Various Non-Thermal Technologies and Their Effectiveness against Human Norovirus Surrogates

DISSERTATION

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Abstract

Human norovirus is the most prevalent cause of acute non-bacterial gastroenteritis, causing an estimated 1 in 15 people to become ill each year. Fresh produce such as leafy greens are at high risk for norovirus contamination because they typically undergo little to no processing after harvest. For these fresh foods and other foods where thermal processing is not practical or consumers have decided that they prefer more fresh-like, minimally processed foods, the use of non-thermal technologies for food processing has emerged. The non-thermal technologies that we investigated in this study include electron beam irradiation (e-beam), ozone, pulsed electric field (PEF) processing, and other sanitizers and surfactants. Our results showed that Tulane virus (TV) was able to be reduced to non-detectable levels in lettuce by e-beam irradiation at 8.7 kGy or higher, and in strawberries at 16.3 kGy or higher, but these levels of irradiation are higher than currently allowed by FDA regulations (FDA, 2013). Visual changes to the food were significant at doses of 8.2 or 8.7 kGy or higher in strawberries and lettuce, respectively. We also found that the human norovirus surrogate, murine norovirus-1 (MNV-1), was more resistant to e-beam treatment than Tulane virus. It appeared that the combination of disruption of the structure, viral proteins, and RNA are all essential in viral inactivation by irradiation.
Gaseous ozone was able to significantly reduce two human norovirus surrogates’ viral titer in both liquid virus stock and in strawberries and lettuce. MNV-1 was slightly more resistant to gaseous ozone. Based on SDS-PAGE, electron microscopy, and RT-PCR results, ozone was able to successfully disrupt virion structure and completely degrade viral proteins, while leaving genomic RNA intact in both viruses. Thus, the primary way ozone disrupts norovirus surrogates is by altering the capsid structure and damaging the viral capsid proteins on the surface of the virus.

We evaluated the effectiveness of chlorine, sodium dodecyl sulfate (SDS), levulinic acid, and combinations of these treatments for the removal or inactivation of Tulane virus in fresh produce and in virus stock over time. Modest concentrations of SDS in combination with chlorine was the most effective sanitizer associated with strawberries and virus removal. For lettuce, traditional chlorine solution performed the best, followed by levulinic acid in combination with chlorine. Over a 72 h period incubation with the treatments, SDS with chlorine showed the most virucidal activity, giving 4.8 logs of viral reduction. None of the other treatments tested were able to successfully degrade viral protein or viral RNA, except for SDS in combination with chlorine. Ultimately, chlorine remained the most effective antiviral treatment under our conditions.

Pulsed electric field was used to inactivate both MNV-1 and TV in liquid virus stock. We showed that using square wave 3 μsec pulses, 29.5 kV, and 8.8 A conditions, that we were able to achieve 1.2 and 2.4 log reductions in MNV-1 and TV, respectively. This is the first account of significant foodborne viral reduction by PEF treatment. Each
of these different non-thermal technologies all show great promise in future food safety applications.
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Abbreviations

ANOVA – Analysis of variance
CaCV – Canine calicivirus
CDC – Centers for Disease Control and Prevention
CFR – Code of Federal Regulations
CPE – Cytopathic effect
DMEM – Dulbecco’s modified eagle medium
DNA – Deoxyribonucleic acid
ELISA – Enzyme-linked immunosorbant assay
EM – Electron Microscopy
FBS – Fetal bovine serum
FCV – Feline calicivirus
EDTA – Ethylenediamine tetraacetic acid
EPA – Environmental Protection Agency
EPRI – Electric Power Research Institute
FCV – Feline calicivirus
FDA – Food and Drug Administration
gEq - Genomic equivalents
GRAS – Generally recognized as safe
HAA – Haloacetic acid
HAdV – Human adenoviruses
HAV – Hepatitis A
HBGA – Histo-blood group antigen
HEV – Hepatitis E
HID50 – Hypothermia inducing dose 50
HuNoV – Human norovirus
LLC-MK2 – A rhesus monkey (Macaca mulatta) epithelial kidney cell line
MEM – Minimal eagle medium
MNV-1 – Murine norovirus
MOI – Multiplicity of infection
NIAID – National Institute of Allergy and Infectious Diseases
ORF – Open reading frames
PCR – Polymerase chain reaction
PEF – Pulsed electric field
PBS – Phosphate buffered saline
PFU – Plaque forming units
Ppm – Parts per million
RAW 264.7 – A mouse macrophage cell line
RNA – Ribonucleic acid
RT-PCR – Reverse transcriptase-polymerase chain reaction
SARS - Severe acute respiratory syndrome
SDS- Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TEM – Transmission electron microscopy
THMs - Trihalomethanes
TV- Tulane virus
USDA – United States Department of Agriculture
UV - Ultraviolet
VLP- Virus-like particle
Chapter 1: Literature Review

1.1. Introduction to foodborne viruses

While foodborne illnesses may be caused by viruses, bacteria, fungi, or parasites, viruses are known to be the most prominent cause of acute gastroenteritis. According to the Centers for Disease Control and Prevention (CDC), foodborne pathogens cause an average of 49 million illnesses per year, leading to 128,000 hospitalizations and 3,000 deaths (CDC, 2011). Of the 31 major known pathogens used to establish these numbers, norovirus alone caused 58% of all cases of illness.

Viruses in general are extremely small organisms, ranging in size from 15-400 nanometers. One characteristic of viruses that sets them apart from other microorganisms is that they are strict intracellular parasites- meaning they are only able to replicate inside of a host. This also indicates that the virus cannot replicate on or in foods during storage, unlike bacterial pathogens (Vasikova et al., 2005). Transmission of viruses may be via a number of routes including direct contact, bodily fluids, sexual contact, droplet contact, fecal-oral transmission, transmission by food, transmission by contaminated water, vector transmission, or vertical transmission. Specifically, foodborne viruses cause illness most frequently by the fecal-oral route, or by contaminated food or water.

A foodborne virus generally is a virus that is able to cause illness by way of contaminated food or water. Viruses all have in common their transmission via the fecal-
oral route. These foodborne viruses attack and infect the digestive system, and are subsequently dispersed by vomiting or by shedding into stool (Koopmans and Duzier, 2002; Vasikova et al., 2005). Based on the type of clinical manifestation each virus exhibits, food- and water-borne viruses can be classified into three different categories. The first group of viruses causes gastroenteritis. These include human norovirus, sapovirus, rotavirus, astrovirus, coronavirus, aichivirus, and adenovirus. The second group includes viruses transmitted enterically that cause hepatitis in humans. These include both hepatitis A and E. The third group is the viruses that replicate in the intestine, but have the potential to migrate and affect other parts of the body, such as the human nervous system. Enteroviruses (ex. poliovirus) are viruses that may cause neurological disease in humans. Among foodborne viruses, human norovirus is the most common virus associated with foodborne illness.

While norovirus is the most prominent of the group, all foodborne viruses do share some common characteristics, including: 1) a low number of particles are needed to cause infection, 2) a high titer of virus may be shed in stool of an infected person (up to $10^{11}$), 3) the living host cells are required for viruses to replicate, 4) foodborne viruses are typically very stable in the environment, and 5) they resist the low pH and the enzymes in the gastrointestinal tract and thus can survive in the stomach and cause illness (Koopmans and Duzier, 2004, Rzezutka and Cook, 2004). These characteristics distinguish foodborne viruses from bacterial pathogens (Koopmans and Duzier, 2002). For example, most common foodborne viruses are much more resistant to various current procedures in food processing, preservation, and storage (Rzezutka and Cook, 2004).
In an effort to meet the urgent demand for reliable ways to inactivate foodborne viruses, we carried out the present study with four objectives: (1) to review the literature on the subject of foodborne viruses and the several non-thermal technologies used to inactivate those foodborne viruses, (2) to investigate the efficiency of various non-thermal technologies including electron beam irradiation, ozone, pulse electric field, and surfactants and sanitizers for inactivating norovirus, (3) to evaluate the effectiveness of these technologies for removing norovirus from fresh produce, and (4) to explore the mechanisms underlying the removal or inactivation of the viruses using the selected technologies.

1.2 Foodborne viruses: gastrointestinal

The foodborne viruses that cause gastroenteritis are human norovirus, sapovirus, rotavirus, astrovirus, coronavirus, aichivirus, and adenovirus. Viral pathogens causing acute gastroenteritis always include the manifestation of diarrhea and vomiting. These viruses all share the same characteristic of being able to withstand very low pH in order to survive the gastrointestinal tract and infect their hosts. Because of this characteristic, they are typically resistant to various processing and storage treatments (Grove et al., 2006). These foodborne viruses may also be able to survive in foods for days or weeks without any loss of infectivity (Rzezutka and Cook, 2004).
1.2.1 Norovirus

Norovirus belongs to the family *Caliciviridae*, and is 28-35 nanometers (nm) in diameter as visible by electron microscopy. The virus is a non-enveloped, positive sense single stranded RNA virus which exhibits icosahedral capsid structure. Its genome is linear in shape and 7.7 kb long, containing three open reading frames (ORFs) (Jiang et al., 1993). The first ORF (ORF1) encodes for a nonstructural polyprotein, which can be proteolytically cleaved into several non-structural proteins including p48, NTPase, p22, VPg, 3CLpro, and RNA-dependent RNA polymerase (RdRp), the second ORF (ORF2) instructs the major capsid protein VP1, and ORF3 encodes the minor structural capsid protein VP2 (Hardy, 2006). There are currently six recognized norovirus genogroups, with GI, GII, and GIV affecting humans. Specifically, genotype variants of GII.4 have caused the most outbreaks since 2002 (CDC, 2011).

In the United States alone, norovirus is the leading cause of acute gastroenteritis, causing an estimated 1 in 15 people to become ill each year. Between 2009 and 2012, 4,318 norovirus outbreaks were reported, resulting in 161,253 illnesses (Hall et al., 2014). Noroviruses are also extremely infectious. In a recent study by Atmar et al. (2014), it was suggested that the 50% human infectious norovirus dose is much higher than previously thought. The researchers found that the HID$_{50}$ was approximately 1320 genomic equivalents (gEq) for people who were either blood type A or O and that were secretor positive. No one enrolled in the study that was blood type B became infected with the given doses of norovirus, but if data from these blood group B patients were also used in
the calculation, the HID$_{50}$ would increase to 28,000 gEq needed to cause norovirus infection (Atmar et al., 2014). This is a higher dose than initially demonstrated by Teunis et al. in 2008, but the difference was likely attributed to differences in the statistical approach used to generate the estimates, rather than actual biological differences (Kirby et al., 2014).

Noroviruses typically cause gastroenteritis after a short incubation period of 1-2 days with symptoms lasting an average of 2-3 days (Koopmans et al., 2002). Symptoms of human norovirus infection include diarrhea, vomiting, nausea, dehydration, and a high fever in some cases. Persons who have become infected will shed virus in their stool for the duration of the infection, and sometimes even after full recovery (Greening, 2006; Bresee et al., 2002). The virus is most frequently transmitted by fecal-oral contamination, but can also be transmitted by person to person spread or by ingesting contaminated food or water. Norovirus outbreaks are common in areas such as nursing homes, schools, military bases, child care centers, and cruise ships (CDC, 2009). In foods, norovirus is most commonly associated with causing outbreaks in shellfish, fresh produce, water, ice, and ready-to-eat foods (Greening, 2006).

### 1.2.2. Sapovirus

Sapoviruses are from the *Caliciviridae* family. They are genetically similar to norovirus, but differ in morphology, host range, and epidemiology (Farkas et al., 2004). Sapoviruses are 28-35 nm in diameter, non-enveloped, single stranded positive sense RNA viruses, with a 7.6 kb genome and icosahedral symmetry. There have been seven
genogroups (I-VII) reported thus far based on sequencing, with groups I, II, IV and V causing infection in humans (Farkas et al., 2004).

While this virus causes gastroenteritis among both adults and children, association with foodborne transmission is rare. Sapoviruses are most frequently associated with children and are more likely to be transferred via person to person spread (Greening, 2006). Outbreaks of sapoviruses occurred in similar settings to those of norovirus, including child-care centers, schools, hopsitals, and long-term care centers (Hansman et al., 2007; Nakata et al., 2000; Johansson et al., 2005). Because sapovirus is unable to propagate in cell culture, similar to human norovirus, surrogates are most commonly used for research on sapovirus.

1.2.3. Rotavirus

Rotaviruses are different than the first two enteric viruses discussed above in that they come from a different family of viruses and are significantly larger in size. Rotavirus is a 70 nm, double stranded RNA virus from the family Reoviridae. It also has a segmented genome (11 segments) which encodes for two proteins, P for protease sensitive, and G for glycoprotein. Rotaviruses may be classified into one of seven antigenic groups (A-G), but A is the most common cause of diarrhea cases. Rotavirus is similar to influenza virus in its ability to mutate and cause antigenic shift and have many different possible strains of the virus (Matson, 1997).

The virus is transmitted fecal-orally, spreading from hand to mouth, or less commonly by contaminated food or water. Outbreaks in settings such as daycare centers
and hospitals are very common. Unfortunately, there is no long term immunity to the virus; children may contract the virus repeatedly after initial infection (Committee on Infectious Diseases, 1998; Velazquez et al., 1996). After an incubation period of 2-4 days, children infected with the virus will show symptoms of fever, vomiting, cramping, abdominal pain, and watery diarrhea for several days. The diarrhea that is characteristic of rotavirus has an extremely foul smell (Greening, 2006).

In warm weathered countries, the virus causes infections year-round, but in other countries, it mostly affects people during the winter months. Rotavirus infection is a serious issue in developing countries where it causes up to 600,000 deaths annually among children (Greening, 2006). In the U.S., rotavirus causes almost 4 million infections, but these result in approximately 100 deaths per year (Kapikian et al., 2001; Sattar et al., 2001). Rotavirus infections are not typically recognized as foodborne but outbreaks caused by food and water have been reported in a number of countries (Sattar et al., 2001).

1.2.4. Adenovirus

Adenovirus is an 80-100 nm non-enveloped double stranded DNA virus (Koopmans and Duzier, 2004). The virus has a linear genome and of 28-45 kb, and the virus also exhibits icosahedral symmetry. Adenoviruses belong to the Adenoviridae family and are most commonly known to cause respiratory disease. There have been six species of human adenoviruses discovered (HAdV-A-F) and more than 50 serotypes discovered to date, but serotypes 40 and 41 of the virus are the only ones known to be
enteric. These two serotypes make up the HAdV-F species (van Regenmortel et al., 2000; Greening et al., 2006).

Most of the adenovirus infections that occur in normal healthy adult individuals are relatively mild. The virus may be shed into the feces of those infected for months or years after infection, as adenoviruses can cause persistent infections. The primary route of transmission for enteric adenovirus is the fecal-oral route. While the existence of adenovirus has been reported in a large variety of environmental substrates including wastewater, marine water, shellfish, and drinking water, there have not been any reported foodborne outbreaks from this virus (Greening, 2006).

1.2.5. Aichi virus

Aichi virus is a virus of the family Picornaviridae and genus Kobovirus. These viruses are small non-enveloped single-stranded positive sense RNA viruses that were first discovered in 1989 during small shellfish associated foodborne outbreak (Yamashita et al., 1991). Three genotypes of the virus have been identified thus far (Aichi virus A, B, and C) (Yamashita et al., 2000; Ambert-Balay et al., 2008). Serological studies have indicated that as much as 90% of the human population has been exposed to Aichi viruses by the age of 40 (Reuter et al., 2011). Conversely, previous research studies have suggested that Aichi viruses are transmitted via the fecal oral route and have been the cause of outbreaks in seafood and shellfish, and even sewage (Sdiri-Loulizi et al., 2010; Hansman et al., 2008; Alcala et al., 2010). Additionally, countries such as Asia, South America, Europe, and Africa have showed low incidence of Aichi viruses in people
exhibiting gastroenteritis (Oh et al., 2006; Pham et al., 2007; Yang et al., 2009; Ambert-Balay et al., 2008; Jonsson et al., 2012; Kaikkonen et al., 2010; Reuter et al., 2009; Sdiri-Loulizi et al., 2009). While the typical symptoms for Aichi viruses are similar to those of other enteric viruses, coupled with the low incidence and high sero-prevalance, perhaps the virus may be asymptomatic in some people while still shedding into the stool of those infected allowing it to be transmitted, or it may also co-exist with other enteric viruses thus undetected during screening of the patients (Jonsson et al., 2012).

1.2.6. **Coronavirus**

Coronaviruses are single-stranded positive sense RNA viruses belonging to the family *Coronaviridae*. These viruses are 80-220 nm in diameter and coronaviruses have one of the largest genomes of known RNA viruses, containing 27-32 kb (Enjuanes, 2005). Coronaviruses most frequently cause respiratory infections, such as the massive Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) outbreaks that took in place in Asia in 2003, but their pathogenesis is interesting because they may also cause gastroenteritis. Despite this potential, there have been no reported outbreaks of coronavirus in foods at the time of this writing.

1.2.7. **Astrovirus**

Astrovirus is a positive sense single stranded polyadenylated RNA virus. Its genome is 6.8-7.2 kb in length, and it its implication in the cause of enteric disease has not been well reported or understood. In healthy persons, astrovirus infection leads to
mild or even symptomless infection, but it has also been reported to be the largest cause of infantile gastroenteritis behind rotavirus (Marx et al., 1998). Not only has the virus been reported in infants, but also in immunocompromised and elderly individuals (Madeley, 1979; Wilson and Cubitt, 1988). Transmission of the virus is most commonly by fecal oral route, and symptoms of the virus include diarrhea or soft stools, headache, malaise, and sometimes vomiting and fever. Outbreaks of this virus have reported in shellfish and water (Oishi et al., 1994; Appleton, 2001).

1.3. **Foodborne viruses: hepatic**

Several viruses cause hepatitis, but only hepatitis A (HAV) and E (HEV) are transmitted by the fecal-oral route. These two viruses are listed as “Severe Hazards” by the U.S. Food and Drug Administration’s Food Code for their ability to cause disease of the liver (Cliver, 1997). Both HAV and HEV are worldwide issues and cause many foodborne outbreaks in shellfish (HAV) and problems in areas with inadequate sanitation (HEV).

1.3.1. **Hepatitis A**

Hepatitis A is a 27-32 nm, nonenveloped, positive-sense, single-stranded RNA virus. It has a 7.5 kb genome and icosahedral capsid symmetry similar to many of the other foodborne viruses mentioned previously. The virus is part of the *Picornaviridae* family and is of the genus Hepatovirus. Hepatitis A virus (HAV) has been associated with many outbreaks of foodborne illness, but the incidence of HAV in recent years has
decreased with the improvement of sewage systems and hygiene practices in developing countries or other counties outside the United States (Cliver, 1997; Cromeans et al., 2001). Unfortunately, this has also led to less “herd immunity” where people now have an increased susceptibility to the disease.

Contamination by hepatitis A virus most frequently occurs during preharvest or subsequent food handling steps in the food processing chain. The most outbreaks of HAV have been reported in various shellfish products, with the biggest outbreak being one that occurred in China in 1988 where a staggering 300,000 people became ill after the consumption of HAV-contaminated clams harvested from an area polluted by raw sewage (Halliday et al., 1991). Some other shellfish associated outbreaks include oysters in Australia and Brazil, mussels in Italy, and clams in Spain. Most of these outbreaks were traced back to sewage as the contamination source (Conaty et al., 2000; Coelho et al., 2003; Croci et al., 2000; Bosch et al., 2001). The contamination of fresh fruits and vegetables by HAV has also been reported in countries such as Finland and New Zealand, where both exposure and immunity are low (Peabody et al., 1998; Calder et al., 2003). In these cases, food handlers and irrigation water were most suspected to be the source of contamination. In the United States alone, hepatitis A is the most common cause of hepatitis, contributing to a death rate of 0.3% (Greening, 2006). Fortunately, lifelong immunity occurs after infection by this virus (Lemon et al., 1992) and it is the only foodborne virus that is preventable by commercially available vaccine (Fiore, 2004). The trade names of the four current commercially available vaccines are Havrix®, Vaqta®, Avaxim®, and Epaxal® (Ott et al., 2012).
1.3.2. **Hepatitis E**

Hepatitis E is a major cause of enteric non-A, non-B hepatitis worldwide. Similar to hepatitis A, it is a nonenveloped, positive-sense, single-stranded RNA virus. It is 32-34 nm in diameter with a 7.2 kb linear genome and icosahedral capsid symmetry. Hepatitis E (HEV) was first classified in two other families, but has since settled in the *Hepadnaviridae* family, under the genus Hepevirus.

Foodborne outbreaks of HEV occur most in developing counties with poor environmental sanitation practices. The first reports of HEV in the United States were cases of those who had traveled to HEV-endemic countries and then returned home. Waterborne outbreaks of HEV have also been reported in some Asian countries (Cromeans et al., 2001; Emerson and Purcell, 2003). The disease may indeed be severe in some cases, but the clinical manifestation of hepatitis E virus is typically mild and self-limiting. Symptoms may include viremia (virus infects the blood), nausea, dark urine, and malaise. After the virus infects the liver, the symptoms of hepatitis appear after a 22-60 day incubation period. The major mode of transmission of HEV is contaminated water (Cromeans et al., 2001).

