial spores was related only to total dose, and was not affected by treatment intensity. This suggests that bacteria are able to repair damage caused by UV light at a limited rate, and that at increasing intensities the repair mechanisms are less and less able to cope; spores and viruses are unable to repair, and as such are inactivated after certain structures are damaged.

2.2.4.4 Use of UV in Food Processing

Ultraviolet light is commercially used for air and water treatment as well as for food preservation; unfiltered apple cider (described in Murakami et al., 2005), baked goods, liquid eggs and other food products have been treated this way to ensure safety and extend shelf-life without compromising “fresh” and “uncooked” sensory quality (Siegner, 2014). Ultraviolet light is particularly useful for disinfection of ice chips, since ice is both transparent and temperature sensitive (Heraeus Noblelight, n.d.). Ultraviolet light is used for food-contact surfaces to suppress microbial growth and prevent cross contamination (Bintsis et al., 2000).
2.2.5 Experimental Methods for Nonthermal Decontamination of Blueberries

Experimental techniques for nonthermal decontamination of blueberries tend to fall into two categories: chemical methods, which rely on the action of sanitizers; and physical methods, which rely on washing or light for effectiveness. Mixed methods exist as well, and will be discussed as part of the physical methods category.

2.2.5.1 Chemical Methods

Chemical methods tested to remove *Salmonella* spp. and other contaminants on blueberries include: gaseous and aqueous ozone (Bialka and Demirci, 2007); chlorine dioxide (Wu and Kim, 2007); and hydrogen peroxide and sodium dodecyl sulfate (Li and Wu, 2014. The approaches, and effectiveness of these treatments is summarized in Table 7.
Table 7: Experimental Chemical Treatments for Inactivation of *Salmonella* and *E.coli* O157:H7 on blueberries

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Sanitizer</th>
<th>Process</th>
<th>Time</th>
<th>Sample size (g or # of berries)</th>
<th>Maximum Effectiveness (log&lt;sub&gt;10&lt;/sub&gt; reduction)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em> outbreak cocktail</td>
<td>gaseous ozone</td>
<td>continuous gas flow</td>
<td>2-64 min</td>
<td>18 berries</td>
<td>1.0 (64 min)</td>
<td>Bialka and Demirci, 2007</td>
</tr>
<tr>
<td><em>S. enterica</em> outbreak cocktail</td>
<td>gaseous ozone</td>
<td>pressurized gas contact</td>
<td>2-64 min</td>
<td>18 berries</td>
<td>3.0 (64 min)</td>
<td>Bialka and Demirci, 2007</td>
</tr>
<tr>
<td><em>S. enterica</em> outbreak cocktail</td>
<td>aqueous ozone, 7.9 ppm</td>
<td>gas sparged into water</td>
<td>2-64 min</td>
<td>18 berries</td>
<td>4.9 (32 min)</td>
<td>Bialka and Demirci, 2007</td>
</tr>
<tr>
<td><em>S. enterica</em> Typhimurium</td>
<td>aqueous chlorine, 4 ppm</td>
<td>agitated washing</td>
<td>1, 5 min</td>
<td>4, 80 g</td>
<td>4.1 (5 min)</td>
<td>Li and Wu, 2014</td>
</tr>
<tr>
<td><em>S. enterica</em> Typhimurium</td>
<td>acetic acid + SDS</td>
<td>agitated washing</td>
<td>1, 5 min</td>
<td>4, 80 g</td>
<td>4.0 (5 min)</td>
<td>Li and Wu, 2014</td>
</tr>
<tr>
<td><em>S. enterica</em> Typhimurium</td>
<td>hydrogen peroxide + SDS</td>
<td>agitated washing</td>
<td>1, 5 min</td>
<td>4, 80 g</td>
<td>4.0 (5 min)</td>
<td>Li and Wu, 2014</td>
</tr>
<tr>
<td><em>S. enterica</em> Typhimurium</td>
<td>aqueous chlorine dioxide, 10 ppm</td>
<td>non-agitated contact</td>
<td>10 s -2 hr</td>
<td>10 g</td>
<td>2.28 (2 hr)</td>
<td>Wu and Kim, 2007</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 cocktail</td>
<td>EO water</td>
<td>electrostatic spray</td>
<td>4, 16 s</td>
<td>6 berries, 9-11 g</td>
<td>0.66 (16 s)</td>
<td>Kim and Hung, 2012</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7 cocktail</td>
<td>gaseous ozone</td>
<td>continuous gas flow</td>
<td>1 min</td>
<td>6 berries, 9-11 g</td>
<td>1.64</td>
<td>Kim and Hung, 2012</td>
</tr>
</tbody>
</table>

In the experiments conducted by Bialka and Demirci (2007), blueberries inoculated with *E. coli* or *S. enterica* were exposed to continuously flowing gaseous ozone, pressurized gaseous ozone and aqueous ozone for treatment times ranging from...
2 to 64 minutes in the absence of agitation. Continuous flow of gaseous ozone over the course of 64 minutes resulted in a 1.0-log reduction for Salmonella; shorter treatment times were less effective. Pressurized ozone exposure for 64 minutes was more effective, resulting in a 3.0-log reduction for Salmonella. Aqueous ozone at 7.9 ppm was more effective than gaseous ozone, and produced a 3.5-log reduction after 16 minutes of treatment. The most effective treatment was at 32 minutes, with a 4.9-log reduction.

Sanitizer washing protocols for blueberries tested by Li and Wu (2013) showed that agitated washing in water containing 4 ppm free chlorine can achieve a 3.2-log reduction of a single strain of Salmonella within 5 minutes. Efficacy was not improved with the addition of sodium dodecyl sulfate (SDS), which would be expected to help detach bacteria from the surface of the produce and perhaps increase membrane permeability. The most effective treatment the authors tested was a combination of 500 ppm acetic acid and 5000 ppm SDS, which resulted in a 4.0-log reduction. Li and Wu also showed that hydrogen peroxide at 200 ppm and SDS at 500 ppm seemed to have a synergistic effect on the reduction of Salmonella, which suggests that multiple sanitizers or sanitizing techniques used together are more effective than each treatment used singly.

Sanitizer treatments that do not include active agitation require significantly more time to achieve 3-4-log reductions than treatments that do involve agitation; in experiments conducted by Wu and Kim (2007), 30 minutes of contact time was needed to achieve a 3-log reduction of Salmonella Typhimurium at 10 ppm aqueous chlorine dioxide. Treatment times of 5 minutes at 10 ppm chlorine dioxide showed
only a 0.60-log reduction, which is approximately one fifth of the efficacy of the treatment used by Li and Wu.

### 2.2.5.2 Physical Methods

Physical methods for nonthermal inactivation of pathogens include ultraviolet light, pulsed light, high pressure processing, irradiation, cold plasma processing, and water washing. The first two methods have been used for inactivation of *Salmonella* or *E. coli* O157:H7 on blueberries; the others have been applied to uninoculated blueberries to determine whether the treatment damages the sensory quality of the blueberries.

**Table 8: Experimental Physical and Combined Treatments for Inactivation of *Salmonella* and *E. coli* O157:H7 on blueberries**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Process</th>
<th>Time</th>
<th>Sample size (g or # of berries)</th>
<th>Maximum Effectiveness (log$_{10}$ reduction)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em></td>
<td>Dry Pulsed Light</td>
<td>5-60 s</td>
<td>18 berries</td>
<td>3.8 (60 s)</td>
<td>Bialka and Demirci, 2007</td>
</tr>
<tr>
<td>outbreak cocktail</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td>Dry Pulsed Light</td>
<td>5-60 s</td>
<td>3 berries, 5 g</td>
<td>5.7 (60 s)</td>
<td>Huang and Chen, 2014</td>
</tr>
<tr>
<td>outbreak cocktail</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td>Wet Pulsed Light</td>
<td>5-60 s</td>
<td>3 berries, 5 g</td>
<td>&gt;5.9 (60 s)</td>
<td>Huang and Chen, 2014</td>
</tr>
<tr>
<td>outbreak cocktail</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>UV + Ozone</td>
<td>2 min</td>
<td>6 berries, 9-11 g</td>
<td>4.02</td>
<td>Kim and Hung, 2012</td>
</tr>
<tr>
<td>cocktail</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>High intensity</td>
<td>1-10 min</td>
<td>6 berries, 9-11 g</td>
<td>&gt;4.1 (10 min)</td>
<td>Kim and Hung, 2012</td>
</tr>
<tr>
<td>cocktail</td>
<td>UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Pulsed light processing as tested by Bialka and Demirci (2007) was of reasonable effectiveness (3.8-log reduction), but only when the berries were placed 3 cm away from the quartz window separating the lamp from the treatment chamber. The authors also tested distances of 8 and 13 cm and observed decreasing effectiveness at each treatment time. At 60 seconds treatment time, placement of the berries at 8 and 13 cm resulted in 2.9- and 2.6-log reductions, which indicates that the intensity of the pulsed light has a noticeable effect.