1.4. **Foodborne viruses: other**

Thus far, we reviewed those foodborne viruses that cause gastroenteritis and hepatitis. There are also viruses that may be transmitted via the fecal oral route, but do not typically cause gastroenteritis. After infection, these viruses may migrate into other
areas of the body such as the nerves or muscle to cause further issues in the body. One example of these type of viruses are enteroviruses.

### 1.4.1. Enteroviruses

Enteroviruses are a broad class of viruses belonging to the family *Picornaviridae*. They are 28-30 nanometers, nonenveloped, positive-sense, single-stranded RNA viruses. Their genome is 7.2-8.4 kb and they also exhibit icosahedral symmetry. The family of enteroviruses includes polioviruses, coxsackie A and B viruses, and echoviruses.

Enteroviruses may cause a broad range of problems including viral meningitis, poliomyelitis, acute paralysis, and in rare cases, encephalitis, severe pulmonary edema, and shock (Solomon et al., 2010; Greening et al., 2006). Polioviruses were the first viruses to be shown to be foodborne, but the massive vaccination effort against this virus has since drastically limited the number of wild type strains seen today. There have been outbreaks of coxsackieviruses and echoviruses reported (Cliver, 1997; Sattar and Tetro, 2001). Overall, there have been very few foodborne outbreaks reported to be associated with enteroviruses, even though the virus is ubiquitous in the environment.

### 1.5. Emergence of non-thermal technologies

Thermal processing has long dominated food processing methods. While thermal processing is the most effective way to kill foodborne pathogens including the viruses just discussed, not all foods can be treated thermally. Thermal processing has effects on flavor, color, and texture of foods, and degrades nutritional components in fresh foods.
(Rasmaswamy et al., 2004). Consumers are forced to choose between fresh, healthy foods, which may or may not be safe from foodborne pathogens, or thermally processed “safe” foods which may not have the same nutritional value or be appealing as their fresh counterparts.

Alternative non-thermal technologies are not a new concept, as high pressure processing was proposed as early as 1884 (Earnshaw et al., 1995), but their popularity and investigation for use in foods has increased in the 1990’s in response to consumer demand for foods and food ingredients that are minimally processed, perceived as more fresh and believed to be more natural. These non-thermal or emerging technologies may inactivate microorganisms and extend shelf life while using less energy and causing minimal changes in the nutritional and sensory properties of treated foods (Morris et al., 2007). Some non-thermal technologies include exposure to irradiation, ozone, pulse electric fields, sanitizers or surfactants, and hurdle technologies involving combinations of these treatments. Each non-thermal processing concept is reviewed here reflecting several independent lines of investigation on their respective mechanisms and food applications. Further, very little is reported on viral effects of non-thermal or emerging food process technologies.

1.5.1. Irradiation

1.5.1.1. General characteristics, types of irradiation

Food irradiation is a non-thermal process used for the preservation of food. It is not a new technology, as experiments were reported in the late 1800s. In 1895 and 1986,
W.K. von Roentgen first reported work with x-rays and Henri Becquerel first investigated radioactivity, triggering interest on the effect of radiation on organisms (Molins, 2001; Barbosa-Canovas et al., 1998). Research investigating irradiation to kill bacteria in food products followed in 1905, when British scientists received a patent to do so. Unfortunately, the patent never came to practice due to the limited available radium, needed to produce ionizing radiation (Diehl, 1995). During the 1940s and 1950s, research on food irradiation continued to glow because of the need to provide our soldiers with safe foods in primitive field conditions, and because of increased availability of radioisotope sources needed for irradiation (Barbosa-Canovas et al., 1998). In 1958, the U.S. Food and Drug Administration classified food irradiation as a “food additive” by an amendment to the U.S. Food, Drug, and Cosmetic Act (Molins, 2001). The Joint Food and Agricultural Organization of the United Nations (FAO), International Atomic Energy Agency (IAEA) and the World Health Organization’s Expert Committee on the Wholesomeness of Irradiated Food all recognized irradiation as a safe process in 1976 (Molins, 2001). Irradiation was subsequently approved in the U.S. for several food applications, such as: disinfection and delay of maturation for spices and vegetables in 1986, poultry in 1990, eggs for the control of Salmonella in 2000, meat for the control of pathogens in 1998, and produce to both extend shelf life and control pathogens in 2008 (FDA 2008; Molins, 2001). Today, there are more than 40 countries around the world that have approved irradiation for use in foods (Loaharanu, 1994; Loaharanu, 1995). The list of countries where irradiation is permissible is in Table 1.
Table 1. Countries that have approved the use of food irradiation. (Adapted from Barbosa-Canovas et al., 1998)

<table>
<thead>
<tr>
<th>Country</th>
<th>Country</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>Germany</td>
<td>Philippines</td>
</tr>
<tr>
<td>Argentina</td>
<td>Hungary</td>
<td>Poland</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>India</td>
<td>Russia</td>
</tr>
<tr>
<td>Belgium</td>
<td>Indonesia</td>
<td>South Africa</td>
</tr>
<tr>
<td>Brazil</td>
<td>Iran</td>
<td>Spain</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>Israel</td>
<td>Syria</td>
</tr>
<tr>
<td>Canada</td>
<td>Italy</td>
<td>Thailand</td>
</tr>
<tr>
<td>Chile</td>
<td>Ivory Coast</td>
<td>Ukraine</td>
</tr>
<tr>
<td>China</td>
<td>Japan</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>Croatia</td>
<td>Korea</td>
<td>United States</td>
</tr>
<tr>
<td>Cuba</td>
<td>Mexico</td>
<td>Uruguay</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>Netherlands</td>
<td>Vietnam</td>
</tr>
<tr>
<td>Denmark</td>
<td>New Zealand</td>
<td>Yugoslavia</td>
</tr>
<tr>
<td>Finland</td>
<td>Norway</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Pakistan</td>
<td></td>
</tr>
</tbody>
</table>

There are many benefits and practical applications for using irradiation. It has many uses and is effective against many pathogens, insects, and pests. As mentioned previously, irradiation is a “cold” process, which gives it the potential to treat fresh or raw foods where thermal processing would not be practical (Fellows, 2000; Barbosa-Canovas et al., 1998). Treatment by irradiation does not involve harmful chemicals nor does it produce any harmful residues after treatment. Concerns about unique radiolytic products in food have proven unfounded. Lastly, foods can be treated directly in their final packaging, minimizing post-process contamination and allowing the direct distribution of foods immediately after treatment (Roberts, 2014).

In irradiation processing, the target dose may be different than the actual absorbed dose. The target dose is the dose sought by this treatment, while the absorbed dose is the
dose absorbed by the food or substance you are treating (Barbosa-Canovas et al., 1998). The unit of measurement for irradiation dose is the kilogram (kGy). One kGy is equal to 1,000 grays (Gy), and 1 Gy is equivalent to 1 joule/kg (Hansen and Shaffer, 2001; Barbosa-Canovas et al., 1998).

There are three types of irradiation energy that may be applied on foods: gamma rays, electron beams, and x-rays. The three techniques of irradiation are shown in Figure 1. Typically only the gamma rays or electrons are used in the irradiation of foods because the x-ray process is very inefficient and much of the energy is lost as heat (Hansen and Shaffer, 2001; Diehl, 1995).

![Figure 1. The three techniques of irradiation used for food processing. (a) electron beams, (b) x-rays, and (c) gamma rays (Koch and Eisenhower, 1965; Jay et al., 2005).](image-url)
Gamma irradiation is produced from radioactive isotope sources ($^{60}$Co or $^{137}$Cs) although Cs has never been used for food irradiation and is not readily available (Hansen and Shaffer, 2001). $^{60}$Co is formed by neutron bombardment in a nuclear reactor by $^{59}$Co pellets absorbing an additional neutron (Stewart, 2001; Hansen and Shaffer, 2001). Gamma rays are emitted when the isotopes, which are already in an excited state after gaining an additional neutron, transition down to the ground state (Hansen and Shaffer, 2001). One common drawback of gamma irradiation is that the rays are emitted in all directions (Figure 1, Figure 2), and they cannot be shut off when not in use. Because of this, the rays must be shielded by a minimum of 27 feet of water or behind a wall of concrete when not in use (Fellows, 2000). Another disadvantage of using gamma irradiation is the relatively short 5.27 year half-life of $^{60}$Co. The isotope source must be changed periodically to maintain adequate levels of gamma emissions (Jay et al., 2005). This drawback may be overcome by using $^{137}$Cs (30 year half-life), but as mentioned before, this radioisotope may only be extracted after nuclear fission by a limited number of processing facilities in the world, so its use is rare (Hansen and Shaffer, 2001).
Figure 2. Visualization of how different types of irradiation interact with matter. Ionizing radiation, whether from radioisotope sources or from an electron accelerator, has the ability to cause a series of localized events during tracking through the matter. Free radicals are also created that may form additional delta rays. Irradiation never travels in a straight line through matter being treated.

(Hansen and Shaffer, 2001).

Electron beams (e-beams) and x-rays are not from radioisotopic decay but are instead generated using electron accelerators. Although different types of equipment are used to produce these types of irradiation, the major difference when applied to food is in their penetration depths and therefore the sizes and types of foods for which they are best suited. E-beams are most commonly generated from a generator that is contained inside a pressurized tank of sulfur hexafluoride (SF₆). Accelerated electrons are emitted from a heated cathode after passing through a high-voltage electrostatic field in an accelerator.
tube (Diehl, 1995; Fellows, 2000; Stewart, 2001). The emitted beam of electrons is then deflected by a scanning magnet which helps to direct the beam over a target treatment area (Diehl, 1995). X-rays use the same electron accelerator as e-beams. The only difference is the presence of a water-cooled target converter plate, composed of a heavy metal such as copper or tungsten (Diehl, 1995). When the accelerated electrons hit the plate, x-rays are produced (Fellows, 2000). The energy conversion of this process may range anywhere from 1-30% and most of the energy is lost as heat from the converter plate (Hansen and Shaffer, 2001; Diehl, 1995).

1.5.1.2. Irradiation effects on viruses, mechanism of inactivation

Ionizing radiation has been reported to have both direct and indirect effects on microorganisms. The manner in which ionizing radiation affects microorganisms is by damage to genetic material, either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). According to Dickson (2001), photons or electrons will strike the genetic material in a random fashion causing breaks or lesions. If the genetic material is double-stranded, single strand lesions may not be lethal, but multiple lesions may render the microorganism non-viable (Dickson, 2001). Irradiation may also cause indirect damage to the microorganism by disrupting other atoms or molecules. When water molecules are irradiated, they lose an electron forming a positively charged water molecule ($\text{H}_2\text{O}^+$) and a free electron ($\text{e}^-$). These products have the ability to combine with other water molecules or each other to form a variety of reactive free radicals (Fellows, 2000). The most detrimental of these radical products are the hydroxyl radicals and hydrogen peroxide,
which may break bonds in nucleic acids. Ionizing radiation may also affect the structure of membranes, enzymes, plasmids, and proteins, all vital to living microorganisms (Dickson, 2001).

The effect of irradiation on both bacteria and parasites in foods has been widely researched. To date, there have been significantly fewer studies on the interaction of irradiation with foodborne viruses. A summary of previous viral studies and their findings is shown in Table 2. Based on the extremely varied data reported by these researchers, the results suggest that foodborne viruses are relatively resistant to irradiation, and each virus exhibits markedly different resistance and sensitivity.

**Table 2. Summary of published data on the inactivation of foodborne viruses by irradiation.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inactivation (log10)</th>
<th>Dose (kGy)</th>
<th>Medium</th>
<th>Irradiation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>poliovirus</td>
<td>2</td>
<td>6</td>
<td>fish filets</td>
<td>gamma</td>
<td>Heidelbaugh and Giron, 1969</td>
</tr>
<tr>
<td>coxsackie virus</td>
<td>1</td>
<td>7.5</td>
<td>frozen ground beef (-30)</td>
<td>gamma</td>
<td>Sullivan et al., 1973</td>
</tr>
<tr>
<td>coxsackie virus</td>
<td>1</td>
<td>7.1</td>
<td>frozen ground beef (-60)</td>
<td>gamma</td>
<td>Sullivan et al., 1973</td>
</tr>
<tr>
<td>coxsackie virus</td>
<td>1</td>
<td>6.8</td>
<td>frozen ground beef (-90)</td>
<td>gamma</td>
<td>Sullivan et al., 1973</td>
</tr>
<tr>
<td>hepatitis A virus</td>
<td>1</td>
<td>2</td>
<td>clams and oysters</td>
<td>gamma</td>
<td>Mallet et al., 1991</td>
</tr>
<tr>
<td>rotavirus SA11</td>
<td>1</td>
<td>2.4</td>
<td>clams and oysters</td>
<td>gamma</td>
<td>Mallet et al., 1991</td>
</tr>
<tr>
<td>hepatitis A virus</td>
<td>1</td>
<td>2.97</td>
<td>strawberries</td>
<td>gamma</td>
<td>Bidawid et al., 2000</td>
</tr>
<tr>
<td>hepatitis A virus</td>
<td>1</td>
<td>2.72</td>
<td>lettuce</td>
<td>gamma</td>
<td>Bidawid et al., 2000</td>
</tr>
<tr>
<td>avian influenza</td>
<td>1</td>
<td>2.4</td>
<td>PBS</td>
<td>e-beam</td>
<td>Brahmakshatriya et al. 2009</td>
</tr>
<tr>
<td>avian influenza</td>
<td>1</td>
<td>1.6</td>
<td>egg whites</td>
<td>e-beam</td>
<td>Brahmakshatriya et al. 2009</td>
</tr>
<tr>
<td>avian influenza</td>
<td>1</td>
<td>2.6</td>
<td>ground turkey</td>
<td>e-beam</td>
<td>Brahmakshatriya et al. 2009</td>
</tr>
<tr>
<td>poliovirus</td>
<td>1</td>
<td>0.46</td>
<td>PBS</td>
<td>gamma</td>
<td>Jung et al., 2009</td>
</tr>
</tbody>
</table>

(Continued)
(Table 2 Continued)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>t value</th>
<th>Pathogen</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>poliovirus</td>
<td>2.84 MEM</td>
<td>gamma</td>
<td>Jung et al., 2009</td>
<td></td>
</tr>
<tr>
<td>poliovirus</td>
<td>2.94 raw oysters</td>
<td>gamma</td>
<td>Jung et al., 2009</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.77 2.8</td>
<td>gamma</td>
<td>Jung et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.4 2.8</td>
<td>romaine lettuce</td>
<td>gamma</td>
<td>Jung et al., 2011</td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.31 2.8</td>
<td>strawberries</td>
<td>gamma</td>
<td>Jung et al., 2011</td>
</tr>
<tr>
<td>MS2</td>
<td>5 0.2 water</td>
<td>gamma</td>
<td>de Roda Husman et al., 2004</td>
<td></td>
</tr>
<tr>
<td>CaCV</td>
<td>2.4 0.2 water</td>
<td>gamma</td>
<td>de Roda Husman et al., 2004</td>
<td></td>
</tr>
<tr>
<td>feline calicivirus</td>
<td>1.6 0.2 water</td>
<td>gamma</td>
<td>de Roda Husman et al., 2004</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.12 2 PBS</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>0.85 2 DMEM</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>0.33 2 cabbage</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>0.12 2 strawberries</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>2.37 4 PBS</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.4 4 DMEM</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>0.7 4 cabbage</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>0.37 4 strawberries</td>
<td>e-beam</td>
<td>Sanglay et al., 2011</td>
<td></td>
</tr>
<tr>
<td>feline calicivirus</td>
<td>1 2.95 lettuce</td>
<td>e-beam</td>
<td>Zhou et al., 2011</td>
<td></td>
</tr>
<tr>
<td>rotavirus</td>
<td>1 1.03 lettuce</td>
<td>e-beam</td>
<td>Espinoza et al., 2011</td>
<td></td>
</tr>
<tr>
<td>rotavirus</td>
<td>1 1.29 spinach</td>
<td>e-beam</td>
<td>Espinoza et al., 2011</td>
<td></td>
</tr>
<tr>
<td>poliovirus</td>
<td>1 2.32 lettuce</td>
<td>e-beam</td>
<td>Espinoza et al., 2011</td>
<td></td>
</tr>
<tr>
<td>poliovirus</td>
<td>1 2.35 spinach</td>
<td>e-beam</td>
<td>Espinoza et al., 2011</td>
<td></td>
</tr>
<tr>
<td>TV</td>
<td>1.4 4.1 strawberries</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
<tr>
<td>TV</td>
<td>1.3 3.9 lettuce</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.28 4.8 PBS</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
<tr>
<td>MNV-1</td>
<td>1.1 4.8 DMEM</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
<tr>
<td>TV</td>
<td>1.5 4.8 PBS</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
<tr>
<td>TV</td>
<td>1.8 4.8 Opti-MEM</td>
<td>e-beam</td>
<td>Predmore et al., 2014</td>
<td></td>
</tr>
</tbody>
</table>

1.5.1.3. Irradiation in the food industry

As mentioned previously, irradiation is currently allowed in over 40 countries across the world (Jay et al., 2005). In 1986, Nham, a type of fermented pork sausage was irradiated at minimum of 2 kGy and sold in Bangkok for the first time. In 1986, Puerto
Rican mangoes were irradiated at 1 kGy and shipped and sold in Miami, Florida (Loaharanu, 1989). In May of 1990, the USDA approved the treatment of poultry with up to 3.0 kGy of irradiation, and September 2, 1993, irradiated poultry was sold in a grocery store in Illinois for the first time (Pszczola, 1993). Strawberries are another food that was irradiated and sold in 1992 in the state of Florida. Three years later in 1995, both Maine and New York repealed the ban that they had on the sale of irradiated foods, allowing them to then be sold in these states. Inhibition of sprouting and disinfestation of insects are still two of the most common applications of food irradiation (Jay et al., 2005). A list of currently approved food irradiation applications and limits set by the U.S. FDA is in Table 3.

Table 3. Doses of ionizing radiation allowed by the U.S. FDA for specific food products (21 CFR 179.26)

<table>
<thead>
<tr>
<th>Food Uses for Ionizing Radiation</th>
<th>Regulatory Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of <em>Trichinella spiralis</em> in pork</td>
<td>Minimum dose: 0.3 kGy Not to exceed 1 kGy</td>
</tr>
<tr>
<td>Control growth and maturation of fresh foods</td>
<td>Not to exceed 1 kGy</td>
</tr>
<tr>
<td>Insect disinfestation in foods</td>
<td>Not to exceed 1 kGy</td>
</tr>
<tr>
<td>Control of foodborne pathogens in fresh or frozen, uncooked poultry products</td>
<td>Not to exceed 1 kGy</td>
</tr>
<tr>
<td>Control of <em>Salmonella</em> in fresh shell eggs</td>
<td>Not to exceed 3 kGy</td>
</tr>
<tr>
<td>Control of foodborne pathogens and extension of shelf life in fresh iceberg lettuce and spinach</td>
<td>Not to exceed 3 kGy</td>
</tr>
<tr>
<td>Control of foodborne pathogens in, and extension of shelf life of, refrigerated or frozen, ground meat or meat byproducts</td>
<td>Not to exceed 4.5 kGy (refrigerated) Not to exceed 7.0 kGy (frozen)</td>
</tr>
<tr>
<td>Control of <em>Vibrio</em> and other foodborne microorganisms in fresh or frozen molluscan shellfish</td>
<td>Not to exceed 5.5 kGy</td>
</tr>
<tr>
<td>Control of microbial pathogens on seeds for sprouting</td>
<td>Not to exceed 8.0 kGy</td>
</tr>
<tr>
<td>Microbial disinfection of dry or dehydrated enzyme preparations</td>
<td>Not to exceed 10 kGy</td>
</tr>
<tr>
<td>Microbial disinfection of spices</td>
<td>Not to exceed 30 kGy</td>
</tr>
<tr>
<td>Sterilization of frozen, packaged meats used for NASA programs</td>
<td>Minimum dose: 44 kGy</td>
</tr>
</tbody>
</table>
By the beginning of 2003, there were over 7,000 grocery stores across the U.S. selling irradiated ground beef, according to the American Council of Science and Health. Additionally, the U.S. Centers for Disease Control and Prevention (CDC) estimated that if half of all of the ground beef, poultry, pork, and processed lunch meats in the U.S. were irradiated, there would be almost 900,000 fewer cases of foodborne illness outbreaks (Food Protection Trends, 2003). In a presentation given by Moreria (2014) at the Annual International Non-Thermal Processing Workshop, it was reported that several companies in the U.S. sell irradiated ground beef including Wegmans supermarkets, Publix supermarkets, Omaha Steaks, and Schwan’s. Moreira also reported that globally, the top five irradiated food items are 1) spices and dry vegetables, 2) grains and fruit, 3) meat and seafood, 4) garlic and potatoes, and 5) others (Moreria, 2014).

Any food that is irradiated must be labeled as regulated by the U.S. Government’s Code of Federal Regulations (CFR). First, any irradiated food product must have the Radura symbol (Figure 3) visible on the package. Following that symbol, either the statement “Treated with irradiation” or “Treated by irradiation” must also be present. If irradiated foods are shipped to a manufacturing plant or processing company after treatment, their invoices or bills must contain either the phrase “Treated with irradiation – do not irradiate again” or “Treated by irradiation – do not irradiate again (21 CFR 179.26).
There are many benefits for using irradiation for foods. It is a safe, energy efficient process, with the ability to extend shelf life and eliminate harmful pathogens, all while causing minimal changes to the nutritional quality and sensory quality of foods. (Fellows, 2000; Tauxe, 2001; Farkas, 1998). Research should be conducted in order to optimize treatment conditions without causing any negative effects or changes to the food being treated.

1.5.2. Ozone

1.5.2.1. Properties, generation, and degradation

Ozone is simply a triatomic oxygen molecule (O₃) that exists as a bluish gas and has a pungent, characteristic odor. It has a molecular weight of 48, it boils at 111.9°C, and it melts at -192.7°C (Merck Index, 1989). Ozone is naturally occurring in the stratosphere and troposphere in minute amounts (0.05 mg/L), but the gas is very unstable and will decompose in air quickly. Even these extremely small amounts of stratospheric
ozone play a major role in the global environment. The ozone layer absorbs a portion of high energy radiation from the sun’s rays, preventing it from reaching the earth’s surface. Without this layer of ozone, more harmful ultraviolet B (UVB) rays would reach the earth’s surface. These UVB rays have been linked with skin cancer and cataracts in humans, along with negative impacts on crops and marine life (EPA, 2010). In the atmosphere, ozone is generated either by action of solar UV irradiation of oxygen, or by photochemical reactions between hydrocarbons, oxygen, and nitrogen. Ozone may also be generated for use by industry or research settings (Horvath et al., 1985).

When ozone is generated by humans it is generally done at the point of application and in closed systems. The corona discharge system is the most common method of ozone generation to produce large amounts. During corona discharge, a high-voltage alternating current is applied across a discharge gap between two electrodes in the presence of either air or pure oxygen. The oxygen electrons will excite and thus induce the splitting of other oxygen molecules. Atoms from these split oxygen molecules will then combine to produce ozone, O₃. Voltage, current frequency, property and thickness of the dielectric surfaces, discharge gap, and absolute pressure within the discharge gap all influence ozone production. An efficient heat removal system is also needed for ozone production. Pure oxygen is more efficient and yields higher final concentrations of ozone than using dried air as the starting gas. The use of dried gas helps to minimize corrosion and prevent arcing of metal surfaces in the system (Rosen, 1972).

In addition to the photochemical and electrical discharge methods of generation, ozone may also be produced by other methods including chemical, thermal,
chemonuclear, and electrolytic methods (Horvath et al., 1985). In 1998, a facility in College Station, Texas, Lynntech Inc., employed a novel approach to produce ozone. They split water into hydrogen and oxygen atoms by electrolysis. One advantage of this approach is the claimed resulting ozone concentrations of 10-18% w/w, which is 3-4 times higher than what is possible using the corona discharge method (Lynntech, 1998).

Another study found that antibody-coated neutrophils, after activation, produced an oxidant thought to be O₃ based on its chemical signature and ability to oxidize vinylbenzoic acid to 4-carboxybenzylaldehyde. Neutrophils are the most abundant leukocytes in the bloodstream, and because they have the potential to produce O₂ and bind antibodies, the researchers speculated this was a biological source of ozone (Babior et al., 2003). While these data are not definitive, it suggests there may be a naturally occurring biological means to create ozone.

Ozone degradation is by multiple mechanisms. In the upper atmosphere, UV irradiation is consumed when it degrades ozone, thus stratospheric ozone protects terrestrial life from harmful UV rays from the sun. In solution, ozone decomposes in a more step-wise manner (Adler et al., 1950; Grimes et al., 1983; Hoigne et al., 1975). When ozone decomposes in solution, it first produces hydroperoxyl (HO₂), hydroxyl (OH), and superoxide (O₂⁻) radicals. The hydroxyl ions are important because they are transient and chain-propagating, where hydroxyls will then interact with many other substrates (Hoigne et al., 1975). These chain reaction products are likely responsible for ozone’s ability to disrupt biological systems.
1.5.2.2. Ozone effectiveness against viruses, mechanism of inactivation

Attempts to use ozone to inactivate viral pathogens have been widely documented. A summary of these findings is in Table 4. Ozone was shown to give significant reduction of a number of viruses.

Table 4. Summary of published data on the inactivation of foodborne viruses by ozone

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inactivation, log10</th>
<th>Treatment, min</th>
<th>Concentration, mg/L</th>
<th>pH</th>
<th>Temp °C</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enveloped</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human immunodeficiency virus (HIV-1)</td>
<td>&gt;11</td>
<td>120</td>
<td>1,200 mg/L</td>
<td>20</td>
<td>cell culture media</td>
<td>Wells et al., 1991</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus (VSV)</td>
<td>&gt;2.0</td>
<td>0.25</td>
<td>7</td>
<td>25</td>
<td>phosphate buffer</td>
<td>Burelson et al., 1975</td>
<td></td>
</tr>
<tr>
<td>Non-enveloped</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus AD40</td>
<td>2.63</td>
<td>0.25</td>
<td>0.49 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
<td>Thurston-Enriquez et al., 2005</td>
</tr>
<tr>
<td>Adenovirus AD40</td>
<td>3.28</td>
<td>2</td>
<td>0.49 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
<td>Thurston-Enriquez et al., 2005</td>
</tr>
<tr>
<td>Adenovirus AD40</td>
<td>3.04</td>
<td>0.5</td>
<td>0.30 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
<td>Thurston-Enriquez et al., 2005</td>
</tr>
<tr>
<td>Adenovirus AD40</td>
<td>3.55</td>
<td>10</td>
<td>0.30 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
<td>Thurston-Enriquez et al., 2005</td>
</tr>
<tr>
<td>Bacteriophage f2</td>
<td>0.7</td>
<td>10</td>
<td>0.1 mg/L</td>
<td>7.2</td>
<td>20</td>
<td>activated sludge effluent</td>
<td>Harakeh et al., 1985</td>
</tr>
<tr>
<td>Bacteriophage f2</td>
<td>&gt;4.3</td>
<td>0.16</td>
<td>0.41 mg/L</td>
<td>7</td>
<td>20</td>
<td>water</td>
<td>Boyce et al., 1981</td>
</tr>
<tr>
<td>Bacteriophage f2</td>
<td>2.72</td>
<td>0.5</td>
<td>0.68 mg/L</td>
<td>7</td>
<td>25</td>
<td>demand free water</td>
<td>Kim et al., 1980</td>
</tr>
<tr>
<td>Bacteriophage MS2</td>
<td>7</td>
<td>1</td>
<td>1.76 mg/L</td>
<td>6.9</td>
<td>22</td>
<td>phosphate buffer</td>
<td>Finch and Fairbairn, 1991</td>
</tr>
<tr>
<td>Bacteriophage MS2</td>
<td>5.68</td>
<td>1</td>
<td>1.29 mg/L</td>
<td>6.9</td>
<td>22</td>
<td>phosphate buffer</td>
<td>Finch and Fairbairn, 1991</td>
</tr>
<tr>
<td>Bacteriophage MS2</td>
<td>2.96</td>
<td>1</td>
<td>0.60 mg/L</td>
<td>6.9</td>
<td>22</td>
<td>phosphate buffer</td>
<td>Finch and Fairbairn, 1991</td>
</tr>
</tbody>
</table>

(Continued)
## (Table 3 Continued)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt;</th>
<th>Concentration</th>
<th>Temperature</th>
<th>Medium</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriophage MS2</td>
<td>3.5</td>
<td>5</td>
<td>0.37 mg/L</td>
<td>7</td>
<td>5</td>
<td>water</td>
</tr>
<tr>
<td>Coxsackie virus B5</td>
<td>4</td>
<td>2.5</td>
<td>0.4 mg/L</td>
<td>7.2</td>
<td>20</td>
<td>sludge effluent</td>
</tr>
<tr>
<td>Coxsackie virus A9</td>
<td>1.7</td>
<td>0.16</td>
<td>0.035 mg/L</td>
<td>7</td>
<td>29</td>
<td>water</td>
</tr>
<tr>
<td>Enteric virus</td>
<td>&gt;1.7</td>
<td>29</td>
<td>4.1 mg/L</td>
<td>7.8</td>
<td>18</td>
<td>raw wastewater</td>
</tr>
<tr>
<td>Feline calicivirus (FCV)</td>
<td>4.28</td>
<td>0.25</td>
<td>1.0 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
</tr>
<tr>
<td>Feline calicivirus (FCV)</td>
<td>&gt;4.74</td>
<td>1.2</td>
<td>1.0 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
</tr>
<tr>
<td>Feline calicivirus (FCV)</td>
<td>1.85</td>
<td>0.25</td>
<td>0.06 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
</tr>
<tr>
<td>Feline calicivirus (FCV)</td>
<td>2.77</td>
<td>5</td>
<td>0.06 mg/L</td>
<td>7</td>
<td>5</td>
<td>demand free water</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>4</td>
<td>3</td>
<td>0.1 mg/L</td>
<td>8</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>4</td>
<td>&gt;3</td>
<td>0.5 mg/L</td>
<td>8</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>4</td>
<td>0.87</td>
<td>1.0 mg/L</td>
<td>8</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>4</td>
<td>0.9</td>
<td>2.0 mg/L</td>
<td>8</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>2.7</td>
<td>0.02</td>
<td>0.25 mg/L</td>
<td>7.2</td>
<td>20</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Human rotavirus</td>
<td>8-9 log10 TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1</td>
<td>25 ug/mL</td>
<td>7</td>
<td>25</td>
<td>water</td>
</tr>
<tr>
<td>Human rotavirus</td>
<td>0.7</td>
<td>10</td>
<td>0.31 mg/L</td>
<td>7.2</td>
<td>20</td>
<td>sludge effluent</td>
</tr>
<tr>
<td>Human rotavirus type 2</td>
<td>5</td>
<td>1</td>
<td>0.5 mg/L</td>
<td>6</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Human rotavirus type 2</td>
<td>5</td>
<td>1</td>
<td>0.5 mg/L</td>
<td>7</td>
<td>4</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Murine norovirus (MMV-1)</td>
<td>&gt;2</td>
<td>2</td>
<td>1.0 mg/L</td>
<td>5.6</td>
<td>4</td>
<td>water</td>
</tr>
<tr>
<td>Murine norovirus (MMV-1)</td>
<td>&gt;2</td>
<td>2</td>
<td>1.0 mg/L</td>
<td>5.6</td>
<td>20</td>
<td>water</td>
</tr>
<tr>
<td>Murine norovirus (MMV-1)</td>
<td>&gt;2</td>
<td>2</td>
<td>1.0 mg/L</td>
<td>7</td>
<td>4</td>
<td>water</td>
</tr>
<tr>
<td>Murine norovirus (MMV-1)</td>
<td>&gt;2</td>
<td>2</td>
<td>1.0 mg/L</td>
<td>7</td>
<td>20</td>
<td>water</td>
</tr>
<tr>
<td>Murine norovirus (MMV-1)</td>
<td>4.1</td>
<td>10</td>
<td>72 mg/L</td>
<td>7</td>
<td>22</td>
<td>cell culture media</td>
</tr>
</tbody>
</table>

(Continued)
(Table 3 Continued)

<table>
<thead>
<tr>
<th>Virus/Species</th>
<th>C</th>
<th>T</th>
<th>Concentration (mg/L)</th>
<th>pH</th>
<th>Cell Type/Media</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine norovirus (MNV-1)</td>
<td>4.4</td>
<td>20</td>
<td>71.4</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Murine norovirus (MNV-1)</td>
<td>6.7</td>
<td>30</td>
<td>65.1</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Murine norovirus (MNV-1)</td>
<td>9</td>
<td>40</td>
<td>53.5</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Norwalk virus</td>
<td>&gt;3.0</td>
<td>5</td>
<td>0.37</td>
<td>7</td>
<td>water</td>
<td>Shin and Sobsey, 2003</td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>3</td>
<td>5</td>
<td>0.37</td>
<td>7</td>
<td>water</td>
<td>Shin and Sobsey, 2003</td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>2</td>
<td>10</td>
<td>0.2</td>
<td>7.2</td>
<td>activated sludge effluent</td>
<td>Harakeh et al., 1985</td>
</tr>
<tr>
<td>Poliovirus 3</td>
<td>1.63</td>
<td>4</td>
<td>0.60</td>
<td>6.9</td>
<td>phosphate buffer</td>
<td>Finch and Fairbairn, 1991</td>
</tr>
<tr>
<td>Poliovirus 3</td>
<td>3.52</td>
<td>1</td>
<td>1.76</td>
<td>6.9</td>
<td>phosphate buffer</td>
<td>Finch and Fairbairn, 1991</td>
</tr>
<tr>
<td>Tulane virus (TV)</td>
<td>0.5</td>
<td>10</td>
<td>72</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Tulane virus (TV)</td>
<td>1.1</td>
<td>20</td>
<td>71.4</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Tulane virus (TV)</td>
<td>2.8</td>
<td>30</td>
<td>65.1</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
<tr>
<td>Tulane virus (TV)</td>
<td>4.4</td>
<td>40</td>
<td>53.5</td>
<td>7</td>
<td>cell culture media</td>
<td>Predmore et al., 2015</td>
</tr>
</tbody>
</table>

1.5.2.3. Ozone use in the food industry

Ozone has been the primary method for water disinfection in Europe for many years. Other uses for ozone in the food industry include disinfection of bottled water and wastewater treatment. In 1982, the FDA granted generally recognized as safe (GRAS) status for the use of ozone in bottled water disinfection (FDA 21 CFR 173.368). A GRAS status classification by the FDA indicates that the substance has been adequately proven to be safe under the conditions of its intended use (FDA, 2014). Later in 1997, the USDA approved the use of ozone for use in reconditioning recycled poultry chilling water (USDA, FSIS Directive 7120.1). Also in 1997, an expert panel assembled by the Electric Power Research Institute (EPRI) self-affirmed ozone was a GRAS substance for use as a
disinfectant or sanitizer in foods. Because there was no objection to this affirmation by
the panel, the USDA approved the use of ozone as a disinfectant or sanitizer in foods and
food processing in the United States. Today, ozone may be further safely used as an
antimicrobial agent, or in the treatment storage, and processing of foods (FDA 21 CFR
173.368).

Ozone is an attractive disinfectant or sanitizer for the food industry because it is
environmentally friendly and decomposes to elemental oxygen, leaving behind no
residues and minimal toxic byproducts. For this reason, it is being investigated as an
alternative to chlorine in many aspects of industry where subsequent human exposure is
likely (Rice et al., 2002). Chlorine may effectively reduce foodborne pathogens both
water and foods, and it has been the preferred disinfectant of the food industry for many
years, but one major drawback of chlorine is its tendency to combine with organic
compounds to form carcinogenic byproducts. These toxic byproducts include
trihalomethanes (THMs) and haloacetic acid (HAA), which are both mutagens and
carcinogens (Trussel and Umprhes, 1978). Not only are they harmful for drinking, but
also are toxic to aquatic life and not desired in the environment. Chlorine is highly
corrosive to food processing equipment. The benefits of chlorine as a disinfectant in
terms of food and water safety still outweigh the risk of chlorine reaction products,
allowing the use of chlorine to continue. The discovery of new sanitizers and
disinfectants that are not harmful to the environment or to humans, and are effective
against foodborne pathogens is still desperately needed.
While ozone has been mainly used in the disinfection of water sources in the United States, much research has been done to evaluate its other potential uses. In foods, the ozone demand will differ depending on the organic constituents present which will compete with microorganisms for the ozone. For example, a 100 gram portion of meat would require a higher dosage of ozone to inactivate microorganisms than 100 grams of fruit or vegetables (Kim et al., 2003).

Ozone has been used in fruits and vegetables both to increase shelf life and as a strong antimicrobial agent to reduce microbial load (Karaca and Velioglu, 2007; Kim et al., 1999). Researchers have reported that ozone would be useful in enhancing the nutritional properties of pineapple and bananas, but that ozone would cause negative effects on the properties of guava fruit (Alothman et al., 2010). They suggested in their study that ozone could have a promising future for use in the minimally processed fruit industry to enhance the antioxidant content of these fruits. Ozone was reported to improve the quality and extend storage life of broccoli, cucumbers, and blackberries (Barth et al., 1995; Skog and Chu, 2001), and was reported to give more reduction in microbial load than water washing alone for red peppers, strawberries, and watercress (Alexandre et al., 2011). In fruits and vegetables, the future use of ozone is very promising, but the higher concentrations of ozone which may be used in processing may be self-limiting by deleterious effects on physiology and sensory quality of the produce itself (Karaca and Velioglu, 2007).

Ozone has also been investigated for use in meats, eggs, fish, and other dry foods (Fan et al., 2007). In 1997, ozone was approved for the use in reconditioned poultry
chilling water by the USDA (Guzel-Seydiem et al., 2004). There has been research investigating the effects of ozone as an antimicrobial agent for poultry carcass disinfection, extension of poultry shelf life, and both the disinfection and preservation of meats (Chen et al., 1992; Fabrizio et al., 2002; Sheldon and Brown, 1986; Vadhanasin et al., 2004; Yang and Chen, 1979). There is still much research remaining to validate and optimize ozone applications for a wide variety of foods.

1.5.3. Pulsed electric field

1.5.3.1. General characteristics, system design

Pulsed electric fields (PEFs) are another non-thermal process that can be used to inactivate foodborne pathogens in foods and beverages. Pulsed electric field processing is conducted by passing short high voltage electrical pulses through a fluid. These short electrical pulses may range anywhere between microseconds up to milliseconds, and the food may be processed at either ambient or refrigerated temperatures. The pulse process typically occurs in milliseconds, but the localized heating of the food from this short pulse is significant and should be measured or controlled.

In order to be successful, food preservation requires the inactivation of not only spoilage microorganisms and pathogenic microorganisms, but also enzymes that catalyze unwanted chemical degradation in foods limiting their shelf life. PEF has the advantage of being able to inactivate both enzymes and microorganisms (Sale and Hamilton, 1967). To understand how pulsed electric fields work we describe here the design of a normal PEF system and its components.
A PEF processing system consists of a power source, capacitor bank, switch, treatment chamber, thermal control, and measurement of voltage, current, flow and temperature. The power source charges the capacitors and the switch discharges energy from the capacitors across the fluid in the treatment chamber. Originally when PEFs were first tested, static chambers were used, but with Ohio State University patents, both continuous and coaxial chambers have advanced the design enabling solid state instead of vacuum tube electronic components. Continuous chambers are generally preferred for industrial applications. The voltage, current, and electric strength may all be varied and controlled and visualized on an oscilloscope. A diagram of the general setup of the PEF machine used in this dissertation is shown below.

![Diagram of PEF machine setup](image)

Figure 4. General set-up of PEF machine, Howlett Hall 062, The Ohio State University (Charles-Rodriguez et al., 2007).
There are three basic pulse types for PEFs—square, sinusoid, and exponential. Each of these different pulse shapes exhibit different efficiencies and different results. Square wave forms have been reported to be the most efficient wave shape at an efficiency of about 80% (Kempkes, 2014). In addition to pulse shape, PEF’s efficiency is based on other critical process factors such as field strength, treatment time, temperature, and type of microorganism the treatment is used for (Mohamed and Eissa, 2012).

1.5.3.2. Effectiveness of PEF against viruses, proposed theories of microbial inactivation

The use of PEF to inactivate or control various microorganisms has been evaluated. Many of these studies are limited to investigation of PEF’s effects on bacteria. Sale and Hamilton (1967) were the first to conduct a large study of the effects of PEFs on the inactivation of microorganisms. Two major findings were that electric field strength and treatment time were the two most important factors affecting microbial inactivation. Treatment time itself is a product of the number of pulses and pulse width. Inactivation of microorganisms or enzymes by PEF occurs when threshold electric field intensity is exceeded. Sale and Hamilton (1967) found that treatment with an applied electric field in excess of 1V were able to cause lysis of the cell membrane.

Based on a similar theory called the “dielectric rupture theory,” proposed by Zimmerman (1986), the external electric field will induce a change in transmembrane potential (TMP) across the cell membrane of a target organism. When this TMP reaches a
threshold value, pore formation or electroporation of the cell membrane may occur. This will in turn induce an increase in cell membrane permeability, which can be lethal to the cell. It may be hypothesized that the mechanism would be somewhat similar in viruses, where PEF may affect the outermost protein capsid of the virus, allowing its genetic material to escape the cell making it non-infectious.