Pulsed light processing of blueberries in the absence of a washing process causes considerable heating on the surface of the berries, melting the waxy “bloom” on the surface and causing softening and darkening (Huang and Chen, 2014). The effectiveness of this treatment was very high (5.7- vs 3.8-log reduction), exceeding what was observed by Bialka and Demirci (2007). Combining the pulsed light process with a water- or sanitizer-washing process improved effectiveness (5.7- vs >5.9-log reduction) prevented thermal damage, rotated the berries to expose all sides, and removed pathogens from the surface where they are rapidly inactivated.

Ultraviolet light processing of blueberries inoculated with *E. coli* O157:H7 has been tested by Kim and Hung (2012). In their experiments, blueberries inoculated on the skin or calyx were held on a rack 0.9 cm away from a UV lamp, resulting in an intensity of 20 μW/cm². For skin-inoculated berries, most of the inactivation occurred in the first minute of treatment, with a 3.1-log reduction observed after 1 minute, and a 3.7-log reduction observed after 5 minutes of exposure (no survivors were detected after 10 minutes). For calyx-inoculated berries, only a 1.5-log reduction was observed after 1 minute, a 1.82-log reduction after 5 minutes, and a 2.14-log reduction after 10 minutes. The authors attributed this strongly decreased effectiveness to the shadowing
effect of the calyx which limits the number of bacteria exposed to the UV light. These results suggest that bacteria not directly in the path of UV light can be inactivated, albeit slowly or that a thick layer of inoculum can have a protective effect for bacteria closest to the berry. A combination of UV treatment and ozone was faster than either treatment alone; 1 min UV treatment at 7.9 μW/cm² followed by 2 min ozone resulted in a similar effectiveness (log reductions of 4.02 vs 4.1) for skin-inoculated berries as a UV treatment 2.5 times more intense and 10 times longer. No changes in sensory quality were noted, despite the very high intensity of the light and the possible thermal effects which could have resulted from close contact with the lamp.

High pressure processing of blueberries could be a promising option for decontamination; sensory tests on processed berries conducted by Lou et al. (2011) showed that the color and appearance were unaffected, but softening was observed. It is possible that cultivars with firmer fruit might be better suited to high pressure processing, since softening of a very firm variety might not be noticeable to consumers. Furthermore, high pressure processing at low temperatures might be a useful way to decontaminate and freeze (or at least pre-chill) blueberries by adiabatic cooling.

Electron-beam and gamma irradiation of blueberries has been tested by Moreno et al. (2007) and Miller et al. (1994), respectively. Electron beam irradiation at up to 1.6 kGy was found to have minimal impact on the sensory quality of the blueberries, besides softening. The physical appearance, chemical composition, pH, and other sensory qualities were essentially unaffected. However, gamma irradiation at all of the levels tested (0.75-3.0 kGy) noticeably damaged blueberries to varying degrees, with damage to overall sensory quality and cell wall structure increasing with
dose. Miller et al. (1994) suggested that doses on the order of 0.75 kGy might be suitable for preservation of blueberries, rather than any of the higher doses; it is possible that an even lower dose would be better.

Physical methods alone were capable of achieving significant reductions; however, addition of a sanitizer or use of water washing process improved effectiveness and/or shortened the time needed to achieve nearly complete elimination.
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Chapter 3

INACTIVATION OF MURINE NOROVIRUS ON GREEN ONIONS AND IN SALSA BY HIGH HYDROSTATIC PRESSURE

3.1 Abstract

High pressure processing was tested as a method for inactivation of a surrogate for human norovirus (murine norovirus) in green onions and salsa. Existing nonthermal methods for decontamination of green onions rely on sanitizer washing, which is effective only against contaminants on the surface of green onions and is infeasible for salsa. Conditions for high pressure treatment, inactivation kinetics at two pressure levels, and persistence testing in salsa at as-received and acidified pH were tested. Over the range of 1-20°C, 1°C was most effective and addition of water to the treatment package was found to improve effectiveness. The most effective treatment for green onions was 350 MPa at 1°C with water in the package for 3 minutes, resulting in complete elimination of the virus (>5.5 log reduction). Decimal reduction times calculated at 300 and 350 MPa (66 s and 36 s, respectively) indicate increasing barosensitivity at higher pressures. A 300-MPa treatment in salsa for 2 minutes resulted in a 2.2-log reduction that was not dependent on pH over the range 3.8-4.0; storage trials lasting 24 and 72 h resulted in ~0.5-log reductions, demonstrating that murine norovirus is insensitive to acidic pH both during pressure treatment and during storage. These results demonstrate the effectiveness of high pressure processing as a useful method for decontamination of green onions and salsa.
3.2 Introduction

Norovirus, most often linked to fresh produce, affects tens of millions of Americans each year and is the source of more than half of all incidents of foodborne illness in the United States (Hall et al., 2012). Green onions and fresh salsa made with green onions are typically consumed raw and without significant food safety interventions beyond washing (Wheeler et al., 2005). Green onions and fresh salsa containing green onions were the source of a 2003 outbreak of hepatitis A in Pennsylvania which sickened more than 600 people (Wheeler et al., 2005). Norovirus outbreaks connected to these commodities have occurred in similar ways (CDC, 2014).

These and other outbreaks could be prevented by the use of high pressure processing. High pressure processing is a nonthermal process by which foods are exposed to pressures exceeding 200 MPa typically for a few minutes (Cheftel, 1995). High pressure processing is used for a wide variety of food products (Oey et al., 2008), such as juice, jam, luncheon meats, guacamole and salsa (Torres and Velazquez, 2005; Bermúdez-Aguirre and Barbosa-Cánovas, 2011). The intense pressures used (as high as 600-800 MPa) denature proteins but leave small molecules, such as flavor and aroma compounds, intact (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). Foods processed with high pressure retain “fresh”, “raw” sensory quality, especially compared to thermally-processed foods (Kovač et al., 2010).

High pressure processing of green onions and salsa represents an opportunity to reduce the spread and incidence of norovirus among the general public, but also more specifically in healthcare and cruise ship settings. These settings, along with daycare centers, are most vulnerable to norovirus outbreaks (Kanerva et al., 2009; Khanna et al., 2003; Kosa et al., 2014).
Human norovirus is not culturable, but existing work indicates that an infectivity assay is a more reliable measure of risk than genome copy assays (Baert et al., 2008, Tang et al., 2010). Fortunately, murine norovirus is a culturable norovirus (Wobus et al., 2004) that has been used frequently as a surrogate for human norovirus (Bae and Schwab, 2008; Huang et al., 2014; Richards, 2012; Tang et al., 2010). The objective of the present study is to determine the effectiveness of high pressure processing for inactivating murine norovirus in green onions and salsa, and to determine strategies for improving the effectiveness of pressure treatments in these foods.

3.3 Methods and Materials

3.3.1 Virus and Cell Culturing Methods

Murine norovirus (MNV-1) was propagated in RAW 264.7 cells maintained in Dulbecco’s Modified Eagle Medium (DMEM; Becton Dickinson Life Sciences, Hunt Valley, MD) supplemented with 10% Fetal Bovine Serum (FBS; BD). Virus stock solution was prepared by infecting 80% confluent monolayers until most of the cells were lysed. The progeny virus was separated from cell debris by three freeze-thaw cycles at -80 °C. The cell debris was removed by centrifugation.

Virus was enumerated by plaque assay using the procedure described by Li et al (2013). Briefly, RAW cells were grown to 80% confluent monolayers in 6-well plates, then treated with 400 μL of sample diluted in DMEM supplemented with 4% FBS for one hour. The plates were swirled every 5 minutes to prevent drying of the cells and to improve viral binding. The inoculation medium was removed and replaced with Minimal Essential Medium (MEM; Difco) overlay supplemented with 5% (v/v)
FBS, 1% sodium bicarbonate, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 2 mM glutamine (GIBCO), and 0.5% agarose (SeaPlaque, Lonza Group Ltd.) the overlay was hardened for at least 2 hours at 4 °C, then incubated at 37°C. After 48 hours of incubation, the cells and virus were fixed with 10% formalin solution in PBS. The overlay was removed and the plaques were visualized with 0.05% aqueous crystal violet solution in 10% ethanol.

3.3.2 Food Sample Preparation

3.3.2.1 Green Onions

Green onions from a local market were washed, trimmed or sliced and dried on petri dishes in a biosafety cabinet. Sections were trimmed to 5 cm and peeled as necessary to reach a target weight of 3 g; 3-mm slices were prepared and assembled to sample weights of 3.00 g. After 2 hours of drying, 20 μL (for sections) or 100 μL (for slices) of murine norovirus stock solution was spot-inoculated (as a series of small droplets) onto the outer or cut surfaces of the green onion. The inoculated samples were dried an additional two hours in a biosafety cabinet to permit viral attachment. The green onions were packaged with or without 1 mL sterile ultrapure water in sterile stomacher bags. The packaged green onions were kept on ice or at ambient temperature (for 20 °C treatments) before and during the treatment. After treatment, the green onions were removed from the bags and placed in 50-mL conical centrifuge tubes (BD) with 20 mL sterile phosphate buffered saline solution. The green onion sections or slices were vortexed for 30 seconds at 10,000 RPM to elute the virus from the surface. Aliquots of PBS were removed for serial dilution in DMEM supplemented with 4% FBS.
3.3.2.2 Salsa

The “medium heat” flavor of a nationally available brand of jarred salsa (See Appendix A for label) was used to guarantee consistency from sample to sample, and also as a representative example of salsa as it is formulated and consumed in the United States. Samples were sealed into stomacher bags and stomached at 260 RPM for 2 minutes to reduce the size of vegetable chunks; remaining chunks were crushed manually. Aliquots were frozen at -18°C until needed for experiments. The pH of each aliquot was checked with a UV-sanitized pH meter before inoculation; commercially available bottled lemon juice was used to adjust the pH to 3.80 as necessary (the pH of the salsa as-received was 4.00 in every case). A 25-mL serological pipette with the tip cut off was used to dispense 10 mL of salsa into sealed bags for inoculation and pressure treatment. In each case, 100 µL of virus stock solution was used as inoculum. All samples were double bagged and each bag was double-sealed. Inoculated samples were kept on ice before and after processing, which was performed at 1 °C and 300 MPa for 2 minutes. Stability tests were conducted in 50-mL conical centrifuge vials stored at ambient or refrigerated temperature for 24 or 72 hours. Salsa was diluted in PBS (Corning Cellgro, Corning, NY) and vortexed; 1 mL of liquid was withdrawn and centrifuged 22,000 x g for 1 minute to clarify. The clarified supernatant was serially diluted in DMEM + 4% FBS as above.

3.3.3 Pressure Treatment

Double-bagged samples were treated at initial sample temperatures of 1, 4, 10 or 20 °C and 300 or 350 MPa for 0.5-3.0 minutes using an Avure PT-1 pressure unit (Avure Technologies, Kent, WA) with temperature control and with water as a hydrostatic medium. The pressure come-up rate was approximately 22 MPa/s and
pressure release time was < 4 s. The pressurization times reported did not include the pressure come-up or release times.

3.3.4 Enumeration

Viral titer was determined by serial dilution of virus stock solution onto prepared 6-well plates. Plaque counts were converted to pfu/g, and log reductions were determined relative to the titer of the stock solutions, multiplied by a recovery factor that was determined separately for green onion sections (0.20) and slices (0.47), and for salsa (0.85) using control samples that were prepared but did not undergo experimental treatment (data not shown). Three samples were prepared for each set of treatment conditions. Statistical analysis was conducted using JMP; Tukey’s HSD Test was used to determine significance at the P<0.05 level.

3.4 Results and Discussion

3.4.1 Green Onion HPP Treatment

The goal of the first stage of this project was to test the effectiveness of pressure treatments on green onions. The first round of treatments was conducted at 4 and 20 °C and 350 MPa for 2 minutes to establish a baseline. Samples were tested with and without water to determine whether the addition of water improved effectiveness (Table 9).

<table>
<thead>
<tr>
<th></th>
<th>Log_{10} reduction in pfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without water</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
</tr>
<tr>
<td>temperature</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>1.7±0.4 a</td>
</tr>
<tr>
<td>20°C</td>
<td>1.6±0.1 a</td>
</tr>
</tbody>
</table>

Table 9: Reduction of MNV on Green Onions at two Temperature and Moisture Conditions (350 MPa, 2 min). Data with different lowercase letters are significantly different (P < 0.05)
Statistical analysis showed that there was a significant difference between inactivation at 4 and 20 °C, as well as a difference between dry and wet processing; this finding agrees with those of Li et al. (2013) who found that effectiveness of HPP against Tulane virus was improved with the addition of water. With this result, subsequent treatments were conducted with water added to the treatment package. To further distinguish the effect of temperature, pressure treatments were conducted at 1, 4 and 10°C at 300 MPa to improve the accuracy of reduction calculations by preventing data censoring (Table 10).

Table 10: Reduction of MNV on Green Onions at Three Temperatures (300 MPa, 2 min). Data with different lowercase letters are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Treatment Temperature (°C)</th>
<th>Reduction ± StdDev (PFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.78±0.37a</td>
</tr>
<tr>
<td>4</td>
<td>2.03±0.19a</td>
</tr>
<tr>
<td>10</td>
<td>1.23±0.47a</td>
</tr>
</tbody>
</table>
From this data, 1°C was chosen as the most effective treatment temperature for subsequent trials. The difference between the effectiveness of the treatments is approximately linear with temperature over this range. Time curves were prepared at 300 and 350 MPa (Figure 1, Figure 2) to gain insight into the kinetics of inactivation, and to yield D-values. Time curves were generated by performing pressure treatments at 300 or 350 MPa and 1°C for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 minutes, using sliced green onions to better simulate high pressure treatment in salsa or as a value added, pre-sliced product for foodservice operations.

Figure 1: Inactivation of MNV as a function of time at 300 MPa, 1 °C
Figure 2: Inactivation of MNV as a function of time at 350 MPa, 1 °C

The D-value at 300 MPa was 66.0 seconds; D-value at 350 MPa was 36.0 seconds, indicating a large increase in sensitivity for a relatively small change in pressure level. This result suggests that higher pressures might be effective (>4 log) in very short treatment times, on the order of 1 minute.

Taken as a whole, the green onion results indicate that it is possible to reduce or eliminate norovirus on green onions using relatively mild high-pressure treatments. The processing conditions which result in most effective decontamination are those which are most practical in industry; cold, moist, and at moderate pressure level. This means that the green onions need not be dried of leftover wash water before processing, nor warmed to ambient temperature for processing (and then chilled afterwards); and the pressure level required is lower than the usual maximum level of 600 MPa. This could result in both capital and energy savings since lower pressure
levels require less compression energy to reach, and less expensive pressure chambers to maintain (Pereira and Vincente, 2010). The time curve experiments indicate that processors can adjust treatment time and pressure according to meet regulatory or customer requirements for reduction of norovirus with confidence. Previous work by Hirneisen and Kniel (2014) indicated that at lower pressures such as 250 MPa, MNV is relatively insensitive, but that at higher pressures, such as 400 and 500 MPa, MNV is very sensitive to pressure.

3.4.2 **Salsa Treatments**

Pressure treatments were slightly more effective for salsa than for green onions under the same conditions (1°C, 300 MPa); salsa pH in the range of 3.80 – 4.00 had no real effect on reduction (Table 11).

<table>
<thead>
<tr>
<th>Salsa pH</th>
<th>Reduction ±Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.80</td>
<td>2.35±0.41a</td>
</tr>
<tr>
<td>4.00</td>
<td>2.23±0.20a</td>
</tr>
</tbody>
</table>

During storage, the independence of viral inactivation with respect to pH remained. Even at long storage times, the virus was only slightly reduced (Table 12); approximately 70% of the virus was inactivated after 3 days, which is insufficient as a food-safety intervention. It is likely as long as any foodservice operation would be willing to store fresh salsa before consumption for space and quality reasons (Wheeler et al., 2005).
Table 12: Inactivation of MNV in salsa at pH 3.8 and 4.0 after 24 and 72 hours.

<table>
<thead>
<tr>
<th>Salsa pH</th>
<th>Reduction (24 hrs)</th>
<th>Reduction (72 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.80</td>
<td>0.38±.04a</td>
<td>0.55±.03a</td>
</tr>
<tr>
<td>4.00</td>
<td>0.43±.13a</td>
<td>0.51±.14a</td>
</tr>
</tbody>
</table>

This demonstrates that a pH decrease from 4.0 to 3.8 is not sufficient to eliminate the threat of norovirus in salsa. Further decreases in pH would likely result in unpalatable salsa; increases in pH would make the salsa more hospitable to spoilage microbes and other pathogens.