Table 5. Summary of existing data on the inactivation of foodborne viruses by PEFs

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inactivation (log10)</th>
<th>Pulse frequency (Hz)</th>
<th>Pulse duration (μsec)</th>
<th>Field strength kV/cm</th>
<th>Total duration of pulses (μsec)</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>human rotavirus</td>
<td>0 log10 TCID50</td>
<td>1000</td>
<td>3</td>
<td>20</td>
<td>145.6</td>
<td>cell culture media</td>
<td>Khadre &amp; Yousef, 2002</td>
</tr>
<tr>
<td>human rotavirus</td>
<td>0 log10 TCID50</td>
<td>1000</td>
<td>3</td>
<td>20</td>
<td>145.6</td>
<td>cell culture media</td>
<td>Khadre and Yousef, 2002</td>
</tr>
<tr>
<td>human rotavirus</td>
<td>1 log10 TCID50</td>
<td>1000</td>
<td>3</td>
<td>25</td>
<td>145.6</td>
<td>cell culture media</td>
<td>Khadre and Yousef, 2002</td>
</tr>
<tr>
<td>human rotavirus</td>
<td>0 log10 TCID50</td>
<td>1000</td>
<td>3</td>
<td>29</td>
<td>145.6</td>
<td>cell culture media</td>
<td>Khadre and Yousef, 2002</td>
</tr>
<tr>
<td>murine norovirus</td>
<td>0.3</td>
<td>2000</td>
<td>2.2</td>
<td>30</td>
<td>94</td>
<td>cell culture media</td>
<td>Predmore et al., unpub.</td>
</tr>
<tr>
<td>murine norovirus</td>
<td>1.2</td>
<td>2000</td>
<td>2.2</td>
<td>30</td>
<td>94</td>
<td>cell culture media</td>
<td>Predmore et al., unpub.</td>
</tr>
<tr>
<td>Tulane virus</td>
<td>0.3</td>
<td>2000</td>
<td>3</td>
<td>30</td>
<td>94</td>
<td>cell culture media</td>
<td>Predmore et al., unpub.</td>
</tr>
<tr>
<td>Tulane virus</td>
<td>2.4</td>
<td>2000</td>
<td>3</td>
<td>30</td>
<td>94</td>
<td>cell culture media</td>
<td>Predmore et al., unpub.</td>
</tr>
</tbody>
</table>
1.5.3.3. Use of PEF in the food industry

Pulsed electric field may be one of the most promising of the non-thermal technologies discussed thus far. PEFs are used in the industry mainly to improve the quality or shelf life of foods including milk, orange juices, liquid eggs, and apple juices (FDA, 2011). The successful application of PEF technology has been demonstrated for all of these products. PEF processing is limited to food products that are free of air bubbles, have a low electrical conductivity, and have a particle size small enough that it is smaller than the gap of the treatment region. PEF is a continuous processing method so it is only applicable for foods that are pump-able (Mohamed and Eissa, 2012).

The first commercial PEF application in the U.S. was installed in 2005 for fruit juice preservation (Clark, 2006). Genesis Juice Corporation used PEF to process their raw juices until they received a warning letter from the FDA. After treatment by PEF, they achieved a 4 week shelf life under refrigeration temperatures. Investigation of other uses for PEF treatment of food products is ongoing, as there were around twenty facilities around the world working on PEF in 1999, and perhaps that number has grown in the past 15 years due to the promise that this non-thermal technology shows for several applications in the food industry (Barbosa-Canovas et al., 1999).

1.5.4. Sanitizers/surfactants

1.5.4.1. General Characteristics

Sanitizers play an important role in food processing. Many federal agencies recognize the importance of controlling foodborne viruses and enhancing public health,
but still many of the research studies published involving food safety focus on bacterial contamination with little focus given to the most common cause of foodborne illness: viruses. The survival and inactivation of foodborne viruses in fresh fruits and vegetables, for example, is still not fully understood.

Chlorine is the most commonly used sanitizer for fresh foods, including leafy greens, that is well studied and reported. Elemental chlorine (Cl) does not exist in a free state on earth, but exists as salts of sodium, potassium, calcium or magnesium (Dychdala, 2001). Gaseous chlorine may be generated by passing electrical current through salt solution by the chemical reaction below (Eifert and Sanglay, 2002):

\[ \text{NaCl} + 2 \text{H}_2\text{O} + \text{electricity} \rightarrow \text{Cl}_2 + 2 \text{NaOH} + \text{H}_2 \]

The resulting products are sodium hydroxide, hydrogen gas, and chlorine gas. The gaseous chlorine can be dried, chilled, pressured, or converted into liquid (Eifert and Sanglay, 2002). If this gaseous or liquid Cl\(_2\) is added to water, the following reactions will occur:

\[ \text{Cl}_2 + \text{H}_2\text{O} \rightarrow \text{HOCl} + \text{HCl} \]
\[ \text{HOCl} \leftrightarrow \text{H}^+ + \text{OCl}^- \]

This chlorine becomes hydrolyzed by the water molecule to form hypochlorous acid and hydrochloric acid (Marriot and Gravani, 2006; Gerba, 2009). Furthermore, the hypochlorous acid (HOCl), the active antimicrobial form of chlorine, will dissociate in water to form a hypochlorite ion and a free hydrogen ion. At pH of 7 or below, the concentration of HOCl is typically greater than that of the hypochlorite ion (OCl\(^-\)), and at
pH higher than 7, the opposite is true (Gerba, 2009). HOCl is considered the most effective of the chlorine compounds. The presence of these chlorine compounds in water in the absence of nitrogenous compounds is referred to as the free available chlorine (Dychdala, 2001; Gerba, 2009). When HOCl combines with ammonia or other organic compounds to form monochloramines, dichloramines, or trichloramines, this is called combined chlorine. These secondary formed chloramines do have some disinfection power, but not greater than HOCl (Gerba, 2009).

Chlorine is listed on the FDA Approved Food Additives List and is permissible at concentrations of 200 parts per million (ppm) or less for sanitization purposes (FDA 21 CFR 187.1010). While chlorine shows significant reduction in bacterial pathogens, its effectiveness against viral pathogens such as norovirus is limited. Chlorine typically gives only a moderate reduction of about 1-1.2 logs in fresh produce (Baert et al., 2009; Dawson et al., 2005; Predmore et al., 2011). While chlorine has variable effects on foodborne viruses, it is highly effective against bacteria, fungi, and yeasts (Marriot and Gravani, 2006). It can be used in a variety of industries and situations, such as for drinking and wastewater, recreational water, fresh produce washing, and poultry chilling (Dychdala, 2001). Chlorine residue does not have to be rinsed off at concentrations lower than 200 ppm, and it is an inexpensive, readily available sanitizer (Marriot and Gravani, 2006). Chlorine has some notable drawbacks to its use. Chlorine is highly corrosive to stainless steel and other metals, it is irritating to skin and mucous membranes of those using it, its effectiveness is greatly diminished by high amounts of organic matter, and it is less efficient at higher pHs. Also, the most important drawback to using chlorine is the
fact that it forms potentially carcinogenic trihalomethane (THM) compounds and harmful disinfection byproducts (DBPs). These THMs are formed when chlorine reacts with several organic compounds (Dychdala, 2001; Marriot and Gravani, 2006).

Still, chlorine is widely used in the food industry, because its benefits further outweigh the drawbacks. These issues have led to the investigation of many other types of novel sanitizers, surfactants, and acids for use in the food industry.

Surfactants are amphipathic compounds that have a variety of uses in our everyday lives. That is, they have both a hydrophobic and hydrophilic present to interact with the substance they are in contact with by affect the interfacial free energy of the interface. This interfacial free energy is defined as the amount of work it takes to create that interface. The interfacial or surface tension is also a measure of the difference in nature of two phases meeting at the interface. Basically, the greater the dissimilarity of the natures will induce a greater surface tension between the two. Surfactants are usually used to lower the surface tension between two things, but there are some instances where they can be used to increase it (Rosen and Kunjappu, 2012).

Based on their chemical structure, surfactants may be classified as either ionic or non-ionic, and the ionic surfactants may either be anionic or cationic. For this dissertation, the surfactant that was chosen for experiments was sodium dodecyl sulfate (SDS). SDS is an anionic surfactant which is considered an FDA approved food additive as a means of sanitizing food processing equipment (FDA 21 CFR 187.1010), but its use for the direct processing of food has not yet been established. SDS has been widely used
in products such as toothpastes, cosmetics, and shampoos and soaps for detergent activity (Ostroumuv, 2006).

Levulinic acid, or 4-oxypenanoic acid, is an organic acid that has recently been used in combination with SDS in attempts to inactivate pathogens on both food contact surfaces and in foods (Zhao et al., 2009a, Zhao et al., 2009b; Zhao et al., 2011a; Zhao et al., 2011b; Aydin et al., 2013; Cannon et al., 2012). Most of these studies focused on bacterial pathogens, so the effects of levulinic on foodborne viruses are still not well understood.

1.5.4.2. Viral inactivation and mechanism of inactivation by surfactants and sanitizers

It has been reported that surfactants may interact with viral proteins. The interaction of surfactants and viruses may cause protein folding, refolding, denaturation, or aggregation. Viruses may also have an effect on the viral envelope of enveloped viruses (Hartman et al., 2006; Howett et al., 2009; Koopmans and Duzier, 2004; Banat et al., 2000). Previous research reports that surfactants are able to successfully inactivate various enveloped viruses (Howett et al., 2009; Howett and Kuhl, 2005; Song et al., 2010). However, the effects of surfactants on foodborne viruses, which are non-enveloped viruses, are less understood. Published data for the inactivation of foodborne viruses by surfactants is shown in Table 6.
Table 6. Summary of previous research involving the effects of chlorine, sodium dodecyl sulfate, and levulinic acid on foodborne viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inactivation (log10)</th>
<th>Sanitizer</th>
<th>Concentration/Conditions</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNV</td>
<td>-0.13</td>
<td>SDS</td>
<td>0.05%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>0.13</td>
<td>SDS</td>
<td>0.5%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>0.03</td>
<td>SDS</td>
<td>1%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>-0.23</td>
<td>SDS</td>
<td>2%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>-0.12</td>
<td>SDS</td>
<td>0.05%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>-0.09</td>
<td>SDS</td>
<td>0.5%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>-0.05</td>
<td>SDS</td>
<td>1%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>-0.05</td>
<td>SDS</td>
<td>2%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>0.09</td>
<td>levulinic acid</td>
<td>0.05%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>-0.04</td>
<td>levulinic acid</td>
<td>0.5%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>-0.06</td>
<td>levulinic acid</td>
<td>1%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>-0.09</td>
<td>levulinic acid</td>
<td>2%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>0.09</td>
<td>levulinic acid</td>
<td>0.05%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>0.32</td>
<td>levulinic acid</td>
<td>0.5%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>0.51</td>
<td>levulinic acid</td>
<td>1%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>0.43</td>
<td>levulinic acid</td>
<td>2%, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>1.02</td>
<td>SDS/levulinic acid</td>
<td>0.5% LVA + 0.05% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>3.04</td>
<td>SDS/levulinic acid</td>
<td>0.5% LVA + 0.5% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MNV</td>
<td>4.21</td>
<td>SDS/levulinic acid</td>
<td>2% LVA + 1% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>3.04</td>
<td>SDS/levulinic acid</td>
<td>0.5% LVA + 0.05% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>3.04</td>
<td>SDS/levulinic acid</td>
<td>0.5% LVA + 0.5% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>FCV</td>
<td>3.04</td>
<td>SDS/levulinic acid</td>
<td>2% LVA + 1% SDS, 1 min</td>
<td>virus stock</td>
<td>Cannon et al., 2012</td>
</tr>
<tr>
<td>MS2</td>
<td>1.8</td>
<td>SDS/acetic acid/thymol</td>
<td>0.2 mg/ml THY + 5% SDS + 2 mg/ml AA</td>
<td>grape tomatoes</td>
<td>Lu, 2011</td>
</tr>
<tr>
<td>Influenza A H3N2</td>
<td>1.73</td>
<td>SDS/levulinic acid</td>
<td>0.5% LVA + 0.5% SDS, 1 min</td>
<td>eggshells</td>
<td>Aydin et al., 2013</td>
</tr>
</tbody>
</table>

(Continued)
Chlorine’s effect against microorganisms has been studied extensively, but the mechanism of inactivation remains unclear. It is suspected that chlorine either interacts with the virus’s genetic material or the capsid proteins (Gerba, 2009; Wigginton and
Kohn, 2012). One study by O’Brien and Newman (1979) investigated the mechanism of inactivation of poliovirus by chlorine, by evaluating the sedimentation characteristics of the virus before and after treatment. They found that the sedimentation coefficient of chlorine-treated poliovirus was similar to that of a virus with empty capsid. Therefore, they hypothesized that the mechanism of inactivation was by the leakage of the viral RNA out of the virus. They further investigated whether this RNA release was the primary action, or if there was a primary event before this such as capsid deformation. They found that the decrease in infectivity was not correlated with a loss of RNA, showing that the release of RNA was not the primary event of inactivation. In another study done by Li et al., (2002), the researchers found that for Hepatitis A virus inactivation by chlorine that different regions of the nucleic acid showed different levels of resistance to chlorine. Coding regions were more resistant than non-translating regions. In addition, they found that the antigenicity of HAV disappeared after loss of infectivity, suggesting that the chlorine’s first target was the genetic material rather than the capsid proteins (Li et al., 2002). Both of these studies gave good insight into the mechanism of inactivation as we know that chlorine can affect the nucleic acid of a virus, but the details of this interaction are still unclear.

The investigation of levulinic acid for viral inactivation is still a very new concept, and the mechanism of inactivation of foodborne viruses by levulinic acid is yet to be discovered. Cannon et al. (2012) reported that the reaction of levulinic acid and viral pathogens was pH dependent. The lower the pH, the higher the amount of
inactivation of the virus was achieved. The authors also suspected that the genome was not the primary target of action (Cannon et al., 2012).

1.5.4.3. Use of surfactants and sanitizers in the food industry

Chlorine has many uses in the food industry. It is recognized as an indirect additive by the U.S. FDA and its use is permitted as a no-rinse sanitizer for food contact surfaces (FDA 21 CFR 178.1010). It is predominantly used for treatment and disinfection of drinking water and wastewater in the United States (Vaughn and Novotny, 1991). Drinking water has a longstanding association with viral contamination and infections. A study by Grabow et al. (2001) found water treated with chlorine was contaminated 23% of the time by viruses, compared to untreated water which is contaminated with viruses 73% of the time. In the fresh produce industry, chlorine is used as either a spray or as an additive to wash water to prevent cross-contamination. Typical concentrations of chlorine for these uses range from 50-200 ppm (Hirneisen et al., 2010). As mentioned previously, some of the disadvantages of chlorine use include the formation of harmful chlorine byproducts, and the effect of organic matter on the effectiveness of chlorine. Chlorine is also corrosive to processing equipment (Hirneisen et al., 2010). The benefits of chlorine use far outweigh the drawbacks, so its use is widespread in the industry.

Surfactants such as SDS are approved by the U.S. FDA for a variety of uses as a food additive. SDS is permitted for use as an emulsifier in egg white solids, frozen egg whites, and liquid egg whites, as a whipping agent for marshmallows, as a surfactant for beverage bases and fruit juice, and as a wetting agent for vegetables oils and animal fats
(FDA 21 CFR 172.822). SDS is also considered an indirect additive for foods by the FDA, where it may be used as a component for food coatings (FDA 21 CFR 175.320). The use of SDS specifically for the removal or inactivation of foodborne viruses such as norovirus has not yet been approved.

Levulinic acid is classified as a food additive by the U.S. FDA. It may be directed added to foods as a synthetic flavoring or adjuvant susbstance (FDA 21 CFR 172.515). It has several other industrial uses such as a resin, plasticizer, textile, animal feed, coating, and even in antifreeze (Ghorpade, 1997).

Sections (from to 1.5.1-1.5.4) covered the current knowledge on foodborne viruses and various novel and emerging non-thermal technologies used to inactivate those viruses. In the subsequent chapters, the use of gaseous ozone, e-beam irradiation, surfactants, and pulsed electric fields and their effects on two human norovirus surrogates, murine norovirus (MNV-1) and Tulane virus (TV), are discussed.
Chapter 2. Electron Beam Inactivation of Tulane virus on Fresh Produce, 
and Mechanism of Inactivation of Human Norovirus Surrogates by Electron Beam 
Irradiation

2.1 Abstract

Ionizing radiation, whether by electron beams or gamma rays, is a non-thermal processing technique used to improve the microbial safety and shelf-life of many different food products. This technology is highly effective against bacterial pathogens, but data on its effect against foodborne viruses are limited. A mechanism of viral inactivation has been proposed with gamma irradiation, but no published study discloses a mechanism for electron beam (e-beam). This study had three distinct goals: 1) evaluate the sensitivity of a human norovirus surrogate, Tulane virus (TV), to e-beam irradiation in foods, 2) compare the difference in sensitivity of TV and murine norovirus (MNV-1) to e-beam irradiation, and 3) determine the mechanism of inactivation of these two viruses by e-beam irradiation. TV was reduced from 7 log10 units to undetectable levels at target doses of 16 kGy or higher in two food matrices (strawberries and lettuce). MNV-1 was more resistant to e-beam treatment than TV. At target doses of 4 kGy, e-beam provided a 1.6 and 1.2 log reduction of MNV-1 in phosphate buffered saline (PBS) and Dulbecco’s Modified Eagle Medium (DMEM), compared to a 1.5 and 1.8 log reduction of TV in PBS and Opti-MEM, respectively. Transmission electron microscopy revealed
increased e-beam doses negatively affected the structure of both viruses. Analysis of viral proteins by SDS-PAGE found that irradiation also degraded viral proteins. Using RT-PCR, irradiation was shown to degrade viral genomic RNA. This suggests the mechanism of inactivation of accelerated electrons by e-beam was likely the same as radiolytic gamma irradiation as the damage to viral constituents led to inactivation.

2.2. Introduction

Foodborne viruses remain important pathogens in the realm of food safety, and of this group, human norovirus is the most prevalent. In the United States alone, norovirus is the leading cause of acute gastroenteritis, causing an estimated 1 in 15 people to become ill each year. Between 2009 and 2012, 4,318 norovirus outbreaks were reported, resulting in 161,253 illnesses (Hall et al., 2014). Noroviruses are also highly infectious. A study by Atmar et al. (2014), suggested the 50% human infectious norovirus dose is much higher than previously thought. The researchers found that the HID50 was approximately 1320 genomic equivalents (gEq) for people who were either blood type A or O and that were secretor positive. No subjects who were blood type B became infected with the given doses of norovirus, so if these blood group B patients were included in the calculation, the HID50 would increase to 28,000 gEq needed to cause norovirus infection. This is a much higher dose than initially demonstrated by Teunis et al. (2008), but the difference was likely due to differences in the statistical approach used to generate the estimates, rather than actual biological differences (Kirby et al., 2014). Norovirus is transmitted via the fecal oral route and the incubation period for the virus is typically 1-3 days, but
symptoms may develop in as quickly as 12-24 hours (Bresee et al., 2002; Koopmans et al., 2002). Common symptoms of norovirus gastroenteritis can include projectile vomiting, non-bloody diarrhea, low-grade fever, and nausea (Greening, 2006; Koopmans et al., 2002). High titers of virus can be shed in feces, with up to 108 viral particles per gram of stool (Koopmans and Duizer, 2004). The illness is acute, self-limiting, and generally lasts for 12-60 hours, but fecal shedding of the virus can last for days or even weeks after the illness subsides which can lead to further transmission of the disease (Bresee et al., 2002; Greening, 2006). One study found that during a norovirus challenge, virus shedding by RT-PCR 18 hours after initial inoculation, and continued between 13 and 56 days (Atmar et al., 2008).

The most common route of transmission of norovirus is the fecal-oral route. This can occur via the consumption of foods or liquids contaminated with norovirus, or by coming into contact with any surface or object contaminated with norovirus and then ingesting viral particles (CDC, 1999; Koopmans et al., 2002). The virus can also be transmitted via direct contact with an infected person, such as a food handler that did not practice proper hygiene, sharing food utensils with an infected person, or by aerosols generated by vomiting or diarrhea (Greening, 2006; Koopmans et al., 2002; Seymour and Appleton, 2001). Infection tends to occur in socially dense, enclosed environments such as cruise ships, schools, daycare centers, nursing homes, restaurants, military barracks and hospitals (CDC, 2009; Wobus et al., 2006). With regard to food, the virus is most commonly associated with shellfish, fresh produce, water and ice, ready-to-eat deli meats, sandwiches, and baked products (Greening, 2006).
One major issue that hinders human norovirus research is that human noroviruses are currently non-cultivable in the laboratory, therefore closely related surrogate viruses are used to give insight on how susceptible or resistant the virus is to treatments such as irradiation. In the past, the primary surrogates used have been feline calicivirus (FCV) and murine norovirus (MNV-1). Recently, an attractive new surrogate, Tulane virus (TV), has been identified. Tulane virus was isolated from the stools of rhesus macaques at Tulane National Primate Research Center in New Orleans, Louisiana in 2008 (Farkas et al., 2008). The virus may serve as an improved surrogate for future norovirus research because it is genetically similar to human norovirus, it uses histo-blood group antigens as receptors, and causes diarrhea in its host, all similar to human norovirus (Farkas et al., 2008; Farkas et al., 2010). No other surrogate thus far has all of these characteristics, making TV an extremely useful surrogate if studies suggest that its resistance to various technologies is greater than MNV-1.