The salsa results show that pressure treatment is approximately as effective for salsa as for green onions against murine norovirus. Furthermore, lowering the pH of salsa within reasonable limits did not increase effectiveness of pressure treatment nor decreased stability of the virus during storage. Therefore, reformulation with additional acid (i.e., to move from pH 4.0 to 3.8) is not a sufficient hurdle to inactivate murine norovirus. This is perhaps unsurprising since norovirus typically passes through the stomach of its hosts before infection can occur, and since previous results from Lee et al. (2012) in dongchimi fermentation did not show large decreases in MNV titer over the course of a 20-day lactic acid vegetable fermentation. The rate of decline was faster than what was observed in groundwater by Bae and Schwab (2008), but this is unsurprising since salsa is a less hospitable environment than groundwater, and the acids present in salsa likely denature proteins in the viral capsid. Previous high pressure work in salsa by Hirneisen et al. (2014) indicated that MNV can be reduced below the detection limit (10 virions; also, this is probably below an infectious dose) by high pressure processing at 400 and 500 MPa.
3.5 Conclusions

High pressure processing is an effective method for inactivating MNV in salsa and green onions. Green onions can be treated at their storage temperature (1 °C) and in water, which is a boon for industry; green onions can be chilled and washed, then cut into sections or sliced, packaged and processed. Pressure-treated green onion slices or sections could then be sold as a value-added product that would reduce prep times and reduce the risk of norovirus (and other pathogens) in foodservice or restaurant settings, especially cruise ships or nursing homes. Pressure treated salsa is available commercially under a variety of brands, including “Wholly Salsa”, which has seen double-digit growth year after year. If norovirus is a concern for salsa, existing high pressure processes can be adjusted to deal with the threat.
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Chapter 4

INACTIVATION OF SALMONELLA ON BLUEBERRIES BY COMBINED WASHING AND ULTRAVIOLET LIGHT PROCESS

4.1 Abstract

A novel combined washing and ultraviolet light process was tested to inactivate Salmonella on blueberries. This combined process inactivates bacteria on blueberries and prevents cross contamination by washing water. Existing static UV processes are severely limited by shadowing and are only effective on one surface of the berry at a time. In this study, simple water washing, ultraviolet light-assisted washing, and combined UV-sanitizer washing treatments lasting 1 and 2 minutes were tested on blueberries inoculated with a four-strain cocktail of Salmonella. The robustness of these treatments was tested with the addition of crushed berries and blueberry juice to the washing water and with skin-, calyx- and dip-inoculated blueberries. Effectiveness of the treatments ranged from a 0.74±0.55-log reduction for calyx inoculated berries exposed to UV for 1 minute in medium containing blueberry juice to a 4.91±1.56 log reduction for a 2-minute combined chlorine and UV-light washing process. Use of hydrogen peroxide as sanitizer reduced the risk of cross contamination but did not increase inactivation on the blueberries; addition of chlorine to washing water improved effectiveness except in the presence of blueberry juice. The results of these treatments indicate that ultraviolet light is a promising technology for decontamination of blueberries when coupled with a washing process.
4.2 Introduction

Fresh blueberries (*Vaccinium corymbosum*) and other fresh fruits are becoming increasingly popular among consumers in the United States (US Highbush Blueberry Council, n.d.) and around the world (Australian Blueberry Growers Association, n.d.). They are valued for their flavor as well as their proven and perceived health benefits compared to cooked and dried fruits (Wang et al, 2009).

Blueberries are an increasing source of foodborne illness (Calder et al., 2003). Outbreaks of *Salmonella* Muenchen and *Salmonella* Newport as well as hepatitis A virus have been traced to fresh blueberries (CDC, 2014; CSPI, 2014). The outbreak of hepatitis A on blueberries, reported by Calder et al. (2003), occurred because workers did not have access to hand-washing facilities, and because latrines were dug near sources of irrigation water.

Blueberries are currently picked and packed into clamshell containers in the field without any food safety intervention; and since consumers do not reliably handle and wash produce properly (Li-Cohen and Bruhn, 2002), pathogens on the surface of the blueberries could easily infect consumers. Therefore, interventions are urgently needed to enhance food safety. Experimental interventions for blueberries have included sanitizer processes (Bialka and Demirci, 2007; Li and Wu, 2014), high pressure processing (Li et al, 2013), and pulsed light treatment (Bialka and Demirci, 2007; Huang and Chen, 2014). These treatments have produced mixed results, with the most experimental success seen with pulsed light.

Ultraviolet light is a proven technology for inactivation of pathogens in air, water and on food-contact surfaces in a variety of settings (Bintsis et al., 2000) but is still an emerging technology for inactivation of pathogens and spoilage microbes in food (Seitz-Wald, 2014). A pioneering use of UV for food safety was by Murakami et
al. (2005) who developed a UV pasteurization process for apple cider. UV light is used for shelf-life extension of baked goods, potatoes, tortillas, cheese, liquid eggs, and other products (Siegener, 2014). The effectiveness of UV treatments is limited to directly exposed surface of solid foods, and for short penetration depths in liquid foods (dependent on turbidity). For some solid foods, such as tortillas, complete exposure of all surfaces is relatively simple; for other foods, such as berries, this is more difficult (Bialka and Demirci, 2007). The process tested in this study uses a combination of ultraviolet light and agitated water, which overcomes the existing problems with UV light for solid foods with shapes more complicated than tortillas.

Many produce items are washed in preparation for further packing (as in bagged salad mixes) or further processing (such as freezing) to remove soil, debris, and spoilage microbes (FDA, 2009). Sanitizers are used in washing processes to prevent cross-contamination with the secondary goal of reducing pathogen counts on food surfaces (FDA, 2009). Chlorine is the most commonly used sanitizer; hydrogen peroxide, peroxyacetic acid and other sanitizers have also been used commercially. In this study, chlorine or hydrogen peroxide was added to the wash water in an attempt to enhance the inactivation efficacy of the wet UV light system. In addition, to simulate turbid water used in the fresh produce industry, 2% juice or 5% crushed berries were added to the washing water. Ultraviolet light combined with a washing process offers a safe, chemical- and residue-free method to prevent cross-contamination during washing and to decontaminate produce itself, especially small fruits such as blueberries.

In this study, treatments using UV light combined with chlorine and hydrogen peroxide have been tested on berries inoculated on the skin, calyx and by dipping with
a four-strain cocktail of *Salmonella* strains (Montevideo, Newport, Saintpaul, and Stanley) isolated from produce outbreaks, and washed in water containing blueberry juice and crushed blueberries.

The objectives of this study were as follows. First, to determine effectiveness of washing and sanitizers for inactivation of outbreak strains of *Salmonella* on blueberries. Second, to determine effectiveness of ultraviolet (UV) light combined with water- and sanitizer-washing procedures for inactivation of outbreak strains of *Salmonella* on blueberries. Third, to test the effectiveness of sanitizer-based and UV-washing systems with the presence of organic matter and turbidity for inactivation of outbreak strains of *Salmonella* on blueberries. Fourth, to test the effectiveness of sanitizer-based and UV-washing systems with blueberries spot inoculated on the calyx, as well as blueberries that have been dip-inoculated.

### 4.3 Methods and Materials

#### 4.3.1 Bacterial Strains and Culturing Methods

Four nalidixic-acid-resistant outbreak strains of *Salmonella* were cultured in tryptic soy broth (Difco Laboratories, Sparks, MD) plus 0.6% yeast extract (BD) and 50 μg/ml nalidixic acid (TSB-YE-NA) (Fisher Scientific, Hampton, NH) using standard bacterial culturing techniques (Huang and Chen, 2014). The strains used were isolated from produce-related outbreaks; details are shown in Table 13.
Table 13: *Salmonella enterica* strains used in this study. Adapted from Huang and Chen (2014).

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Strain#</th>
<th>Associated Commodity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montevideo</td>
<td>G4639</td>
<td>Tomato</td>
</tr>
<tr>
<td>Newport</td>
<td>H1275</td>
<td>Sprouts</td>
</tr>
<tr>
<td>Saintpaul</td>
<td>02-517-1</td>
<td>Cantaloupe</td>
</tr>
<tr>
<td>Stanley</td>
<td>HO588</td>
<td>Sprouts</td>
</tr>
</tbody>
</table>

Strains were cultured separately and combined for inoculation; cultures were maintained at 4 °C on tryptic soy agar plus 0.6% yeast extract (TSAYE). Each strain was transferred from a single colony on a TSAYE plate to TSB-YE-NA, then incubated 24 h at 35°C. An aliquot of 100μL was transferred into fresh TSB-YE-NA and incubated 24 h a second time. Inoculum was prepared by combining 2 mL of each culture in a centrifuge tube, followed by centrifugation for 10 minutes at 2450 x g; the pellet was resuspended in 1-2 mL of 0.1% peptone (BD).

4.3.2 Blueberry Sample Preparation

Blueberries were acquired from a local market. Berries were refrigerated overnight, then weighed into 60 gram samples (40-60 berries). From each sample, 10 berries were chosen for inoculation by spot inoculation onto the skin or calyx, or by dip inoculation. Before inoculation, blueberries were exposed to UV light for at least 10 minutes to reduce background microbiota. Skin inoculation was performed by placing 4-6 small droplets of total volume 20 μL *Salmonella* cocktail onto the skin of each blueberry. Calyx inoculation was performed by placement of a single, 20-μL droplet into the calyx of each blueberry. Dip inoculation was performed on all 10
berries in each sample at once. Large centrifuge tubes (50 mL; BD) were loaded with 200 μL inoculum and 1 mL of 0.1% peptone water, mixed, then loaded with the 10 blueberries. The centrifuge tube was shaken and rolled axially for 30 seconds to distribute the inoculum over the surfaces of the blueberries. The blueberries were unloaded to a petri dish. In all cases, inoculated blueberries were allowed to dry for 2 hours in a running biosafety cabinet at ambient temperature. Starting inoculum for skin and calyx inoculation ranged from 5 x 10^5 to 4 x 10^7 cfu; dip inoculation starting inoculum was lower, from 5 x 10^5 to 8 x 10^6 cfu.

### 4.3.3 Washing Medium Preparation

Washing medium, consisting of 1000 mL of deionized or tap water, was chilled overnight in covered containers. Shortly before treatment (<20 minutes), the medium was modified by the addition of 3 grams manually crushed un-inoculated blueberries (representing 5% of the total blueberry weight), 20 mL blueberry juice (Knudsen Farms) (resulting in approximate 2% juice in the washing medium) or left unmodified. Sanitizers, such as 7.9% sodium hypochlorite solution (Clorox), or 3% hydrogen peroxide (Shoprite Brands) were added immediately before treatment (<30 seconds). Sodium hypochlorite was added to an effective concentration of 10 or 100 ppm free chlorine, and adjusted to pH 6.5 with citric acid solution (for unmodified medium and crushed berry medium) or sodium hydroxide solution (for 2% blueberry juice). For treatments with 2% blueberry juice, 25 ppm of chlorine was added to compensate for the reducing effects of blueberry juice. Hydrogen peroxide was added to the washing medium to a final concentration of 1%. The absorbance of the diluted blueberry juice at 254 nm was 0.054 Absorbance units/cm (see Appendix C). The chemical oxygen demand was 2.47 g/L. Addition of crushed berries did not noticeably
change the color of the bulk medium. A photograph of the crushed berries and 2% blueberry juice is shown below in Figure 3, which shows that the crushed berry medium has large particles but does not have the dark anthocyanin pigments from the blueberry skin.

![Figure 3: Photograph of 2% blueberry juice (left) and 5% crushed berry solution (right). Samples are illuminated from below. Taken by the author.](image)

4.3.4 Processing and Sampling Methods

An UV unit (UVC Test System, Reyco Systems, Meridian, ID) was run for 10 minutes to stabilize the output of the UV lamps and sanitize the treatment vessel (8 x 8 inch 1.9-L Pyrex baking dish). A photograph of the UV unit is shown below in Figure 4.
Blueberries were added to the empty treatment vessel with an 80-mm stir bar, and the prepared washing medium was poured over the berries to submerge them before treatment began. The treatment vessel was placed in the UV unit and on the top of a stir plate. The height of the stir plate was adjusted from 25 to 9.5 cm to change the UV intensity from 5 μW/cm$^2$ to 13 μW/cm$^2$; the distance from the lamps to the surface of the water was 21 and 5.5 cm. The door to the UV unit was closed and the stir plate was turned on. UV treatment began once all of the berries were in motion (<5 seconds). Treatment times of 1 minute and 2 minutes were tested. At the conclusion of treatment, the vessel was removed from the unit and a water sample of 1 mL or 100 μL (when high counts were expected) was taken from roughly the geometric center of the vessel. The berries were removed and placed into a filter stomacher bag containing 200 mL Dey-Engley (D/E) Neutralizing Broth (Difco). The sampled blueberries were crushed for 2 minutes at 260 RPM. The stomached blueberry slurry was either spread-plated directly or serially diluted in 0.1% peptone water (Difco) before plating onto TSA-YE-NA. The water sample was plated onto TSA-YE-NA. An untreated control
sample of inoculated blueberries was prepared and plated to determine the initial inoculation levels.

4.3.5 Statistical Analysis

Each experiment was performed at least three times; analysis was conducted using JMP (SAS Institute, Cary, NC). Colony counts were converted to CFU/g of berries and the log reduction was determined relative to the control sample in each trial. Tukey’s HSD Test was used to determine significance at the P<0.05 level.

4.4 Results

4.4.1 Skin Inoculation Treatments

4.4.1.1 Simple Treatments

In the first round of treatments, the berries were treated with either UV light or sanitizers. The results are shown in Table 14.
Table 14: Effect of single treatments on inactivating *Salmonella* spot-inoculated on blueberry skin. Blueberries skin spot-inoculated with *Salmonella* were washed with DI water or sanitizers or treated with low (5 mW/cm²) or high (13 mW/cm²) intensity of UV light while being immersed in agitated DI water. Data represent mean of at least three replicates ± one standard deviation.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Log Reduction of <em>Salmonella</em> on Berries (log CFU/g)</th>
<th><em>Salmonella</em> Survivors in Washing Water (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI water for 2 min</td>
<td>2.45±0.75a</td>
<td>2040±2290a [4/4]</td>
</tr>
<tr>
<td>Low UV for 1 min</td>
<td>4.48±1.02a</td>
<td>0.00±0.00a [0/4]</td>
</tr>
<tr>
<td>Low UV for 2 min</td>
<td>4.74±0.58a</td>
<td>0.00±0.00a [0/4]</td>
</tr>
<tr>
<td>High UV for 1 min</td>
<td>4.44±1.38a</td>
<td>0.00±0.00a [0/4]</td>
</tr>
<tr>
<td>High UV for 2 min</td>
<td>4.73±0.94a</td>
<td>0.00±0.00a [0/4]</td>
</tr>
<tr>
<td>10 ppm chlorine for 2 min</td>
<td>3.81±1.36a</td>
<td>0.14±0.38a [1/7]</td>
</tr>
<tr>
<td>100 ppm chlorine for 2 min</td>
<td>3.74±1.02a</td>
<td>0.00±0.00a [0/6]</td>
</tr>
<tr>
<td>1% H2O2 for 2 min</td>
<td>2.03±0.68a</td>
<td>25.7±22.7a [3/3]</td>
</tr>
</tbody>
</table>

These treatments were done to test the main effect of each of the treatment factors before adding interaction effects. This table shows that ordinary deionized water and water plus hydrogen peroxide are not very effective for treatment on their own; chlorinated water, however, is effective at both 10 and 100 ppm free chlorine. The treatments with UV light are of similar effectiveness on the blueberries despite a nearly-threefold difference in intensity; this suggests that the UV intensity is not the controlling factor in a clear medium. Low intensity UV light and 10 ppm chlorine were not able to completely eliminate bacteria in the wash water, but no survivors were detected when high intensity UV light or 100 ppm chlorine were used. Deionized water washing in the absence of UV light was not effective for preventing cross contamination, but did remove 99% of the bacteria from the surface. Hydrogen peroxide was not helpful for reducing bacteria on the surface, but did reduce the number of the survivors in the medium.
4.4.1.2 Combined Treatments

In a commercial setting, it might be beneficial to add ultraviolet light to improve the effectiveness of an existing chlorine or hydrogen peroxide washing process, or to add chlorine or hydrogen peroxide to an ultraviolet process.
Table 15: Effect of combined treatments on inactivating *Salmonella* spot-inoculated on blueberry skin. Blueberries skin spot-inoculated with *Salmonella* were treated with low (5 mW/cm²) or high (13 mW/cm²) intensity of UV light while being immersed in agitated 10 ppm chlorine or 1% hydrogen peroxide solution. Data represent mean of at least three replicates ± one standard deviation; fraction of water samples that showed any growth in brackets.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Log Reduction of <em>Salmonella</em> on Berries (log CFU/g)</th>
<th><em>Salmonella</em> Survivors in Washing Water (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ppm chlorine</td>
<td>1% H₂O₂</td>
</tr>
<tr>
<td>Low UV for 1 min</td>
<td>3.29±1.75aA</td>
<td>4.15±0.83aA</td>
</tr>
<tr>
<td>Low UV for 2 min</td>
<td>4.01±1.62aA</td>
<td>4.45±1.80aA</td>
</tr>
<tr>
<td>High UV for 2 min</td>
<td>4.46±1.17aA</td>
<td>4.91±1.56aA</td>
</tr>
</tbody>
</table>

Initial bacteria populations (log CFU/g) were 1.4 x 10⁶ – 1.7 x 10⁷. Within the same category of “10 ppm Chlorine” and “1% H₂O₂”, data in the same column with different lowercase letters are significantly different (P < 0.05). Within the same category of “Log Reduction of *Salmonella* on Berries (log CFU/g)” and “*Salmonella* Survivors in Washing Water (CFU/mL)”, data in the same row with different uppercase letters are significantly different (P < 0.05).

The combined treatments are of similar effectiveness to the simple treatments. Chlorine and ultraviolet light are less effective than either treatment alone. Hydrogen peroxide reduced effectiveness of all treatments except when combined with high intensity UV light for 2 minutes. Neither sanitizer was completely effective for eliminating survivors in the medium in all cases; however, both eliminated all survivors in most treatments.
4.4.1.3 Treatments with Blueberry Juice

To better simulate an industrial processing environment, experiments were run with 2% bottled blueberry juice (Knudsen Farms—see Appendix B for product label) added to the treatment medium. This decreased the effectiveness of the UV treatments, while leaving the water wash treatment essentially unaffected. Hydrogen peroxide was more effective for controlling cross-contamination than chlorine.