Food irradiation was proposed over a century ago, but it has renewed interest as an emerging technology for preservation and microbial inactivation in foods. The three types of irradiation include electron beam, gamma irradiation, and x-ray. E-beam irradiation is machine-accelerated electrons. It can be shut off when not in use, the beam can be directed toward the food allowing for a more controlled application, and treatment times are generally shorter than gamma irradiation (Barbosa-Canovas et al., 1998; Diehl, 1995; Fellows, 2000; Hansen and Shaffer, 2001; Sanglay et al., 2011). This is because dose rate of e-beam (103-105 Grays\(^{-1}\)) may be much higher than gamma (0.1-1 Grays\(^{-1}\)) (Tahergorabi et al., 2014). However, a disadvantage of e-beam is a limited penetration
depth of 3 to 10 cm (Barbosa-Canovas et al., 1998; Diehl, 1995). There is no literature demonstrating the e-beam mechanism of viral inactivation, although Feng et al. (2011) reported similarity to gamma irradiation.

Few studies have examined the efficacy of gamma irradiation or e-beam irradiation on norovirus surrogates and human norovirus virus like particles. Feng et al. (2011) demonstrated only a 1.7 to 2.4 log reduction of human norovirus surrogate MNV-1 in different types of fresh produce treated with 5.6 kGy of gamma irradiation. The researchers also demonstrated that irradiation can disrupt the MNV-1 virion structure, degrade the viral proteins, and degrade genomic RNA. Sanglay et al. (2011) observed that an e-beam dose of 4 kGy, the current maximum allowed for fresh iceberg lettuce and spinach by the FDA, provided a 1 log or less reduction in cabbage and strawberries, respectively (FDA, 2013). They concluded the food matrix may have provided a protective effect for the virus against irradiation. Zhou et al. (2011) tested e-beam irradiation and found the D10 value of FCV inoculated into lettuce was 2.95 kGy. No studies have investigated the effects of e-beam irradiation on TV as a new surrogate for human norovirus. In addition, only a handful of studies have investigated the inactivation of TV or compared the differences between MNV-1 and TV directly (Hirneisen and Kniel, 2013a; Hirneisen and Kniel, 2013b; Li et al., 2013; Tian et al., 2013; Wang et al., 2013).

Tian et al. (2013) investigated the resistance of TV to many physical and environmental conditions, and they found that the maximum reduction in viral titer was a 5 log reduction for heat denaturation and alcohol experiments, and a 4 log reduction for
UV, chlorine, and pH stability experiments. Hirneisen and Kniel (2013a; 2013b) completed two studies that compared TV and MNV-1 directly. One study evaluated the stability of the virus surrogates under varied treatments of heat, pH, chlorine, and survival in tap water at room temperature and refrigeration temperature for up to 30 days (Hirneisen and Kniel, 2013b), while the other study compared the survival of human norovirus, TV, and MNV-1 on spinach during preharvest growth during a 7 day period (Hirneisen and Kniel, 2013a). Wang et al. (2013) investigated the survival of TV, MNV-1, and hepatitis A virus (HAV) on alfalfa sprouts during storage and germination. Lastly, Li et al. (2013) compared the sensitivity of TV and MNV-1 to pH changes and high pressure processing (HPP). This study fills the knowledge gap of how e-beam irradiation affects with two different surrogates, MNV-1 and TV, and allows the direct comparison of sensitivities to irradiation.

The objectives were to: 1) evaluate the effectiveness of e-beam irradiation on inactivating TV in food matrices 2) compare the sensitivities of MNV-1 and TV to e-beam irradiation from 0 to 30 kGy in liquid solutions, and 3) determine the mechanism of viral inactivation of these viruses by e-beam using transmission electron microscopy (TEM), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and reverse transcriptase polymerase chain reaction (RT-PCR).

2.3 Materials and Methods

2.3.1. Cell culture and virus stock. Murine norovirus 1 (MNV-1) was kindly provided by Dr. Herbert W. Virgin IV (Washington University School of Medicine, St.
Louis, MO) MNV-1 was propagated in the RAW 264.7 cell line (mouse leukemic monocyte macrophage cell line; American Type Culture Collection, Manassas, VA). RAW 264.7 cells were cultured in 150 cm² tissue culture flasks (Corning Inc., Corning, NY) containing high-glucose Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Grand Island, NY), 25 mM HEPES buffer, and 2 mM Gluta-MAX-1. The growth medium was removed and cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. Flasks were incubated at 37°C, 5% CO2 for 1 h with agitation every 15 min. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO2 for 24 h. When extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles to lyse cells and release virus particles. The cell and virus suspensions were dispensed into 50 ml conical centrifuge tubes (USA Scientific, Ocala, FL) and were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter, Brea, CA) at 3,000 rpm for 20 min to remove any remaining cellular debris. The supernatant fluid was collected and stored at -80°C until ready for use.

TV was kindly provided by Dr. Xi Jiang (Cincinnati Children’s Hospital, Cincinnati, OH). TV was propagated in the LLC-MK2 cell line (ATCC, Manassas, VA). Cells were cultured in 150 cm² tissue culture flasks (Corning) containing Opti-MEM (minimum essential media) supplemented with 2% fetal bovine serum (Invitrogen) and 2mM GlutaMAX-1 (Invitrogen). The growth medium was removed from confluent flasks of LLC-MK2 cells and infected with TV at a MOI of 10. Flasks were incubated at 37°C
and 5% CO2 for 1h, with agitation every 15 min. Opti-MEM was added to the flasks and they were incubated at 37ºC and 5% CO2 for 48h. When CPEs were observed, flasks were subjected to freeze-thaw cycles, centrifugation, and re-suspension as described above. The titer of the virus stock was 106 PFU/mL.

**2.3.2. Sample preparation.** For liquid samples, 100 µl of MNV-1 or TV were inoculated into either 900 µl of 1X phosphate buffered saline (PBS; 0.85% NaCl, 0.12% Na2HPO4, 0.022% NaH2PO4, pH 7.4) or growth media (DMEM or Opti-MEM with no serum, respectively). Samples were placed into Bitran S-Series polyethylene and SARANEX specimen bags (5.1 × 10.2 cm, 3 mil; Com-Pac International, Carbondale, IL) and were heat sealed using an AIE-200 impulse sealer at setting 4 (American International Electric, Whittier, CA). For pH measurement, 2 ml of PBS or DMEM were placed in separate specimen bags and heat sealed. Samples were stored at 4°C and transported on ice.

For food samples, strawberries and romaine lettuce were purchased from a local grocery store. The hulls and leaves of the strawberries were removed, and were cut into pieces no larger than 20 × 20 mm. The lettuce was torn into pieces no larger than 20 by 20 cm. Ten grams of each food were placed into separate polyethylene bags as described above. Samples were inoculated with 1mL of thawed MNV-1 or TV virus stock to provide an approximate titer of 6 log10 PFU/mL or g in the liquid or food respectively. Pouches were heat sealed as described above. For the liquid samples, air was pushed out of the bags manually. Samples were stored at 4°C until e-beam treatment the next day.
For the mechanistic study (TEM, SDS-PAGE, and RT-PCR), 50 µl of purified MNV-1 or TV was added to 50 µl of PBS (1:1, v/v). These samples were placed into sample bags and heat sealed, and then stored on ice during transport.

**2.3.3. E-beam irradiation.** E-beam irradiation was carried out as described previously (Sanglay et al., 2011). Samples were transported on ice to the NEO Beam facility in Middlefield, OH. The facility uses a Dynamitron electron beam accelerator (Radiation Dynamics Inc., Edgewood NY). Electrons are magnetically scanned over a distance of 120 cm at a frequency of 100 Hz at the exit aperture of the accelerator. Samples were secured to cardboard sheets that were secured to a cart conveyor system (Figure 5). Samples were treated to the target doses 0, 4, 8, 16, 24, and 30 kGy under these following conditions: 4.5 MeV (beam energy) and 5 mA (beam current). The accelerator has a 27 cart exposure system (120 × 180 cm trays; SI Handling Systems, Easton, PA) with speeds ranging from 10.5 to 1.7 m/min to vary dose by varying exposure time (Table 7). Cart speed was calculated from $D = k \frac{I}{v}$, where $D$ is the target dose (kGy), $I$ is the beam current (mA), $v$ is the speed (m/min), and $k$ is a constant determined by dosimetry data, equal to $8.22 \text{kGy} \times \text{m/(min*mA)}$. The speed needed for each dosage and number of passes needed is included in Table 7, along with actual absorbed doses achieved for each target dose.
Figure 5: Fixed sample arrangement on a flat paperboard surface for exposure in the e-beam tunnel. Four alanine dosimeters were put into thin sleeves and were placed above and below sample bags (bottom left). An irreversible temperature sensor (bottom center) monitored temperature increase.

Table 7: Target e-beam dosages were achieved by varying conveyor speeds ($v = \text{m/min}$) and a second pass under the electron source.

<table>
<thead>
<tr>
<th>Target Dose (kGy)</th>
<th>Absorbed Dose (kGy) Liquid</th>
<th>Absorbed Dose (kGy) Strawberry</th>
<th>Absorbed Dose (kGy) Lettuce</th>
<th>Speed (m/min)</th>
<th>Number of Passes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4.8</td>
<td>3.9</td>
<td>4.1</td>
<td>10.5</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>8.6</td>
<td>8.2</td>
<td>8.7</td>
<td>5.2</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>16.9</td>
<td>16.3</td>
<td>17.2</td>
<td>2.6</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>26.5</td>
<td>25.2</td>
<td>26.1</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>32.7</td>
<td>30.7</td>
<td>30.6</td>
<td>2.7</td>
<td>2</td>
</tr>
</tbody>
</table>
To ensure samples received the desired dose, four BioMax alanine dosimeter films (Eastman Kodak Co., Rochester, NY) were placed on the top and bottom of the bags, as established in prior work (Sanglay et al., 2011). Preliminary experiments verified all areas of the sample received the same e-beam dose, within the uncertainty of the dosimeter (6%). After e-beam treatment, dosimeters were read using a Bruker e-scan electron spin spectrometer (Bruker BioSpin Corporation, Billerica, MA). The absorbed dose in each sample was calculated as the average of the dose obtained at the top and bottom of the samples. Single-use temperature indicators (GEX Corporation, Denver, CO) were placed on the cardboard sheets to correct for temperature effects on the response of the dosimeter.

After e-beam treatment, samples were repackaged into coolers with frozen ice packs and transported to The Ohio State University for microbial analysis. Samples were held at 4°C until testing.

2.3.4. Plaque assays. Sample bags were aseptically opened in a biosafety hood and were serially diluted using PBS blanks. Confluent monolayers of RAW 264.7 or LLC-MK2 cells were grown in 6-well CellBIND plates (Corning) containing DMEM plus 10% FBS or Opti-MEM plus 2% FBS for 24 h at 37°C, 5% CO2. The growth medium was removed, and 0.2 ml of each sample dilution was applied to the wells in duplicate. Plates were incubated for 1 h at 37°C, 5% CO2, with agitation every 15 min to evenly disperse the virus and to allow viral attachment to cells. Each well was overlaid with 2 ml MEM (Earle’s balanced salts; Invitrogen) supplemented with 5% FBS, 1.6%
sodium bicarbonate (7.5% w/v; Fisher), 0.5% penicillin-streptomycin (10,000 units of penicillin and 10,000 μg/ml of streptomycin in 0.85% saline; Invitrogen), 2.5% HEPES (Sigma-Aldrich, St. Louis, MO), 1% glutamine (Sigma-Aldrich), and 1.5% low melting point agarose (Invitrogen). Plates were refrigerated (4°C) for at least 30 min or until the MEM overlay was solidified. Plates were then incubated at 37°C, 5% CO2 for 48 h for MNV-1 and 72 h for TV.

After incubation, each well was fixed with 2 ml of 10% formaldehyde (Fisher Scientific) in PBS for at least 2 h. The formaldehyde and overlay was removed, and each well was stained with 0.05% (w/v) crystal violet for at least 1 h to visualize plaques.

2.3.5. Purification of MNV-1 and TV. Purification of MNV-1 was performed as described previously, with minor modifications (Katpally et al., 2008). To generate large stocks of purified MNV-1 or TV, 20 confluent flasks of RAW 264.7 or LLC-MK2 cells were infected with MNV-1 at an MOI of 0.01 or with TV at an MOI of 1, respectively. Flasks were incubated at 37°C, 5% CO2 for 1 h with agitation every 15 min. DMEM supplemented with 2% FBS, or Opti-MEM supplemented with 2% FBS was added to the flasks and incubated at 37°C, 5% CO2 for 48 h. When extensive CPEs were observed, flasks were subjected to three freeze-thaw cycles to lyse cells and release virus particles. The cell and virus suspensions were centrifuged using an Sorvall RC-5C Plus centrifuge (Kendro Lab Products, Newtown CT) with a Sorvall SS-34 rotor at 12,000 rpm for 10 min to completely remove cellular debris. The supernatant was collected and digested with DNase I (10 μg/ml; Roche Diagnostics, Indianapolis, IN) and MgCl2 (5 mM;
Sigma-Aldrich) at room temperature for 1 h. DNase activity was halted by addition of 1% lauryl sarcosine (Sigma-Aldrich) in 10 mM of EDTA (Sigma-Aldrich). The virus suspension was centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, Fullerton, CA), using a Ty 50.2 rotor, at 30,000 rpm for 6 h at 4°C. The supernatant was discarded, and the viral pellets were re-suspended in 200 µl of PBS overnight at 4°C on ice. Viruses were further purified by ultracentrifugation in a sucrose gradient (7.5 to 45%) in a SW55 Ti swinging bucket rotor (Beckman Coulter) at 41,000 rpm for 6 h at 4°C. The virus pellets were re-suspended in 100 µl of PBS overnight at 4°C on ice. The purified virus suspension was stored at -80°C.

To determine the virus titer of purified MNV-1 and TV, plaque assays were performed as described above. The titer of purified MNV-1 was $10.22 \pm 0.06$ log PFU/ml, and TV was $9.92 \pm 0.4$ log PFU/ml. Viral protein content was measured by the Bradford assay, and the protein concentrations were 1 mg/ml for purified MNV-1 and 0.2 mg/ml for purified TV.

2.3.6. Transmission electron microscopy (TEM). Untreated and treated e-beam samples (using purified virus) were analyzed using TEM to see if there was visual physical damage to the virus particles. Briefly, 20 µl aliquots of sample were fixed on copper grids (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 1% ammonium molybdate (Sigma-Aldrich). Fixed samples were analyzed using a FEI Tecnai G2 Spirit transmission electron microscope at 80 kV at the Microscopy and
Imaging Facility at The Ohio State University. TEM pictures were taken using a MegaView III side-mounted charge-coupled-device (CCD) camera.

2.3.7. Analysis of viral proteins by SDS-PAGE. 5 µl of untreated and e-beam treated purified virus suspensions were boiled in loading buffer (1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl [pH 6.8], and 5% glycerol) for 5 minutes. Samples were loaded onto a 12% polyacrylamide gel and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Viral proteins on the gel were visualized using Coomassie blue staining.

2.3.8. Reverse transcription PCR (RT-PCR). MNV-1 or TV genomic RNA, in both untreated and e-beam treated samples, were extracted using an RNeasy Minikit (Qiagen, Valencia, CA) using the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR kit (Qiagen, Valencia, CA). Two primers targeting the MNV-1 VP1 capsid gene and the TV VP1 capsid gene were used, and these primers are listed in Table 8. The 50µl RT-PCR reaction mixture consisted of 400 µM each deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer, 4 µl of RNA template, 5 units of RNase inhibitor, and 2 µl of RT-PCR enzyme mix. The amplified PCR products were analyzed using 1% agarose gel electrophoresis.
Table 8. Primer sets used for MNV-1 and TV RT-PCR (Feng et al., 2011).

<table>
<thead>
<tr>
<th>MNV-1 Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-ATGAGGATGAGTGATGGC-3’ (forward)</td>
</tr>
<tr>
<td>5’-TTATTGGATTTGAGCATTG-3’ (reverse)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TV Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-CATAGACATGGAAAACAG-3’ (forward)</td>
</tr>
<tr>
<td>5’-GCCAGCCATTATCTAAAGA-3’ (reverse)</td>
</tr>
</tbody>
</table>

2.3.9. **Color/Texture analysis.** Color of strawberry and lettuce samples was measured using a Hunter Spectrophotometer (Hunter Lab, Color Quest XE, Virginia, USA) with reflectance specular included (RSI), a 1 inch viewing, and a 60 degree observer angle. These CIELAB (L*a*b*) based measurements were done in triplicate. Texture profile analysis (TPA) of three strawberries from each respective treatment dosage was carried out using a TA.XT-PLUS Texture Analyzer (Stable Micro Systems Ltd., UK) fitted with a cylinder plunger SMS-P/10 CYL Delrin probe (10 mm diameter). Samples were compressed twice to a depth of 2 mm and time between strokes was 5 s. Pre-test, test, and post-test speeds were set at 5 mm/s, 1 mm/s and 8 mm/s, respectively. The texture parameters determined from the force–time profile included firmness (hardness), resilience, springiness, gumminess, and chewiness.

2.3.10. **Statistical analyses.** E-beam viral inactivation experiments were performed three times. Virus survival before and after e-beam treatment was reported as mean log plaque forming units (PFU) per milliliter + one standard deviation. Color and
texture experiments were also performed in triplicate. Data were analyzed using the General Linear Model function and Tukey’s pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

2.4. Results and Discussion

2.4.1. E-beam effect on TV in food matrices. The inactivation kinetics of TV after various doses of e-beam treatment is illustrated in Table 9. TV was reduced to non-detectable levels at 8.7 kGy and above in lettuce, and at 16.3 kGy and above in strawberries. This may have been due to differences in food matrix. The target goal of starting titer on the strawberries and lettuce was 6 log PFU/g, but the actual titer for these foods was 4.4 + 0.6 and 3.7 + 0.6 log PFU/g, respectively. The dosage currently allowed by the FDA is 1 kGy in fresh foods for the purpose of controlling insects or other arthropods, and to inhibit maturation of the food, but the dosage allowed specifically for fresh spinach and iceberg lettuce is set at 4 kGy (FDA, 2013). However, the high tolerance of foodborne viruses to these low dosages of ionizing radiation may warrant new government regulations with higher maximum doses for other types of fresh produce. At 3.9 kGy in strawberries, TV was reduced by 1.4 logs, and at 4.1 kGy in lettuce, there was a 1.3 log reduction. This data showed a much higher log reduction than what Sanglay et al. found using MNV-1. Sanglay et al. (2011) saw a 0.7 log reduction in cabbage and a 0.4 log reduction in strawberries at 4 kGy of e-beam irradiation. In the study conducted by Sanglay et al., (2011), the authors were able to achieve a higher
starting titer in the food, and there may have been differences in storage time or other factors. Therefore, it is difficult to directly compare these two studies, but we suggest that TV is more sensitive to e-beam than MNV-1. Most of the dosages used in our experiments are higher than allowed by the FDA, but they allow observation of visual changes, and allow direct comparison of dosages and mechanisms.

Table 9. E-beam inactivation of TV (log PFU/ml ± standard deviation) in strawberries and lettuce. Data are the means of three replicates. Means within columns with different lowercase letters are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>E-beam Absorbed Dose (kGy)</th>
<th>TV (log PFU/ml ± SD)</th>
<th>E-beam Absorbed Dose (kGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td>Strawberries</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.7 ± 0.6 a</td>
<td>4.4 ± 0.6 a</td>
</tr>
<tr>
<td>3.9</td>
<td>2.4 ± 0.6 b</td>
<td>3.0 ± 0.5 b</td>
</tr>
<tr>
<td>8.7</td>
<td>ND</td>
<td>1.8 ± 0.3 c</td>
</tr>
<tr>
<td>17.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>26.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30.7</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

63
Previous research demonstrates that e-beam irradiation at low doses was effective at reducing the microbial population and extending the shelf life of many foods, including strawberries, both iceberg and romaine lettuce, cantaloupe, mangoes, and blueberries, among others (Han et al., 2006; Moreno et al., 2006; Moreno et al., 2007; Palekar et al., 2004; Yu et al., 2006). While studies have shown irradiation’s effectiveness against various types of bacteria in foods including Salmonella, Escherichia coli, Listeria monocytogenes (Gliemmo et al., 2014; Kundu et al., 2014; Song et al., 2014), only a limited number of studies have shown how irradiation affects noroviruses in foods (Feng et al., 2011; Nair et al., 2013; Praveen et al., 2013; Sanglay et al., 2011). Therefore this research is important for understanding how irradiation affects not only MNV-1, but the newer surrogate, TV, in food.