Table 16: Effect of single and combined treatments on inactivating Salmonella spot-inoculated on blueberry skin in the presence of 2% blueberry juice. Blueberries skin spot-inoculated with Salmonella were treated with low (5 mW/cm²) or high (13 mW/cm²) intensity of UV light while being immersed in agitated DI water, 10 ppm chlorine or 1% hydrogen peroxide solution with 2% blueberry juice. Data represent mean of at least three replicates ± one standard deviation; fraction of water samples that showed any growth in brackets. Limit of detection in water samples is 0.9 cfu/mL.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Log Reduction of Salmonella on Berries (log CFU/g)</th>
<th>Salmonella Survivors in Washing Water (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No UV</td>
<td>Low UV</td>
</tr>
<tr>
<td>DI water for 1 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.47±0.73aA</td>
<td>3.19±0.60aA</td>
</tr>
<tr>
<td>1% H₂O₂ for 1 min</td>
<td>ND</td>
<td>2.93±0.54aA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ppm chlorine for 1 min</td>
<td>2.43±0.37aA</td>
<td>2.21±0.34aA</td>
</tr>
</tbody>
</table>

Initial bacteria populations (log CFU/g) were 1 x 10⁶--1 x 10⁷ Within the same category of “No UV”, “Low UV” and “High UV”, data in the same column with different lowercase letters are significantly different (P < 0.05). Within the same category of “Log Reduction of Salmonella on Berries (log CFU/g)” and “Salmonella Survivors in Washing Water (CFU/mL)”, data in the same row with different uppercase letters are significantly different (P < 0.05).
Blueberry juice reduced the effectiveness of all treatments, but was most detrimental to the treatments with chlorine. This is likely caused by the pH adjustment using sodium hydroxide meant to prevent the chlorine from off-gassing during treatment, as well as the reaction of the chlorine with the organic matter of the blueberry juice. Chlorine is known to react quickly with organic matter in washing processes; this is accelerated by the relatively high concentrations of organic matter (Gallard and von Gunten, 2002; Gil et al, 2009). The low pH of the blueberry juice itself seems to have a sanitizing effect, and the pH increase required for the use of chlorine negated the effectiveness of the blueberry juice but did not increase transparency. Hydrogen peroxide did not seem to interfere with the effectiveness of the blueberry juice as much, but was still less effective than deionized water alone for reduction on the blueberries. The numbers of survivors in the wash water tell a similar story: the addition of chlorine to the treatment medium decreased effectiveness dramatically and greatly increased the risk of cross contamination. Though hydrogen peroxide was not more effective than deionized water for reductions of *Salmonella* on berries, at both high and low UV intensity it was able to reduce the number of survivors in the treatment medium as compared to deionized water.

### 4.4.2 Calyx Inoculation Treatments

Calyx inoculation represents the “worst case” scenario for surface contamination; the bacteria are protected inside the calyx of the blueberry and are protected from continuous exposure to the UV light and the shear forces exerted by the medium. The most effective treatments from the skin inoculation study were used
with calyx-inoculated blueberries to compare effectiveness. Results for these treatments are shown in Table 17.
Table 17: Effect of single and combined treatments on inactivating *Salmonella* spot-inoculated on blueberry calyx. Blueberries calyx spot-inoculated with *Salmonella* were treated with high (13 mW/cm²) intensity of UV light while being immersed in agitated tap water, 10 ppm chlorine or 1% hydrogen peroxide solution with/without 2% blueberry juice or 5% of crushed berries. Data represent mean of at least three replicates ± one standard deviation; fraction of water samples that showed any growth in brackets. Limit of detection in water samples is 0.9 cfu/mL.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Log Reduction of <em>Salmonella</em> on Berries (log CFU/g)</th>
<th><em>Salmonella</em> Survivors in Washing Water (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% Juice</td>
<td>5% Crushed Berries</td>
</tr>
<tr>
<td>High UV for 1 min</td>
<td>0.74±0.55aA</td>
<td>1.62±0.42aA</td>
</tr>
<tr>
<td>High UV for 2 min</td>
<td>1.04±0.29abA</td>
<td>1.94±0.34aAB</td>
</tr>
<tr>
<td>10 ppm chlorine for 2 min</td>
<td>1.35±0.52abA</td>
<td>1.29±0.34aA</td>
</tr>
<tr>
<td>High UV + 10 ppm chlorine for 2 min</td>
<td>1.17±0.33abA</td>
<td>1.73±0.47aA</td>
</tr>
<tr>
<td>1% H₂O₂ for 2 min</td>
<td>1.91±0.52abA</td>
<td>1.74±0.77aA</td>
</tr>
<tr>
<td>High UV + 1% H₂O₂ for 2 min</td>
<td>2.34±0.78bA</td>
<td>2.32±1.03aA</td>
</tr>
</tbody>
</table>

Initial bacterial populations (log CFU/g) were 5 x 10⁵ - 2 x 10⁷. Within the same category of “2% Juice”, “5% Crushed Berries” and “Tap Water”, data in the same column with different lowercase letters are significantly different (P < 0.05). Within the same category of “Log Reduction of *Salmonella* on Berries (log CFU/g)” and “*Salmonella* Survivors in Washing Water (CFU/mL)”, data in the same row with different uppercase letters are significantly different (P < 0.05).
The treatments of calyx-inoculated berries were all significantly less effective than even deionized water washing of skin-inoculated berries. As was the case with skin-inoculated berries, addition of blueberry juice reduced the effectiveness of UV treatments over tap water by 1.18 and 1.22 log; chlorine treatments were less affected by the presence of the blueberry juice but effectiveness was still reduced by 0.39 log and 0.65 log for the treatments without and with UV. Crushed berries in the medium decreased effectiveness of the treatment slightly but acted as a haven for bacteria in the treatment medium which prevented the action of ultraviolet light and chlorine. The treatment medium results with blueberry juice show that increasingly transparent treatment medium and longer treatment times result in fewer and fewer survivors in the medium. As was the case with the skin-inoculated berries, the addition of chlorine to blueberry juice increased the number of survivors in the treatment medium, though the action of the UV light was able to reduce that effect. The most effective treatment was combination of high intensity UV light and hydrogen peroxide, which resulted in a 2.74±0.66 log reduction with no detected survivors in the washing water.

4.4.3 Dip Inoculation Treatments

Dip inoculation incorporates effects of both skin and calyx inoculation. The most effective treatments from the skin and calyx inoculation were used for dip inoculation; 2-minute treatments were tested to maximize effectiveness.
Table 18: Effect of single and combined treatments on inactivating *Salmonella* dip-inoculated on blueberries. Blueberries dip-inoculated with *Salmonella* were treated with high (13 mW/cm²) intensity of UV light while being immersed in agitated tap water, 10 ppm chlorine or 1% hydrogen peroxide solution with/without 2% blueberry juice or 5% of crushed berries. Data represent mean of at least three replicates ± one standard deviation; fraction of water samples that showed any growth in brackets. Limit of detection in water samples is 0.9 cfu/mL.

Initial bacteria populations (log CFU/g) were $6 \times 10^5$ – $8 \times 10^6$. Within the same category of “2% Juice”, “5% Crushed Berries” and “Tap Water”, data in the same column with different lowercase letters are significantly different ($P < 0.05$). Within the same category of “Log Reduction of *Salmonella* on Berries (log CFU/g)” and “*Salmonella* Survivors in Washing Water (CFU/mL)”, data in the same row with different uppercase letters are significantly different ($P < 0.05$).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Log Reduction of <em>Salmonella</em> on Berries (log CFU/g)</th>
<th><em>Salmonella</em> Survivors in Washing Water (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% Juice</td>
<td>5% Crushed Berries</td>
</tr>
<tr>
<td>High UV for 2 min</td>
<td>2.02±0.40aA</td>
<td>2.24±0.30aA</td>
</tr>
<tr>
<td>10 ppm chlorine for 2 min</td>
<td>1.21±0.15A</td>
<td>1.53±0.78A</td>
</tr>
<tr>
<td>High UV + 10 ppm chlorine for 2 min</td>
<td>1.98±0.91aA</td>
<td>2.15±1.04aA</td>
</tr>
<tr>
<td>1% H$_2$O$_2$ for 2 min</td>
<td>1.55±0.48A</td>
<td>1.48±0.56A</td>
</tr>
<tr>
<td>High UV + 1% H$_2$O$_2$ for 2 min</td>
<td>2.16±0.60aA</td>
<td>2.28±0.76aA</td>
</tr>
</tbody>
</table>
The most effective treatments for the berries of those tested were the tap water combination treatment with hydrogen peroxide and UV light, and the crushed berry combination treatment with UV light and hydrogen peroxide. The effectiveness was still significantly lower than was observed for skin inoculation with the same treatments, but is comparable to calyx inoculation. The low numbers of survivors in the medium, coupled with the low reductions, suggest that the washing process itself was slowed by the inoculation site, and that fewer bacteria left the surface of the berry for the treatment medium. The combination treatments with sanitizer and UV light were the most effective for inactivation of survivors in the medium.

4.5 Discussion

The results obtained for the processing of skin-inoculated blueberries indicate that a combined washing and UV illumination process is effective for inactivating Salmonella. The consistently most important variable for reduction of Salmonella was inoculation site, which suggests that the treatments were mass-transfer limited, rather than UV- or sanitizer-limited. This idea is corroborated by the very small numbers of bacteria found in the washing water for treatments not involving blueberry juice. The rate of bacterial death in the water was likely much faster than the rate at which bacteria are washed off the surface of the berries. This issue is not neatly solved at a large scale, because the rate of mass transfer in turbulent flow (and therefore the rate at which bacteria are removed from the surface) is only weakly dependent on the power input of the agitator (Atiemo-Obeng, 2003). This suggests that combined UV and washing treatments should be designed for duration for reductions on berries and intensity for reductions in washing water. Addition of blueberry juice, which
contributed both acidity and turbidity, reduced effectiveness of the UV treatments but did not affect the simple water-washing treatment (i.e., without sanitizers and UV); the survivor results offer some evidence that blueberry juice at this concentration can itself act as a sanitizer, likely due to the low pH (3.5 when diluted to 2%) and polyphenol content. The absorbance spectrum of 2% blueberry juice, shown in Appendix C, suggests that 254 nm is close to the optimal wavelength for penetration of 2% blueberry juice; other wavelengths in the germicidal range would require a different lamp technology to produce and would be more strongly absorbed.

Skin inoculation is a simulation of the “best-case” scenario for contamination; all of the inoculum is on the surface of the berry, where it is exposed directly to the ultraviolet light and to the shear forces of the wash water. Spot-inoculation of the calyx of the blueberries resulted in a decrease in effectiveness across all treatments. This is likely because the bacteria were trapped inside the calyx and were not exposed or able to diffuse out into the medium; the calyx also shelters bacteria from direct illumination by UV light. Dip inoculation treatments were of similar or lower effectiveness than calyx treatments, which suggests that the limiting factor for these treatments are bacteria caught inside the calyx or porous stem end of the berry. Dip inoculation is also likely the best simulation of an actual contamination event, since bacteria would be present on both the skin of the berry as well as the calyx.

Deionized water was used in the skin-inoculation treatments to prevent the presence of chlorine in the washing water, which could confound results. Later treatments were performed in tap water, which is what would be used in industrial practice. The presence of crushed berries or blueberry juice in the washing water is likely in an industrial scenario, however the levels are not likely to be as high as tested
here. A washing process which would crush the quantity of berries tested here (5% of the weight of the berries) or produce the amount of juice observed here (2% of the washing water volume) would be probably not be profitable; however, extraneous soil and other matter might foul the washing water to a comparable degree as the crushed berry treatment.

Chlorine was effective for clear water treatments and in the presence of crushed berries, but reduced the effectiveness of treatments where blueberry juice was present, and resulted in larger numbers of survivors in the water than without chlorine. As mentioned previously, this could be because of the pH adjustment required to prevent chlorine from off-gassing during the washing process. Very high levels of chlorine might overcome this problem and bleach the UV-blocking phenolic compounds in the juice, but such concentrations of chlorine may be beyond the legally permitted levels. Existing work by Gil et al. (2009) has shown that the effectiveness of chlorine is reduced in the presence of organic matter; the chemical oxygen demand the blueberry juice solution, at 2.47 g O₂/L, was much greater than the amount of free chlorine present (no more than 25 ppm, or 25 mg Cl₂/L).

The results of the treatments tested here are in agreement with the work of Li and Wu (2013) who found that sanitizer washing treatments with chlorine were able to achieve a 4.1-log reduction after 5 minutes of treatment. It is likely that an extended treatment time would help remove more of the bacteria from the surface into the medium for inactivation.

Hydrogen peroxide was ineffective in the absence of UV light, but was not as strongly affected by blueberry juice as chlorine, and was able to strongly reduce or even eliminate bacteria in the treatment medium. In the calyx and dip inoculation
treatments, UV light consistently increased the effectiveness of the treatments and was able to consistently reduce bacteria in the medium regardless of its composition. Hydrogen peroxide is more effective at acidic pH (FDA, 2009), and though the blueberry juice absorbed UV light, it acidified the washing water and boosted the effectiveness of the hydrogen peroxide. The effectiveness of hydrogen peroxide is increased by exposure to light at 254 nm, which accelerates the formation of radicals (Hadjok et al, 2007). The fact that the most effective treatment in each case (shown below in Table 19) was one which combined ultraviolet light and hydrogen peroxide demonstrates the importance of this effect.

Addition of crushed berries slightly reduced the effectiveness of the treatments on the blueberries, but perhaps more importantly prevented the sanitizers and UV light from completely eliminating all Salmonella in the treatment medium. Hydrogen peroxide was only mildly affected by crushed berries; in the case of calyx-inoculated berries, the treatment became less effective; in the case of dip inoculation, the treatment became more effective.

The most effective treatment for each inoculation method shown above is collected in Table 19 for comparative purposes.
Table 19: Most effective treatments for each inoculation method; excerpted from Table 15, Table 17 and Table 18.

<table>
<thead>
<tr>
<th>Inoculation Method</th>
<th>Washing Conditions</th>
<th>UV Conditions</th>
<th>Reduction ± Std Dev</th>
<th>Medium Survivors ± Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Deionized Water, 1% Hydrogen Peroxide</td>
<td>High UV for 2 min</td>
<td>4.91±1.56</td>
<td>41.8±72.6 [2/4]</td>
</tr>
<tr>
<td>Calyx</td>
<td>Tap Water, 1% Hydrogen Peroxide</td>
<td>High UV for 2 min</td>
<td>2.74±0.66</td>
<td>0.00±0.00 [0/3]</td>
</tr>
<tr>
<td>Dip</td>
<td>5% Crushed Berries, 1% Hydrogen Peroxide</td>
<td>High UV for 2 min</td>
<td>2.28±0.76</td>
<td>0.00±0.00 [0/3]</td>
</tr>
</tbody>
</table>

These results show the importance of the location of inoculation on reduction of bacteria in the blueberries and in the medium. These results also demonstrate the effectiveness of hydrogen peroxide coupled with two minutes of high-intensity UV exposure and mixing, and that though this process is most effective with clear washing water, the presence of crushed berries in the washing water does not necessarily mean that the treatment will be made ineffective. Bacteria on the skin are easily removed and destroyed (as evidenced by the high reduction and low survivor counts in most cases), but bacteria trapped in the calyx are not as easily removed, even if they are promptly inactivated once they diffuse into the medium. Furthermore, the most effective treatment in each case was the one with relatively clear medium that allows UV penetration and does not readily harbor bacteria. The most likely contamination scenario is splashing of contaminated water or other liquids onto the surface of a blueberry (Matthews et al., 2014); since some of the contaminant is likely to enter the calyx as well as dry onto the skin, the most realistic inoculation method of the ones tested is dip inoculation.

Alternatives to washing, such as high pressure treatment, could be more effective for inactivation of hard-to-remove pathogens in the calyx or stem scar of the blueberry (Huang et al., 2014; Li et al., 2014), but the capital costs and textural
damage associated with such treatments makes commercial application unlikely in the
produce industry. E-beam and gamma-ray treatments cause cell-wall and other
damage to the blueberries and might be viewed negatively by consumers, who are
expecting a “natural”, “unprocessed” product.

4.6 Conclusions

The addition of blueberry juice into the treatment medium dramatically
reduced the effectiveness of the UV light; addition of chlorine did not help, but
addition of hydrogen peroxide with UV light was consistently effective for reduction
of survivors in the medium. In the absence of UV light, addition of chlorine made
treatments less effective. The presence of crushed berries represents both an economic
loss to the processor as well as a loss of effectiveness for UV and chlorine treatments,
though hydrogen peroxide was not strongly affected. Hydrogen peroxide is a better
sanitizer in the presence of blueberry juice than chlorine. It gives a small increase in
effectiveness over tap water in the presence of organic matter and effectively
eliminates survivors in the washing water. Washing procedures which tend to involve
large amounts of acidic organic matter could benefit from the use of hydrogen
peroxide rather than chlorine.
REFERENCES


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Chapter 5

OVERALL CONCLUSIONS AND FUTURE WORK

5.1 MNV Project

The MNV project is, in some ways, the final part of a larger study started by Dr. Hudaa Neetoo and Dr. Haiqiang Chen who, with others, studied the inactivation of *E. coli* and *Salmonella* on green onions and in salsa using high pressure. The results of this study agree with their results; high pressure is reasonably effective for reduction of pathogens, but does not eliminate all pathogens at the conditions tested. I offer a few suggestions for possible future work in a few categories: additional pathogens for testing, altered sample preparation, altered process conditions, and altered salsa formulation.