The goal of any food processing is to find the perfect dose and concentration with a balance between high microbial kill and low impact on quality or sensory aspects of the food. We have seen the effects of irradiation demonstrated in previous research. Irradiation of produce may result in changes such as discoloration, loss of firmness, and altered respiration rates, which in turn will affect the physiological response (Diehl, 1999; Han et al., 2004). We observed some of these changes at higher doses, especially color change and firmness, and therefore, we do not recommend that lettuce or strawberries be irradiated at doses higher than regulations currently allow.

2.4.2. E-beam effects on texture and color of irradiated food. The visual changes in the produce can be observed in Fig. 6. As the e-beam dose increased in
strawberries, there was a color change from a deeper red to almost white in the 30.7 kGy sample. The image in Fig. 6 was obtained immediately after treatment so the samples were warm and show air bubbles which could be from induced irradiation heating, or from radiolytic products induced by the e-beam. We also observed a softening of the texture the strawberries at higher doses. In the lettuce, as the dosage increased, the color shifted from a light green to dark green and wilted the leaves. To quantitatively measure the texture and color changes caused by higher doses of irradiation, texture profile analysis (TPA) was conducted on the strawberries, and colorimetry was conducted on both the strawberries and lettuce samples. The CIELAB (L*a*b*) values demonstrated by these color changes are shown in Table 10. We observed an overall increase in L* values and decrease in a* chroma for the strawberry samples as doses of irradiation increased. These positive L* values indicated an increased lightness toward white, and the decrease in a* chroma were correlated with this change, moving further from a deep red to a lighter red. The color changes in lettuce were less significant. There was a general trend of decreasing L* values, meaning that the lettuce samples became slightly darker in nature, but the other a* and b* chroma were not as conclusive.
Figure 6. Appearance of lettuce and strawberries before (0 kGy) and after e-beam treatment at five targeted dosage levels of 4, 8, 16, 24, and 30 kGy. The top row (left to right) shows lettuce exposed to 0, 4, and 8 kGy, the middle row (left to right) shows lettuce exposed to 16, 24, and 30 kGy, and the bottom row (left to right) shows strawberries exposed to 0, 4, 8, 16, 24, and 30 kGy.

Table 10. Color values (L*a*b*) of strawberries and lettuce. Data are the means of three replicates.

<table>
<thead>
<tr>
<th>absorbed dose</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>absorbed dose</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 kGy</td>
<td>47.8</td>
<td>21.5</td>
<td>10.0</td>
<td>0 kGy</td>
<td>54.5</td>
<td>-6.0</td>
<td>15.0</td>
</tr>
<tr>
<td>4.1 kGy</td>
<td>49.3</td>
<td>19.7</td>
<td>8.2</td>
<td>3.9 kGy</td>
<td>54.0</td>
<td>-6.1</td>
<td>13.2</td>
</tr>
<tr>
<td>8.2 kGy</td>
<td>53.0</td>
<td>16.8</td>
<td>9.1</td>
<td>8.7 kGy</td>
<td>49.7</td>
<td>-4.8</td>
<td>12.4</td>
</tr>
<tr>
<td>16.3 kGy</td>
<td>50.6</td>
<td>15.3</td>
<td>6.9</td>
<td>17.2 kGy</td>
<td>49.4</td>
<td>-4.8</td>
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</tr>
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<td>25.2 kGy</td>
<td>50.6</td>
<td>13.1</td>
<td>7.7</td>
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<td>44.5</td>
<td>-4.9</td>
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<td>30.6 kGy</td>
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<td>44.5</td>
<td>-5.4</td>
<td>13.6</td>
</tr>
</tbody>
</table>
The texture profile analysis of the strawberry samples is shown in Table 11. We evaluated strawberry samples for hardness, resilience, springiness, gumminess, and chewiness. Firmness can be defined as the value that measures cell wall strength and adhesion (Tiovonen and Brummell, 2008). Hardness values of the strawberries significantly decreased as the e-beam dose increased, and all treated strawberries were significantly different from the control (0 kGy). Gumminess is a parameter that measures the disintegration of the sample for swallowing (Aday et al., 2011). The gumminess of the strawberries significantly decreased as the e-beam dose was increased. Less force was required to cause the strawberry to disintegrate at these higher doses of irradiation. Another notable measurement is chewiness, which is defined as the energy for chewing of food until swallowing, (Huang et al., 2007). Consistent with the other parameters that we evaluated, the chewiness values significantly decreased as the e-beam dose increased. The strawberries underwent major changes in both texture and color at higher doses of e-beam, and the TPA and color analysis further confirmed this. Because texture and color are important characteristics for acceptance of food products, treating fresh produce with higher doses of e-beam than currently permitted by regulation are not practical.
Table 11. TPA of strawberries. Changes in texture were measured by firmness (hardness), resilience, springiness, gumminess, and chewiness. Data are the means of three replicates. Means within columns with different letters are significantly different (p<0.05).

<table>
<thead>
<tr>
<th>kGy</th>
<th>hardness (g)</th>
<th>resilience (%)</th>
<th>springiness (%)</th>
<th>gumminess (g)</th>
<th>chewiness (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>840.1 a</td>
<td>7.1 a</td>
<td>65.2 a</td>
<td>203.7 a</td>
<td>131.0 a</td>
</tr>
<tr>
<td>4.1</td>
<td>275.8 b</td>
<td>8.9 b</td>
<td>84.2 b</td>
<td>95.9 b</td>
<td>82.5 b</td>
</tr>
<tr>
<td>8.2</td>
<td>268.5 c</td>
<td>5.4 c</td>
<td>71.7 c</td>
<td>74.9 c</td>
<td>52.9 c</td>
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<td>16.3</td>
<td>126.5 d</td>
<td>5.5 c</td>
<td>71.9 c</td>
<td>36.8 d</td>
<td>26.1 d</td>
</tr>
<tr>
<td>25.2</td>
<td>99.8 e</td>
<td>4.0 d</td>
<td>62.0 d</td>
<td>28.0 e</td>
<td>17.3 e</td>
</tr>
<tr>
<td>30.6</td>
<td>100.2 e</td>
<td>3.3 d</td>
<td>59.6 d</td>
<td>25.7 e</td>
<td>15.0 e</td>
</tr>
</tbody>
</table>

2.4.3. E-beam inactivation of MNV-1 and TV in liquid. The effect of each increasing e-beam dose on the reduction of viral titers was found to be significant from each other dose (p < 0.05) (Table 12). After 4.8 kGy of e-beam, the MNV-1 titer was reduced by approximately 1.5 logs in PBS, and by 1 log in DMEM. MNV-1 was still detectable in PBS at 16.9 kGy (0.5 logs of virus remaining) and in DMEM (0.8 logs of virus remaining) at 26.5 kGy. MNV-1 was not detectable in media or PBS after treatment of 26.5 to 32.7 kGy. In both PBS and DMEM, significant differences were observed in MNV-1 titers for all dose levels (p < 0.05). TV treated with 4.8 kGy of e-beam resulted in a 1.4 log reduction in PBS, and a 1.8 log reduction in Opti-MEM. At 8.6 kGy, TV was still detectable in both PBS and Opti-MEM (4.1 and 3.2 log10 PFU/ml, respectively). TV was not detectable at doses of 26.5 kGy or higher. Similar to MNV-1, in both PBS and Opti-MEM, significant differences between each dose were observed for TV (p < 0.05).
Table 12. E-beam inactivation of murine norovirus and Tulane virus (log PFU/ml ± one standard deviation) in aqueous solutions. Data are the means of three replicates. Means within columns with different lowercase letters are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>E-beam Absorbed Dose (kGy)</th>
<th>MNV-1 (log PFU/ml ± SD)</th>
<th>TV (log PFU/ml ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>DMEM</td>
</tr>
<tr>
<td>0</td>
<td>7.2 ± 0.2 a</td>
<td>7.2 ± 0.1 a</td>
</tr>
<tr>
<td>4.8</td>
<td>5.6 ± 0.3 b</td>
<td>6.1 ± 0.3 b</td>
</tr>
<tr>
<td>8.6</td>
<td>3.8 ± 0.3 c</td>
<td>5.3 ± 0.1 c</td>
</tr>
<tr>
<td>16.9</td>
<td>0.5 ± 0.5 d</td>
<td>3.3 ± 0.2 d</td>
</tr>
<tr>
<td>26.5</td>
<td>ND</td>
<td>0.8 ± 0.6 c</td>
</tr>
<tr>
<td>32.7</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Based on the viral inactivation data (Table 12), MNV-1 was more resistant to e-beam treatment than TV. The MNV-1 results are consistent with Feng et al. (2011), who observed MNV-1 being stable in aqueous solutions (water, PBS, and DMEM) after 5.6 kGy of gamma irradiation. Sanglay et al. (2011) reported similar results where MNV-1 was more resistant to e-beam irradiation when suspended in DMEM versus PBS. At 12 kGy of e-beam treatment, the MNV-1 titer in PBS was 0.89 log10 PFU/ml, while DMEM was 3.64 log10 PFU/ml (Sanglay et al., 2011). Sullivan et al. (1971) reported the D10
value for coxsackievirus B-2 in Eagle’s minimum essential medium treated with gamma irradiation to be 4.5 kGy, but in water the D10 value was much lower (1.40 kGy). Sullivan et al. (1973) reported similar D10 values for thirty different viruses in Eagle’s minimum essential medium and distilled water treated with gamma irradiation, and all of these values ranged from 4.1 to 5.3 kGy. The irradiation dosage had to be increased by a more than three-fold when treating viruses suspended in Eagle’s minimum essential medium compared to distilled water. Based on the data here and in literature, viral inactivation in liquid media by irradiation is dependent on the chemical composition of the media itself. If the liquid media contains more ingredients, these components provide a protective effect for the viruses against irradiation (Sullivan et al., 1973).

No prior studies have investigated e-beam irradiation sensitivity of TV, and few studies have compared TV and MNV-1 directly. The stability of the surrogate viruses in various settings is of utmost importance when determining the best surrogate to use (Kniel, 2014). One study that has compared these two surrogates found that MNV-1 and TV were both reduced beyond detection limits at 2000 ppm of chlorine, (Hirneisen and Kniel, 2013b), but at 2 ppm chlorine in water, MNV-1 was reduced significantly less than TV (0.38 and 2.11 log reductions, respectively). These have further led to speculation that MNV-1 is still the better surrogate because of its similar behavior to human norovirus which is resistant to low levels of chlorine in drinking water (Seitz et al., 2011). The opposite result occurred when the viruses were subjected to UV light treatment- TV was much more stable than both MNV-1 and FCV at 25-70 mJ/cm2 (Tian et al., 2013). The successful cultivation of human norovirus in cell culture is elusive, so finding the
best surrogate to use in norovirus research and validating its use is imperative. Knowing both TV and MNV-1’s strengths and weaknesses as surrogates will allow researchers to quickly isolate which surrogate they need based on these characteristics.

2.4.4. Effect of e-beam on virus structure. TEM was utilized to determine if e-beam directly disrupted the structure of MNV-1 and TV. Figs. 7 and 8 illustrate the MNV-1 and TV virions, respectively, before and after e-beam treatment. Both MNV-1 and TV are non-enveloped, positive-sense RNA viruses that range from 28 to 35 nm in diameter, and possess an icosahedral shape that is very similar to that of human noroviruses (Wobus et al., 2006).
Figure 7. Transmission electron micrographs of MNV-1 treated with 0 (A), 4.8 (B), 8.6 (C), 16.9 (D), 26.5 (E), and 32.7 kGy (F) of e-beam irradiation.
Figure 8. Transmission electron micrographs of TV treated with 0 (A), 4.8 (B), 8.6 (C), 16.9 (D), 26.5 (E), and 32.7 kGy (F) of e-beam irradiation.

For MNV-1, the intact and untreated virus particles are shown in Fig. 7A. E-beam doses of 4.8 and 8.6 kGy do not appear to cause significant damage to the particles, but the amount of viral particles appears to decrease (Figs. 7B and 7C). At 16.9 and 26.5 kGy, there are sufficiently fewer particles than the lower doses, and it appears that the diameter of the virions is slightly smaller compared to the control (Figs. 7D and 7E). At 32.7 kGy, the MNV-1 capsid appears completely degraded by e-beam, as it is much smaller and no longer resembles an intact particle (Fig. 7F).
For TV, the intact untreated particles are shown in Fig. 8A. At 4.8 and 8.6 kGy, there is not significant visual damage to the viral particles, but the number of virions present decreases. As the dosage increases, similar to MNV-1, the amount of particles continues to decrease, and visual damage becomes more apparent. For example, in Fig. 9E and 9F the virions are no longer distinct spheres; the edges were more jagged and misshapen, showing the outer capsid surface may be degraded.

**2.4.5. Effect of e-beam on viral proteins.** Purified MNV-1 and TV were treated with increasing e-beam doses and subjected to SDS-PAGE analysis to determine if there was any effect on the viral proteins. Fig. 9 represents SDS-PAGE analysis of untreated and e-beam treated MNV-1 and TV. The MNV-1 genome encodes for two structural proteins: a 58.9 kDa major capsid protein (VP1) and a 22.1 kDa basic minor capsid protein (VP2) (Wobus et al., 2006). There are 90 dimers of VP1 that form the structure of the capsid, with 2-3 copies of VP2 found within the intact virion. In Fig. 10A, the VP1 capsid protein was present in the 0 kGy control, and appeared to diminish with increasing e-beam dosage. VP1 was not detectable after 32.7 kGy of e-beam treatment. TV shows a single protein band at about 60 kDa, suspected to be the capsid protein VP1 (Farkas et al., 2008). In Fig. 9B, similar to MNV-1, the 60 kDa band appeared to decrease in intensity with increasing e-beam dosage.
Based on the results of the TEM and SDS-PAGE it appears that e-beam treatment degrades viral proteins. These results are in agreement with Feng et al. (2011). The researchers found degradation of MNV-1 viral proteins by up to 22.4 kGy of gamma irradiation. They observed a decrease in the MNV-1 VP1 capsid protein after irradiation treatment (Feng et al., 2011). With regards to irradiation and its effects on proteins, amino acids vary in their susceptibility. Amino acids containing sulfur (cysteine, cystine, methionine) or aromatic compounds (tyrosine, phenylalanine) tend to be susceptible to irradiation because they act as scavengers and will react with hydroxyl radicals more easily than aliphatic (alanine, leucine, valine) amino acids (Diehl, 1995; Stewart, 2001). Histidine is another example of an amino acid that is highly sensitive to radiation, where
the amino acid undergoes a high rate of deamination (Diehl, 1995; Stewart, 2001). Also, crosslinking or aggregation of proteins can occur during irradiation, which may involve disruption of secondary and tertiary structures and exposure of reactive groups (Stewart, 2001).

2.4.6. Effect of e-beam on viral RNA. Figure 11 illustrates the effect of e-beam irradiation on the genomic RNA of purified MNV-1 and TV. Primers were designed to target the VP1 gene of both MNV-1 and TV. For both MNV-1 (Fig. 10A) and TV (Fig. 10B), as e-beam dose increased, the intensity of the VP1 bands for both viruses decreased. For MNV-1, viral RNA was still detectable up to 16.9 kGy, while no RNA was detected at 26.5 and 32.7 kGy. For TV, viral RNA was detectable all the way up to 32.7 kGy but the intensity of the band was lighter than lower doses.
Figure 10. RT-PCR products of MNV-1 (A) and TV (B) before and after e-beam treatment. The lanes are labeled with each respective e-beam dosage or the DNA ladder.

No band was detected for MNV-1 treated at 26.5 or 32.7 kGy. Dickson (2001) states during irradiation, photons or electrons cause random breaks or lesions in the genetic material of the microorganism. If there are multiple lesions in the genetic material, the result would be lethal for the microorganism (Dickson, 2001). Also, the formation of hydroxyl radicals and hydrogen peroxide during radiolysis of water can react with bonds that bind the nucleic acids together (Dickson, 2001). Our results are also in agreement with Feng et al. (2011). The VP1 gene of MNV-1 was not detected in RT-PCR at doses above 20 kGy, thus suggesting that the VP1 gene was degraded by
irradiation (Feng et al., 2011). For TV, it is interesting that the titer dropped quickly in both foods and liquids, but the RNA was still intact at higher doses during the mechanistic study. This may be due to strand breaks not occurring in the VP1 gene but elsewhere in the RNA, or secondary RNA structure differences. It could also be due to the formation of semi-degraded protein complexes or aggregated protein complexes that have protected the RNA from e-beam treatment.

2.5. Conclusion

Our results showed that TV was able to be reduced to non-detectable levels in lettuce at 8.7 kGy or higher, and in strawberries at 16.3 kGy or higher, but these levels of irradiation are higher than currently allowed by FDA regulations (FDA, 2013). Visual changes to the food were significant at doses of 8.2 or 8.7 kGy or higher in strawberries and lettuce, respectively. We also found that the human norovirus surrogate, MNV-1, was more resistant to e-beam treatment (requiring 26.5 kGy in PBS and 32.7 kGy in DMEM for inactivation) than TV (inactivation after 8.6 kGy in PBS and 16.9 kGy in Opti-MEM), even though TV human norovirus recognizes HBGA receptors similar to its human counterpart. Based on the mechanistic study, e-beam, like gamma irradiation, disrupted virion structure and degraded viral proteins and genomic RNA in both viruses. Viruses tend to be more resistant to ionizing radiation than bacteria, parasites, yeasts, or fungi due to their much smaller size and simple structure. However, it appears that the combination of disruption of the structure, viral proteins, and RNA are all essential in viral inactivation by irradiation.
Chapter 3: Control of human norovirus surrogates in fresh foods by gaseous ozone and a proposed mechanism of inactivation

3.1 Abstract

Fresh produce is a major concern for transmission of foodborne enteric viruses as it is normally consumed with no heat treatments and minimal other processing to ensure safety. Commonly used sanitizers are ineffective at removing foodborne viruses from fresh produce. Thus, the use of gaseous ozone for viral inactivation was investigated. Ozone has great potential for improving food safety because of four benefits: It is a potent oxidizer, it is effective against a wide range of microorganisms, it is permitted for food use as regulated by the U.S. FDA and several other nations, and it spontaneously decomposes to oxygen leaving no residue. This study determined the effectiveness of gaseous ozone for the sanitization of two norovirus surrogates (MNV-1 and TV) from both liquid media and popular fresh foods where viral contamination is common—lettuce and strawberries. Samples were treated with gaseous ozone at 6% wt/wt ozone in oxygen for 0, 10, 20, 30, and 40 min, and surviving viruses were quantified by viral plaque assay. Our results showed that gaseous ozone inactivated norovirus in both liquid media and fresh produce in a dose-dependent manner. These results are promising because ozone treatment significantly reduced two important norovirus surrogates to in both liquid and
food matrices. Viruses are generally more resistant to sanitation treatments than bacteria, thus gaseous ozone is an effective means to improve fresh produce safety.

3.2 Introduction

Foodborne viruses continue to be a primary concern in food safety. In 1999, it was estimated that foodborne disease caused a staggering 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths per year in the United States. Of these 76 million illnesses, norovirus alone caused 23 million illnesses, 50,000 hospitalizations, and 310 deaths (Mead, 1999). More recently in 2011, it was estimated that there are 9.4 million cases of foodborne illness each year, attributing to 55,961 hospitalizations and 1,351 deaths. Of these cases, 58% were caused by norovirus (Scallan, 2011). From these cases, we see there has been some improvement in the overall safety of our food supply due to these decreasing numbers of foodborne illness cases per year, but with millions of people still becoming sickened per year, further improvement is needed. Thus, the investigation of alternative methods for sanitization of viruses from foods is a priority.

Human norovirus remains the leading cause of acute gastroenteritis every year, causing around 60% of all foodborne illnesses reported annually. Norovirus outbreaks are common in any reservoir where many people are in close contact, such as cruise ships, hospitals, restaurants, schools, nursing homes, military bases, or other similar places (Widdowson et al., 2008). Transmission is primarily the fecal-oral route, by ingesting contaminated food or water or by person to person contact. Symptoms of norovirus
include diarrhea, vomiting, fever, chills, and severe dehydration. One major issue that has hampered the research of human norovirus is its inability to propagate in cell culture.

Virus surrogates have been a primary way to understand and predict how human norovirus behaves. The two most extensively used surrogates have been feline calicivirus (FCV), murine norovirus (MNV). While these viruses show varied genetic relatedness to human norovirus, they differ in other important characteristics such as clinical manifestation, susceptible cells, host receptors, pathogenesis, and immunity. The degree of similarity that these surrogate viruses have with human norovirus still remains speculative. In 2008 a new primate calicivirus, Tulane virus (TV), was discovered in the stools of rhesus macaques at Tulane National Primate Research Center. TV, unlike human norovirus, is able to replicate in vitro and also shows a typical cytopathic effect (CPE). Similar to human norovirus, TV binds to histo blood-group antigen (HBGA) receptors and is able to cause diarrhea in rhesus macaques, making it a superior potential surrogate for human norovirus (Farkas et al., 2008).