Additional pathogens to test that have caused outbreaks in produce are *Shigella flexneri* and *Cryptosporidium parvum*, which have each caused an outbreak associated with green onions. So many pathogens have caused outbreaks associated with salsa that there is not space to list them comprehensively here; this is an ongoing food safety issue.

Changes in sample preparation might reveal more information about the most effective methods for decontamination as well as the limits of high pressure processing. Green onion samples could be prepared to include sanitizers in the treatment medium, pathogens could be internalized by vacuum cycling or soaking inoculation of different components of salsa and treatment medium for green onions could contain added green onion juice. Surfactants and organic acids might be
effective as sanitizers that could disrupt viral capsids by denaturation and inactivate the virions. Internalization of pathogens could be accomplished by soak-inoculation methods over long periods of time, or by the application of a vacuum to cause air pockets to expand and then draw in inoculum. Some studies (FDA, 2009) showed that adding warm produce to cold inoculum can cause internalization of pathogens. Perhaps different vegetables in salsa would provide different inactivation environments? In the green onions treatments, sterile, ultrapure water was added to the packages to improve effectiveness; this is not what would be used commercially. Tap water is a far more likely choice, and could be supplemented with green onion juice or puree to help maintain flavors; either of these would contribute ions and other compounds which might affect the rate of inactivation (Sanchez et al., 2011).

At the 2014 meeting of the Institute of Food Technologists, where this work was presented, multiple visitors to the poster commented that their companies use 600 MPa for 2 minutes as the default pressure level and time. It is probable that norovirus would drop to undetectable levels in seconds if exposed to such pressures, but this would probably damage green onions to an unacceptable degree. Most of the components of salsa would be unaffected, except onions or other air-containing vegetables which would be softened.

Fresh salsa can be made with a variety of foods in place of the traditional tomatoes or tomatillos (which themselves might merit examination); in particular, fruits such as papaya and pineapple can be used for a lighter fresh salsa served with fish. Papaya and pineapple contain papain and bromelain, respectively; salsas made with them might rapidly inactivate unenveloped viruses through a combination of low pH and proteolytic enzyme activity. A simple way to determine whether this is
feasible before performing experiments would be to compare published genome sequences for norovirus VP1 and VP2 capsid proteins with known preferred cleaving targets of papain, bromelain, and other common proteases.

5.2 *Salmonella* Project

The *Salmonella* project represents the beginning of a new line of work rather than an end. The results show that UV light coupled with a washing process can effectively decontaminate berries so long as the medium is relatively transparent and the bacteria can be efficiently washed into the treatment medium. Furthermore, the results also show that some sanitizers are made more effective by UV light, while others are unaffected. The results described above, however, have been tested with a specific pathogen-food pair (*Salmonella* cocktail and blueberries). Some simple options for future experiments would be to use different organisms in place of *Salmonella* and different produce items in place of blueberries. Some suggestions are listed below, in no particular order. Possible targets could include: *E.coli* cocktail, *Campylobacter jejuni*, native yeast and mold, hepatitis A virus, *Shigella flexneri*, lactic acid bacteria, and Aichi virus. Possible produce items could include: blackberries, raspberries, small tomatoes (cherry, grape, pear), cherries, raw almonds, baby carrots, leafy greens, and grapes.

Another set of options for future work would involve changes to the process itself rather than the microbes or food item. Some suggestions are as follows: staged washes, changing sanitizer, changing medium volume, and initial water inoculum. Staged washing would be a better approximation of a continuous, counter-current system, and would probably improve the reductions for the dip and calyx inoculation treatments. The two-minute “plain” water wash for skin-inoculated berries would be
significantly more effective, since the berries would reach saturation twice. A staged washing process also opens the door to serial treatments; for instance, the first wash could be in tap water with UV light to simulate a dump tank, followed by a chlorinated or plain tap water rinse. It is possible that a primary washing step in hydrogen peroxide followed by UV exposure could allow hydrogen peroxide to diffuse into the bacterial cells before being activated by UV light, increasing both effectiveness and selectivity.

Changing the sanitizer could improve effectiveness. Both chlorine and hydrogen peroxide were tested in these experiments; hydrogen peroxide was found to be more effective than chlorine when combined with UV light. Peroxyacetic acid or benzoyl peroxide might be more effective than hydrogen peroxide. Organic acids, as tested by Li and Wu (2013) would almost certainly improve effectiveness of UV treatment. Acetic acid, in particular, might be an attractive option for organic farmers and others seeking a “natural” alternative to chlorine. Organic acids would maintain effectiveness in the presence of crushed berries or berry juice.

Reducing the water volume might expose new information about the washing and UV process. Using less medium would increase the power per unit mass from the agitator (accelerating the washing process slightly) but would also cause berries to reach saturation at a lower concentration. Reducing the amount of water used would make the water depth shallower, though this probably would not matter unless the treatment medium was so opaque that the UV light was noticeably attenuated at the bottom of the vessel (2% blueberry juice is not this opaque). Reducing the amount of medium would probably also better simulate an industrial process, where the ratio of berries to water would presumably be higher.
Adding inoculum to the treatment medium and using uninoculated berries would be the ultimate test for cross contamination. The level of inoculum to use could be determined from water washing tests as done here; though more replicates would be a good idea, since multiple statistical comparisons would be made against one number. It is probable that the very effective treatments (high intensity UV, tap or deionized water, and chlorine) would quickly eliminate organisms in the medium and prevent uninoculated berries from being heavily contaminated, but less effective treatments might result in cross-contamination.

Along the same lines, the crushed berry treatments could be performed with berries that were crushed and then inoculated (or vice versa, if there is a repeatable and aseptic way to do so). After all, the berries most likely to suffer damage during a washing process are those already softened by bacterial or mold growth. Another potentially interesting experiment is suggested by the results of section 4.4.2, where all of the crushed berry treatments were found to have surviving bacteria in the treatment medium. Could large particles other than damaged berries serve as a hiding place for bacteria? Could pieces of porous material, such as water-saturated foam, hold and release bacteria? This could simulate the action of large soil particles or twigs in a berry washing process. Another approach might be to use non-porous material, such as aluminum foil cut into regular shapes, as a “shade” which would prevent UV light from reaching certain areas of the medium but would not absorb bacteria; a comparison could be made with the presence of silica particles, which are used to simulate turbidity caused by soil.

Yet another interesting project, perhaps suitable as an undergraduate research project, would be to test the efficacy of the washing treatment that, in the author’s
anecdotal experience, is the one most commonly used by consumers: opening the berry clamshell and holding it under running water for a few seconds until all of the berries on the surface appear thoroughly wet, then blotting the berries dry with a paper towel. Such a project would entail only a few treatments and has more of a University Extension flavor, but would be a useful piece of information for the general public.
Appendix A

SALSA INGREDIENT AND NUTRITION FACTS LABELS

Appendix A

SALSA INGREDIENT AND NUTRITION FACTS LABELS

Nutrition information

<table>
<thead>
<tr>
<th>Amount</th>
<th>% Daily Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories</td>
<td>10</td>
</tr>
<tr>
<td>Fat</td>
<td>0 g</td>
</tr>
<tr>
<td>Saturated</td>
<td>0 g</td>
</tr>
<tr>
<td>Trans</td>
<td>0 g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0 mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>360 mg</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>8 g</td>
</tr>
<tr>
<td>Fiber</td>
<td>1 g</td>
</tr>
<tr>
<td>Sugars</td>
<td>0 g</td>
</tr>
<tr>
<td>Protein</td>
<td>0 g</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>2%</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0%</td>
</tr>
<tr>
<td>Iron</td>
<td>0%</td>
</tr>
<tr>
<td>Calcium</td>
<td>0%</td>
</tr>
</tbody>
</table>

INGREDIENTS:

tomato, onion, vinegar, salt, garlic powder,
chili peppers, citric acid, and natural flavoring.

Mild, Medium and Hot.
Appendix B

BLUEBERRY JUICE NUTRITION FACTS AND LABEL

<table>
<thead>
<tr>
<th>Available Size(s)</th>
<th>32 FL OZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTAINS</td>
<td>100% JUICE</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Serving Size</th>
<th>8 FL OZ (240mL)</th>
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</thead>
<tbody>
<tr>
<td>Servings Per Container</td>
<td>4</td>
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</table>

Amount Per Serving

<table>
<thead>
<tr>
<th>Calories</th>
<th>100</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>% Daily Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fat</td>
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</tr>
<tr>
<td>Potassium</td>
</tr>
<tr>
<td>Total Carbohydrates</td>
</tr>
<tr>
<td>Sugars</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Calcium</td>
</tr>
<tr>
<td>Iron</td>
</tr>
</tbody>
</table>

Not a significant source of calories from fat, saturated fat, trans fat, cholesterol, dietary fiber, vitamin A, and vitamin C.

*Percent Daily Values are based on a 2,000 calorie diet
Appendix C

ABSORBANCE OF 2% BLUEBERRY JUICE MIXTURE