Ozone has gained more attention for its potential use in foods because of possible toxic chlorinated byproducts generated by disinfectant chlorine (Jolley et al., 1984). Ozone is attractive for use on foods because it naturally decomposes to elemental oxygen, is a very powerful oxidant, and has high penetrability and reactivity. While many previous studies show the effectiveness of ozone against bacterial pathogens, research concerning virus reduction on fresh foods, specifically norovirus by ozone, is quite limited.
Ozone ($O_3$) is a triatomic oxygen molecule that exits as a bluish gas with a strong characteristic odor. It is a strong oxidizing agent that is active against bacteria, fungi, spores, protozoa, and viruses. Its effectiveness has been shown to be up to 52% higher than that of chlorine with an oxidizing potential of 2.07 mV (Castell-Perez and Moreira, 2004; Manley and Neigowski, 1967). Ozone is most commonly generated by corona discharge as it is the most energy efficient method. In this method, dry oxygen is passed through two electrically charged plates separated by a dielectric medium and a discharge gap (Rice et al., 1996). Under these conditions oxygen is converted to ozone. When pure oxygen is used as the feed gas, high concentrations of up to 6% ozone can be generated (Rice et al., 1996). Although the initial cost of ozone generators may be high, the application over time may justify the cost (Khadre et al., 2001).

Ozone has been tested for use in the preservation of many foods and food ingredients, for the purification and artificial aging of alcoholic products, disinfection of brewing operations equipment, and several medical applications. It was widely used in water disinfection by European countries as early as 1906 (Rice et al., 1981). In 1982, the US Food and Drug Administration (FDA) gave ozone GRAS (generally recognized as safe) status for use in bottled water disinfection (Guzel-Seydeim et al., 2004). Experts gathered by the Electric Power Research Institute (EPRI) affirmed ozone GRAS status for more broad use in foods and water. Because the FDA did not object to this affirmation, ozone is now allowed as a disinfectant or food sanitizer in the U.S. (Khadre et al., 2002).
Because of ozone’s promising characteristics as a sanitizer, the goals of this research were to 1) evaluate and compare the effects of gaseous ozone on two human norovirus surrogates, MNV-1 and TV, 2) investigate the mechanism of inactivation of these two viruses by gaseous ozone, 3) evaluate the efficacy of ozone on the sanitization of MNV-1 and TV from fresh lettuce and strawberries, and 4) quantify the texture and color changes of strawberries and lettuce after treatments by ozone.

3.3. Materials and Methods

3.3.1. Cell culture and virus stock. Murine norovirus strain MNV-1 was provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine. MNV-1 was propagated in the mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA) as following. RAW 264.7 cells were cultured and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and a 5% CO₂ atmosphere. To prepare MNV-1 stock, confluent cells were infected with MNV-1 at a multiplicity of infection (MOI) of 10. After 1 h of incubation at 37°C, 15 mL of serum free DMEM were added. Two days after infection, the virus was harvested. The flasks were subjected to freeze-thaw three times, and the supernatant was collected after centrifugation at 5,000 rpm for 20 min at 4°C. The titer of the virus stock was 10⁸ PFU/mL.
TV was kindly provided by Dr. Xi Jiang (Cincinnati Children’s Hospital, Cincinnati, OH). TV was propagated in the LLC-MK2 cell line (ATCC, Manassas, VA). Cells were cultured in 150 cm² tissue culture flasks (Corning Life Sciences, Wilkes-Barre, PA) containing Opti-MEM (minimum essential media) supplemented with 2% fetal bovine serum (Invitrogen, Carlsbad, CA) and 2mM GlutaMAX-1 (Invitrogen, Carlsbad, CA). The growth medium was removed from confluent flasks of LLC-MK2 cells and infected with TV at a MOI of 10. Flasks were incubated at 37°C and 5% CO2 for 1 h, with agitation every 15 min. Opti-MEM was added to the flasks and they were incubated at 37°C and 5% CO2 for 48h. When CPEs were observed, flasks were subjected to freeze-thaw cycles, centrifugation, and re-suspension as described above. The titer of the virus stock was 10⁶ PFU/mL.

3.3.2. MNV-1 and TV plaque assay. MNV-1 plaque assay was performed in RAW 264.7 cells as described previously. In short, Raw 264.7 cells were seeded in 6 well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2 × 10⁵ cells per well. After 24 h of incubation, cells were infected with 400 µL from a 10-fold dilution scheme of the virus. After 1 h of incubation at 37°C with agitation every 15 min, overlay containing 2.5 mL of minimal eagle medium (MEM) containing 2% FBS, 1% sodium bicarbonate, 0.1 mg/mL of kanamycin, 0.05 mg/mL of gentamicin, 15 mM HEPES (pH 7.7), 2mM L-glutamine, and 1% agarose was added to the plates. After incubation at 37°C for two days, the plates were fixed with 10% formaldehyde, and the plaques were then visualized by staining with crystal violet. TV plaque assay was performed in the
same way except that LLC-MK2 cells were used in the assays and the plaques were fixed 72 h post-inoculation.

3.3.3. Sample preparation. For liquids, 1 mL of MNV-1 or TV was inoculated into small hexagonal weigh boats (Fisher Scientific, Waltham, MA) so the liquid had the greatest area exposed to gaseous ozone. These samples were then placed directly into the ozone chamber for treatment. The coupons were type 304 stainless steel (McMaster-Carr, Elmhurst, IL), and glass microscope slides (Fisher Scientific, Waltham, MA) were used for the glass surfaces. The stainless steel was cut into coupons by the OSU Machine shop. Both glass and stainless steel coupons were 2.5 x 5 cm. Before use, the coupons were cleaned by deionized water, and then soaked for 10 min in 95% ethanol before autoclaving. 250μl of virus sample was inoculated to each coupon and allowed to air dry completely in a biosafety cabinet before ozone treatment. For virus elution from the coupons, the coupons were added to 10ml of 1X PBS in a 50ml tube and then vortexed for 30 s.

For food samples, strawberries and romaine lettuce were purchased from a local grocery store. The strawberries were left whole and the lettuce was torn into no larger than 20 cm² pieces. Total sample sizes for the strawberries and lettuce were 50g and 25g, respectively. When contaminating strawberries and lettuce with virus, the virus stock was inoculated onto the surface of the strawberries or lettuce by pipetting. The strawberries were also injected with virus in order to test if ozone was effective against internalized viruses. The virus stock was injected via syringe and 21 ½ gauge needle
(Becton Dickenson and Co., Franklin Lakes, NJ) into the center of the strawberries. For example, if the total inoculum was 1 mL and there were two strawberries in the 50g sample, 500 μl were added to each of the two strawberries. The strawberries or lettuce were then placed into large hexagonal weigh boats (Fisher Scientific, Waltham, MA) and treated with ozone.

For the mechanistic study (TEM, SDS-PAGE, and RT-PCR), 50 μl of purified MNV-1 or TV was added to 50 μl of PBS (1:1, v/v). These samples were inoculated into small weigh boats (Fisher Scientific, Waltham, MA) and then treated with each ozone treatment.

3.3.4. Gaseous ozone treatment. A modified standard operating procedure was developed for the newly built commercial ozone generator and equipment specifically for processing food-grade products. The ozone process followed patented parameters (Yousef and Rodriguez-Romo, 2004). Before ozonation, a 10 psig vacuum was drawn. Gaseous ozone at 6% wt/wt ozone in oxygen) was created by corona discharge from extra dry compressed purified (99.6%) oxygen using an ozone generator (OZAT CFS-03 2G, Ozonia). The come up time and degradation was not included in the amount of treatment time, similar to a retorting process. The amount of ozone in the chamber was displayed on the ozone Hi Con meter in g/m3, so we reported the ozone concentration in this unit. The experiments were conducted at room temperature at 25°C. The pH of the MNV-1 virus stock in DMEM + glutaMAX and the TV virus stock in Opti-MEM + glutaMAX was 7.2.
The ozone machine is displayed in Figure 11. Gaseous ozone was pumped into the chamber through an inlet valve until a positive pressure of 15 psig was reached. At this point, by not completely closing the inlet valve and slightly opening the valve leading to the ozone destruct unit, the gas flow was decreased while maintaining constant pressure. At the end of each timed treatment, the pressure was slowly released and residual ozone was directed into a thermal destruct unit and vented through a hood.

![Image of the ozone machine in Figure 11.](image.png)

Figure 11: Gaseous ozone generator and treatment chamber. Gaseous ozone at 6% wt/wt ozone in oxygen was created by corona discharge from extra dry compressed purified (99.6%) oxygen using an Ozonia ozone generator model OZAT CFS-03 2G.
3.3.5. Virus extraction from foods. After ozone treatment, the viruses were recovered from strawberries and lettuce by stomaching with the addition of 50 or 25 mL of 1X PBS, respectively. We used a 1:1 ratio based on the number of grams used per sample. The supernatant containing virus was recovered and then used to quantify the amount of virus by viral plaque assay. The average loss of virus recovered from the foods was between 0.5 and 1 log.

3.3.6. Purification of MNV-1 and TV. To prepare a large stock of MNV-1 or TV, 15 confluent T150 flasks of RAW 267.1 or LLC-MK2 cells were infected with MNV-1 or TV at a MOI of 20 in a volume of 3 ml of DMEM. At 1 h post-absorption, 15 ml of DMEM with 2% FBS was added to the flasks, and infected cells were incubated at 37°C for 48 h. When extensive cytopathic effect (CPE) was observed, cell culture fluid was harvested and subjected to three freeze-thaw cycles to release virus particles. The purification of both MNV-1 and TV was performed using the method described by Katpally et al. (2008) with minor modifications. Virus suspension was centrifuged at 8,000 x g for 15 min to remove any cellular debris. DNase I was used to digest the supernatant solution (10 µg/ml) and MgCl₂ (5 mM) at room temperature. After 1 h incubation, 10 mM EDTA and 1% lauryl sarcosine were added to stop nuclease activity. Virus was then concentration by centrifugation at 82,000 x g for 6 h at 4°C in a Ty 50.2 rotor (Beckman). The pellet was re-suspended in PBS and further purified by centrifugation at 175,000 x g for 6 h at 4°C using a sucrose gradient (7.5 to 45%) in an SW55 Ti rotor (Beckman). Lastly, virus-containing pellets were re-suspended in 100 µl PBS. The virus titer was determined by plaque assay in RAW 264.7 or LLC-MK2 cells.
Viral protein concentration was measured by Bradford reagent (Sigma Chemical Co., St. Louis, MO).

3.3.7. Transmission electron microscopy. Negative staining electron microscopy of purified virions determined whether surfactants damage the virus particles. Fifty μl of highly purified MNV-1 and TV suspension was diluted 1:1 with MilliQ water, and then exposed to 6% gaseous ozone for 0, 10, 20, 30, or 40 min. Viral plaque assay was conducted to confirm the inactivation of virus. Twenty μl aliquots of the samples were fixed in copper grids (Electron Microscopy Sciences, Hatfield, PA), and negatively stained with 1% ammonium molybdate. Virus particles were visualized by FEI Tecnai G2 Spirit Transmission Electron Microscope (TEM) at 80 kV at Microscopy and Imaging Facility at The Ohio State University. Images were captured on a MegaView III side-mounted CCD camera (Soft Imaging System, Lakewood, CO).

3.3.8. Analysis of viral proteins by SDS-PAGE. Five μl of untreated and e-beam treated purified virus suspensions were boiled in loading buffer (1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl [pH 6.8], and 5% glycerol) for 5 minutes. Samples were loaded onto a 12% polyacrylamide gel and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Viral proteins were visualized using Coomassie blue staining of the gel.

3.3.9. Reverse transcription PCR (RT-PCR). MNV-1 or TV genomic RNA, in both untreated and e-beam treated samples, was extracted using an RNeasy minikit.
(Qiagen, Valencia, CA) using the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR kit (Qiagen, Valencia, CA). Two primers targeting the MNV-1 VP1 capsid gene and the TV VP1 capsid gene were used. These were as follows: TV VP1 forward- 5’CATAGACATGGAAAACAGC, TV VP1 reverse- 5’ GCCAGCCATTATCTAAAGA; MNV-1 VP1 forward- 5’ CTACCGTTGCGGTAGAAGAC, MNV-1 VP1 reverse- 5’ GCCCTTCGACCTTGCTCTTG. The 50µl RT-PCR reaction mixture consisted of 400 µM each of deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer, 4 µl of RNA template, 5 units of RNase inhibitor, and 2 µl of RT-PCR enzyme mix. The amplified PCR products were analyzed using 1% agarose gel electrophoresis.

3.3.10. Color/Texture analysis. Color of strawberry and lettuce samples was measured using a Hunter Spectrophotometer (Hunter Lab, Color Quest XE, Virginia, USA) with reflectance specular included (RSI), a 1 inch viewing, and a 60 degree observer angle. These measurements were done in triplicate. Texture profile analysis (TPA) of three strawberries from each respective treatment dosage was carried out using a TA.XT-PLUS Texture Analyzer (Stable Micro Systems Ltd., UK) fitted with a cylinder plunger SMS-P/10 CYL Delrin probe (10 mm diameter). Samples were compressed twice to a depth of 2 mm and 5 s between strokes. Pre-test, test and post-test speeds were set at 5 mm/s, 1 mm/s and 8 mm/s, respectively. The texture parameters determined from the
force–time profile included firmness (hardness), adhesiveness, resilience, cohesiveness, springiness, gumminess, and chewiness.

3.3.11. Statistical analysis. All experiments were done in triplicate. The surviving viruses were expressed as mean log viral titer ± standard deviation. Data were analyzed using the General Linear Model function and Tukey’s pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

3.4. Results and Discussion

3.4.1. Resistance of MNV-1 and TV to gaseous ozone. It has been well documented that ozone is a powerful disinfectant against viruses. Because ozone can directly disrupt various virus constituents such as proteins, nucleic acids, and virus capsid components (Kim et al., 1999), we hypothesized that ozone may be able to inactivate human norovirus surrogates. We tested efficacy against two norovirus surrogates in a simple liquid virus stock and in a complex food environment, specifically fresh produce. We first evaluated possible reduction of MNV-1 and TV in a simple liquid environment with varied time treatments of a constant concentration of gaseous ozone. One mL samples were exposed to 6% gaseous ozone for 0, 10, 20, 30, or 40 min. Surviving viruses after treatment were quantified by viral plaque assay. Figure 12 shows the viral reduction achieved by ozone treatments. The starting titer for MNV-1 was much higher.
than TV, but TV remained more stable over the treatment times used. After 10 min of treatment, MNV-1 showed a 4.1 log reduction while TV only experienced a 0.5 log reduction. The reduction for both viruses was similar during higher treatments, and after 40 min, both viruses were destroyed. The detection limit by our plaque assay method was 10 PFU/ml. TV only showed 1 log PFU/mL remaining after 40 min of gaseous ozone treatment, while MNV-1 was not detectable.

Figure 12: Inactivation of MNV-1 and TV in liquid by ozone. 1 mL samples of virus stock were treated directly with gaseous ozone with one of five different treatments (0, 10, 20, 30, or 40 min). After each time, a serial dilution scheme was set up to quantify the surviving viruses by plaque assay. Data are the means of three replicates.
Ozone is soluble in liquid as explained by Henry’s Law (1803). Under ideal conditions, the saturation concentration of ozone is proportional to its concentration when pressure and temperature are constants (Bocci, 2002; Battino, 1981). In water at 20°C, the solubility of 100% pure ozone is only 570 mg/L (Kinman et al., 1975). In addition to temperature, ozone pressure, and ozone concentration, other factors affecting the solubility of ozone in water during ozone solubility studies are pH, effects of mass transfer, difficulty of the analyses (Roth and Sullivan, 1981). Ozone’s decomposition into hydroxyl, superoxide, and hydroperoxyl radicals contributes to its high oxidizing power. The hydroxyl radicals initiate an oxidative chain reaction, creating secondary intermediates of lower reactivity (Kim et al., 1999). Thus we evaluated whether the amount of viral reduction was different when the liquid virus stocks of MNV-1 and TV were allowed to completely dry on two different surfaces, glass and stainless steel, before ozone treatment. Stainless steel is the primary surface used in many health care and food service establishments. These results are shown in Figure 13.
Figure 13: Inactivation of MNV-1 and TV on stainless steel or glass coupons by ozone. 250 μl samples of virus stock were inoculated onto stainless steel or glass coupons and allowed to dry completely. The coupons were then either untreated by ozone, or treated with gaseous ozone for 30 min. After treatment, a serial dilution scheme was used to quantify the surviving viruses by plaque assay. Data are the results of three replicates.

There were no significant differences between the two untreated controls and their respective treatments with ozone. The amount of reduction from the coupons was significantly lower than in the liquid virus stock solutions that were not dried before treatment. This indicates we achieved less viral reduction when the virus was not in solution. This might be caused by limiting the secondary oxidizing effects in solution. Ozone’s anti-viral action is thought to be by direct oxidation, but it appears there are secondary reactive oxygen species causing further reduction in viruses (Staehelin and
Hoigne, 1985). Because the virus was completely dried, we eliminated aqueous reactive oxygen species from ozone.

### 3.4.2. Viral reduction on strawberries and lettuce by ozone

The reduction of both viruses (TV and MNV-1) in and on two different food matrices, strawberries and lettuce was tested. The results of viral reduction in strawberries are shown in Figures 14 and 15, and the reduction in lettuce can be observed in Figure 16. Ozone has been reported previously to increase the shelf life and to reduce the microbial counts on different fruits and vegetables, including apples, strawberries, lettuce, and also fruit juices such as apple cider and orange juice (Achen and Yousef, 2001; Garcia et al., 2003; Kim et al., 1999; Kniel, 2002; McLoughlin, 2000; Wei et al., 2007; Williams et al., 2004).
Figure 14: Inactivation of MNV-1 on or in strawberries by ozone. 1 mL samples of virus stock were either inoculated onto the surface of strawberries, or injected by syringe into the inside of the strawberries. The 50g samples were then treated with gaseous ozone with one of five different treatments (0, 10, 20, 30, or 40 min). After each treatment, a serial dilution scheme was used to quantify the surviving viruses by plaque assay. Data are the results of three replicates.
Figure 15: Inactivation of TV on or in strawberries by ozone. 1 mL samples of virus stock were either inoculated onto the surface of strawberries, or injected by syringe into the inside of the strawberries. The 50g samples were then treated with gaseous ozone with one of five different treatments (0, 10, 20, 30, or 40 min). After each treatment, a serial dilution scheme was used to quantify the surviving viruses by plaque assay. Data are the results of three replicates.

The internalization of foodborne viruses was also demonstrated in previous research (DiCaprio et al., 2012). Traditional washing methods such as chlorine rinses are ineffective for reaching those viruses that may have penetrated through the surface of foods to be internalized. Thus we tested if ozone was able to penetrate the strawberry exterior and inactivate internalized viruses. To measure this we either injected virus into the center of strawberry samples or inoculated the surface of strawberries before treating...
them with gaseous ozone. The results for MNV-1 are shown in Figure 14. After 40 min of ozone treatment, we saw significantly more reduction in viral titer on the surface of the strawberries (3.3 log) compared to internalized viruses (1.5 log). The TV data (Figure 15) show similar results, where there was again significantly more reduction in surface viruses compared to internalized viruses. It can also be seen that MNV-1 is much more resistant to ozone treatment than TV in food matrices from these two figures. Perhaps MNV-1 binds more tightly to fresh produce than TV because of its binding to sialic acid receptors rather than HBGA receptors, and is more difficult to remove. These results are similar to the liquid virus stock results. Surface inoculated TV was reduced to non-detectable levels after 40 min, where there was still 2.5 log viral titer left after this same treatment of MNV-1. We show that the differences in sensitivities of MNV-1 and TV to different treatments depend on the treatment itself. In the case of irradiation TV is more resistant, but for gaseous ozone we see that MNV-1 is more resistant.
Figure 16: Inactivation of MNV-1 and TV on lettuce by ozone. 1 mL samples of virus stock were inoculated onto the surface of 25g samples of lettuce. The samples were then treated with gaseous ozone with one of five different treatments (0, 10, 20, 30, or 40 min). After each treatment, a serial dilution scheme was set up to quantify the surviving viruses by plaque assay. Data represents the results of three replications.

3.4.3. **Ozone effects on the texture and color of food.** We conducted texture profile analysis (TPA) on the strawberries and conducted colorimetric measurements on both the strawberries and lettuce treated with ozone. It was impractical to conduct TPA of the lettuce samples due to the variability, as some pieces of lettuce contained the romaine hearts and others were much leafier, thus the natural variance within a single lettuce plant was greater than any treatment. The TPA data are shown in Table 13. As the time of treatment with gaseous ozone increased, the hardness of the sample decreased.
significantly with each treatment. The chewiness and gumminess also significantly
decreased in the same fashion. The strawberries became softer with increasing treatment
times of ozone.

Table 1. TPA of strawberries. Changes in texture were measured by firmness
(hardness), resilience, springiness, gumminess, and chewiness. Data are the means of
three replicates. Means within columns with different letters are significantly
different (p<0.05).

<table>
<thead>
<tr>
<th>ozone (min)</th>
<th>hardness (g)</th>
<th>resilience (%)</th>
<th>springiness (%)</th>
<th>gumminess (g)</th>
<th>chewiness (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1072.4 a</td>
<td>14.8 a</td>
<td>52.8 a</td>
<td>380.7 a</td>
<td>202.4 a</td>
</tr>
<tr>
<td>10</td>
<td>1005.1 b</td>
<td>14.1 a</td>
<td>42.1 b</td>
<td>318.2 b</td>
<td>165.8 b</td>
</tr>
<tr>
<td>20</td>
<td>784.6 c</td>
<td>14.5 a</td>
<td>43.1 b</td>
<td>279.4 c</td>
<td>120.3 c</td>
</tr>
<tr>
<td>30</td>
<td>792.2 d</td>
<td>12.1 b</td>
<td>41.0 b</td>
<td>248.0 d</td>
<td>101.9 d</td>
</tr>
<tr>
<td>40</td>
<td>463.9 e</td>
<td>14.3 a</td>
<td>51.1 a</td>
<td>172.4 e</td>
<td>88.3 e</td>
</tr>
</tbody>
</table>

The L*a*b* values are shown in Table 14. In lettuce we saw a slight decrease in
value, hue, and chroma, L* a* and b* respectively. Overall, the lettuce samples became
lighter and some areas became bleached or whiter after longer times of ozone treatment.
The visual changes that occurred in strawberries and lettuce after each different treatment
of ozone are shown in Figure 17. For the strawberries, the reddish color and also the
leaves became lighter with increasing times of ozone treatment. The leaves also became
crunchier at higher times. The strawberries dried out and lost moisture with increasing
ozone treatment. This is evidenced by the L*a*b* values, where a* and b* both increased
as treatment times increased. There was not a statistically significant change in color, but
a general trend is noted.
Table 14. Color values (L*a*b*) of strawberries and lettuce. Data are the means of three replicates.

<table>
<thead>
<tr>
<th>Lettuce</th>
<th>Strawberries</th>
</tr>
</thead>
<tbody>
<tr>
<td>ozone (min)</td>
<td>L*</td>
</tr>
<tr>
<td>0</td>
<td>54.4</td>
</tr>
<tr>
<td>10</td>
<td>48.1</td>
</tr>
<tr>
<td>20</td>
<td>46.6</td>
</tr>
<tr>
<td>30</td>
<td>47.5</td>
</tr>
<tr>
<td>40</td>
<td>43.4</td>
</tr>
</tbody>
</table>

Figure 17: Visual changes of lettuce and strawberries after ozone treatment. 50g samples of strawberries or 25g samples of lettuce were placed in small weigh boats and inoculated with virus. The samples were then treated with gaseous ozone with one of five different treatments (0, 10, 20, 30, or 40 min). Visual changes were observed, and changes were also quantified by TPA and color analysis.
3.4.4. Effect of ozone on viral particles. To determine how the viruses were inactivated by ozone, two highly purified virus stocks (TV, MNV-1) were treated with ozone for 0, 10, 20, 30, and 40 min. The virus particles were visualized by electron microscopy as described previously. Figure 18 shows MNV-1 with or without gaseous ozone treatment. Normally, MNV-1 is a small round-structured virus that ranges in diameter from 27-32 nanometers (Kapikian, 1972). After treatment with varied times of ozone treatment, the number of virus particles decreased, and after 40 min of treatment, there were no intact virus particles visible at all. Like MNV-1, TVs are also small round-structured particles with a mean diameter of 35.8 nanometers (Farkas et al., 2008). As the treatment time with gaseous ozone increased, the number of “empty” virus particles increased, and the particles also became misshapen and jagged. Similar to MNV-1, after 40 min of treatment, no TV particles were visible by EM (Figure 19).
Figure 18: Ozone disrupts MNV-1 particles. Purified MNV-1 was either untreated or treated with 10, 20, 30, or 40 min of gaseous ozone, respectively. Virus particles were visualized by Transmission Electron Microscope. (A) untreated MNV-1; (B) MNV-1 treated by 10 min ozone; (C) MNV-1 treated by 20 min ozone; (D) MNV-1 treated by 30 min ozone; (E) MNV-1 treated by 40 min ozone.

Previous research has suggested that the primary mechanism of inactivation of viruses is by direct damage to the genetic material (Kim et al., 1980; Roy et al., 1981). It is possible that ozone may also cause damage to the outer protein capsid later of the virus. In bacteria, it has been hypothesized that it is the surface that ozone comes into contact with first, causing increased permeability of the membrane and lysis of the cell
(Giese and Christenser, 1954; Murray et al., 1965; Scott and Lesher, 1963). From our micrographs, perhaps a similar mechanism occurs, where there has been damage to the outer protein capsid allowing the viral RNA to leak out, leaving the empty viral particles shown in Figure 19.

![Figure 19: Ozone disrupts TV particles. Like MNV-1, purified TV was either untreated or treated with 10, 20, 30, 40 min of gaseous ozone, respectively. Virus particles were visualized by Transmission Electron Microscopy. (A) untreated TV; (B) TV treated by 10 min ozone; (C) TV treated by 20 min ozone; (D) TV treated by 30 min ozone; (E) TV treated by 40 min ozone.]
3.4.5. Effect of ozone on viral proteins. Both MNV-1 and TV are single-stranded RNA viruses which are surrounded by viral capsid proteins. There are two capsid proteins present—VP1, the major capsid protein which plays a role in virus attachment and host cell entry, and release of the mature virion, and VP2, the minor capsid protein (Jiang et al., 1992; Prasad et al., 1999). We can observe that ozone interacts with the protein capsid layer of both MNV-1 and TV (Figs. 18, 19) but it is still unclear whether ozone is able to directly degrade these viral proteins. To measure this, highly purified MNV-1 and TV were treated with increasing dosages of gaseous ozone and then subsequently analyzed the samples by SDS-PAGE. In Figure 20A and B, we see that in the untreated control MNV-1 and TV, the VP1 capsid protein was present. The VP1 protein of TV has an approximate molecular weight of 58 kDa (Farkas et al., 2008). Interestingly, all other treated samples did not produce a band corresponding to the VP1 protein. This was consistent for both surrogates of human norovirus. Similar to the proposed mechanism of ozone interaction with bacteria, perhaps because the ozone first comes into contact with the proteins comprising the outer layer of the virus and its capsid, and therefore these are most strongly damaged or degraded. This is consistent with previous reports of ozone interacting with the viral capsids of viruses (Cronholm et al., 1976; Riesser et al., 1976).
3.4.6. Effect of ozone on viral RNA. If viral genomic RNA is damaged or degraded, it would be lethal to the virus. To evaluate this, we used RT-PCR to determine if the ozone can degrade viral RNA. Two primers were designed for each virus (MNV-1 and TV) to amplify the VP1 gene from the viral genomic RNA. Experiments were conducted with great caution to ensure that no RNases were introduced into the samples because these may easily degrade any RNA present. As expected, we saw a band for the VP1 gene (with a size of approximately 1.5 kb) was present for both of our untreated
controls (Figure 21A and B). We also observed a slight decrease in the intensity of the bands for increasing treatments of ozone for both viruses.

Figure 21: Ozone treatment degrades viral RNA. Highly purified MNV-1 and TV were treated with ozone. After treatment, RNA was extracted from the virus and RT-PCR was conducted. The RT-PCR products of MNV-1 (A) or TV (B) are shown above.
3.5. Conclusion

Gaseous ozone was able to significantly reduce two human norovirus surrogates’ viral titer in both liquid virus stock and in two food matrices. Visual changes to the food were significant at all doses of ozone for strawberries and lettuce. We found that the human norovirus surrogate, MNV-1, was slightly more resistant to gaseous ozone. Based on mechanistic results, ozone was able to successfully disrupt virion structure and completely degrade viral proteins, while leaving genomic RNA intact in both viruses. Thus the primary way ozone disrupts norovirus surrogates is by altering the capsid structure and damaging the viral capsid proteins on the surface of the virus.
Chapter 4: Reduction and mechanism of inactivation of human norovirus surrogate

Tulane virus in fresh produce by a combination of SDS and other sanitizers

4.1 Abstract

Fruits and vegetables are major vehicles for foodborne virus transmission due to the minimal processing they receive before reaching consumers. Contamination may happen from pre-harvest to post-harvest with foodborne viruses. Previous studies have consistently reported that commonly used sanitizers are not effective in the removal of foodborne viruses from fresh produce. In this study, we systematically evaluated the effectiveness of a number of sanitizers and surfactants on removal of a new human norovirus surrogate, Tulane virus (TV), from both simple and complex matrices. We showed several treatments enhanced the removal of viruses from fresh fruits and vegetables. Aqueous washing with water alone and traditional chlorine solution (200 ppm) did not significantly reduce TV titer in strawberries. The combination of SDS and chlorine was the most effective method of sanitization. Removal of norovirus from lettuce was most effective with traditional chlorine and levulinic acid in combination with chlorine. Both SDS and levulinic acid are GRAS (Generally Recognized As Safe) by U.S. regulation. This sanitization strategy would be an easily implemented and cost effective approach to reduce the total virus load in fresh produce, and a means of improving the overall safety of fresh produce.
4.2. Introduction

Human norovirus remains the most prevalent enteric foodborne virus in the United States. From 2009-2012, there were over 4,300 norovirus outbreaks reported to the NORS (Norovirus Outbreak Reporting System), with those outbreaks resulting in 161,253 illnesses (Hall et al., 2014). Norovirus is a large problem in foods because of its infectious dose and high environmental stability (Duizer et al., 2004; Teunis et al., 2008). Atmar et al. (2014) found the 50% infectious dose of human norovirus to be 1320 genomic equivalents for secretor positive individuals of blood types A and O. Outbreaks of the virus are common in areas of close contact including cruise ships, restaurants, hotels, schools, military installations, nursing homes, and hospitals (Widdowson et al., 2008). Transmission is primarily by the fecal-oral route, either by person to person spread or ingesting food or water that has been contaminated by the virus. Symptoms of norovirus include diarrhea, vomiting, fever, chills, and extreme dehydration. Human norovirus was challenging to study in the past because it is non-cultivable and there is no small animal model for the virus (Wobus et al., 2006). For these reasons, proper surrogates have been continually sought for human norovirus research.

Murine norovirus (MNV-1) and the more recently discovered Tulane virus (TV) are two surrogates that are attractive for study due to several attributes similar to the pathogenic human norovirus. Murine norovirus and Tulane virus both come from the family Caliciviridae, and while both viruses exhibit similar genomic properties, TV binds to histo-blood group antigens (HBGAs) where MNV-1 binds to sialic acid moieties. In
addition, where MNV-1 causes systemic infection in mice, TV has been shown to cause diarrhea in rhesus macaques (Farkas et al., 2008).

Fresh produce is at high risk for contamination by norovirus because it is normally subjected to either little or no processing, and the produce can be contaminated at any step from farm to fork. According to recent outbreak data, fruits and vegetables are major vehicles in the transmission of foodborne illness (Everis, 2004). Outbreaks have been reported in recent years in lettuce, tomatoes, melons, strawberries, raspberries, fresh cut fruits, and other fresh vegetables (Butot et al., 2009, Vandekinderen et al., 2009). One major route for contamination of produce is contaminated irrigation or wash water. Another major source of contamination may be infected workers handling the food (Butot et al., 2009). Because people have strived to be more health conscious in recent years by increasing their consumption of fruits and vegetables, norovirus outbreaks have become a major public health concern (Baert et al., 2008).

In current industrial practice, fresh produce undergoes a brief sanitization step after harvest from the field, and the currently used sanitizers are not effective in removing viruses from fresh produce. The most common sanitizer, 200 ppm of sodium hypochlorite solution, typically gives less than 1.2 logs virus reduction in fresh produce (Predmore and Li, 2011, Baert et al., 2009). Recently, Baert et al. (2009) found that tap water washing gave a modest average of 0.94 logs of reduction in shredded lettuce, while the addition of 200 ppm of sodium hypochlorite only led to an additional 0.48 logs, and the addition of 80 ppm of peroxyacetic acid brought about only 0.77 additional logs of reduction.
Therefore, there is urgent need to develop and implement more effective sanitizers into current commercial practice to remove the norovirus hazards from fresh produce.

Surfactants are surface-active compounds that can reduce the surface tension of a liquid. The addition of surfactants to a wash protocol makes the liquid more miscible and lowers the interfacial tension between the two mediums. These surfactants are useful as detergents, wetting agents, emulsifiers, foaming agents, and dispersants, depending on their nature (anionic, non-ionic, cationic, amphoteric). One common characteristic of surfactants is that they are amphiphilic— they contain both a hydrophilic and hydrophobic group (Rosen, 2004). This feature expedites the release of tightly bound contaminations such as foodborne pathogens. We chose sodium dodecyl sulfate (SDS) which is an anionic surfactant categorized as GRAS (generally recognized as safe) by the U.S. Food and Drug Administration (FDA CFR 172.822). In 2009, Zhao et al. found the combination of SDS and levulinic acid was able to significantly reduce populations of *Salmonella* and *E. coli* on lettuce, poultry, or water containing chicken feces or feathers. They used concentrations of 3% levulinic acid and 1% SDS for lettuce washing, and achieved >6.7 log CFU/g of reduction. In 2011, Predmore and Li found that the combination of SDS and sodium hypochlorite achieved 3 logs of viral reduction in various types of produce. In a later study, Cannon et al. (2012) also found that SDS in combination with levulinic acid may be a potential alternative. Their focus was on more potent concentrations of these sanitizers and their effectiveness on food contact surfaces, thus we performed an evaluation of the effectiveness of much lower concentrations on their ability to sanitize norovirus from fresh produce.
Here we report a systematic evaluation of the effectiveness of SDS alone and combined with other sanitizers for sanitizing a human norovirus surrogate, Tulane virus, from fresh produce. Our results suggest the combination of SDS and chlorine solution is the most effective and practical approach to enhance the safety of fresh produce.

4.3. Materials and methods

4.3.1. Cell culture and virus stock. TV was kindly provided by Dr. Xi Jiang (Cincinnati Children’s Hospital, Cincinnati, OH). TV was propagated in the LLC-MK2 cell line (ATCC, Manassas, VA). Cells were cultured in 150 cm² tissue culture flasks (Corning) containing Opti-MEM (minimum essential media) supplemented with 2% fetal bovine serum (Invitrogen) and 2mM GlutaMAX-1 (Invitrogen). The growth medium was removed from confluent flasks of LLC-MK2 cells and infected with TV at a MOI of 10. Flasks were incubated at 37°C and 5% CO2 for 1 h, with agitation every 15 min. Opti-MEM was added to the flasks and they were incubated at 37°C and 5% CO2 for 48 h. When CPEs were observed, flasks were subjected to freeze-thaw cycles, centrifugation, and re-suspension as described above. The titer of the virus stock was 10⁸ PFU/mL.

4.3.2. TV plaque assay. TV plaque assay was performed in LLC-MK2 cells as described previously. In brief, LLC-MK2 cells were seeded in 6 well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2 × 10⁵ cells per well. After 24 h of incubation, cells were infected with 400 µL from a 10-fold dilution scheme of the virus. After 1 h of incubation at 37°C with agitation every 15 min, the cells were overlaid with
2.5 mL of minimal eagle medium (MEM) containing 2% FBS, 1% sodium bicarbonate, 0.1 mg/mL of kanamycin, 0.05 mg/mL of gentamicin, 15 mM HEPES (pH 7.7), 2 mM L-glutamine, and 1% agarose. After incubation at 37°C for three days, the plates were fixed with 10% formaldehyde, and the plaques were then visualized by staining with crystal violet.

**4.3.3. Inoculation of virus onto fresh produce.** Fresh produce samples (strawberries, romaine lettuce) were purchased from a local supermarket. A sample consisted of 25g placed in a sterile plastic bag. 1 mL of TV stock (10^8 PFU/ml) was added to each sample. The bag was heat-sealed using an AIE-200 Impulse Sealer (American International Electric, Whittier, CA), and the samples were mixed thoroughly by shaking at the speed of 200 rpm at room temperature for 1 h to allow attachment of virus to the sample.

**4.3.4. Sanitization procedure.** SDS (powder), and levulinic acid, (liquid) were purchased from Sigma (St. Louis, MO), and chlorine bleach containing 8.25% sodium hypochlorite was purchased from local supermarket. The TV inoculated fresh produce was sanitized by tap water, 200 ppm of chlorine solution, 50 ppm SDS, 200 ppm levulinic acid, the combination of SDS + chlorine, and the combination of SDS + levulinic acid. For strawberries, the amount of washing solution was 2 L. For lettuce, 4 L of washing solution were used. Freshly-prepared washing solution was used for every replication, and the washing container was cleaned and rinsed out between replications.
Each sample was washed by each sanitizer with gentle agitation for 2 min. After sanitization, the fresh produce was placed into a stomacher bag. The remaining viruses were eluted by addition of 25 mL of PBS solution and stomached for 2 min. The viral survivors were quantified by plaque assay.

4.3.5. **Virucidal assay.** A non-enveloped virus (TV) was used to test whether surfactants and sanitizers can directly inactivate the virus in virus stock. 1 ml of TV (10^8 PFU/ml) stock was incubated with each treatment at 37 °C. At each time point, 50 µl of the virus sample was collected, and the virus survivors were quantified by plaque assay. Because surfactants and chlorine are known to have cytotoxic effect, the inoculum solutions were removed after 1 h of incubation before the overlay was added. The time points where virus was collected were 1, 4, 8, 12, 24, 36, 48, 60, and 72 h. The kinetics of viral inactivation were generated for each treatment.

4.3.6. **Purification of MNV-1 and TV.** Purification of TV was performed as described previously, with minor modifications (Katpally et al., 2008). To generate large stocks of purified TV, 20 confluent flasks of LLC-MK2 cells were infected at an MOI of 1. Flasks were incubated at 37°C, 5% CO₂ for 1 h with agitation every 15 min. DMEM supplemented with 2% FBS, or Opti-MEM supplemented with 2% FBS was added to the flasks and incubated at 37°C, 5% CO₂ for 48 h. When extensive CPEs were observed, flasks were subjected to three freeze-thaw cycles to lyse cells and release virus particles. The cell and virus suspensions were centrifuged using an Sorvall RC-5C Plus centrifuge.
(Kendro Lab Products, Newtown CT) with a Sorvall SS-34 rotor at 12,000 rpm for 10 min to completely remove cellular debris. The supernatant was collected and digested with DNase I (10 µg/ml; Roche Diagnostics, Indianapolis, IN) and MgCl₂ (5 mM; Sigma-Aldrich) at room temperature for 1 h. Dnase activity was halted by addition of 1% lauryl sarcosine (Sigma-Aldrich) in 10 mM of EDTA (Sigma-Aldrich). The virus suspension was centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, Fullerton, CA), using a Ty 50.2 rotor, at 30,000 rpm for 6 h at 4°C. The supernatant was discarded, and the viral pellets were re-suspended in 200 µl of PBS overnight at 4°C on ice. Viruses were further purified by ultracentrifugation in a sucrose gradient (7.5 to 45%) in a SW55 Ti swinging bucket rotor (Beckman Coulter) at 41,000 rpm for 6 h at 4°C. The virus pellets were re-suspended in 100 µl of PBS overnight at 4°C on ice. The purified virus suspension was stored at -80°C. The titer of TV was 9.92 ± 0.4 log PFU/ml. Viral protein content was measured by the Bradford assay, and the protein concentration was 0.2 mg/ml for purified TV.

**4.3.7. Transmission electron microscopy (TEM).** Negative staining electron microscopy of purified virions was performed to determine whether surfactants and sanitizers damage the virus particles. A highly purified TV suspension was incubated with each treatment. Viral plaque assay was conducted to confirm the inactivation of virus. Twenty µl aliquots of either treated or untreated samples were fixed in copper grids (Electron Microscopy Sciences, Hatfield, PA), and negatively stained with 1% ammonium molybdate. Virus particles were visualized by FEI Tecnai G2 Spirit
Transmission Electron Microscope (TEM) at 80 kV at Microscopy and Imaging Facility at The Ohio State University. Images were captured on a MegaView III side-mounted CCD camera (Soft Imaging System, Lakewood, CO).

4.3.8. Analysis of viral proteins by SDS-PAGE. Five µl of untreated and e-beam treated purified virus suspensions were boiled in loading buffer (1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl [pH 6.8], and 5% glycerol) for 5 min. Samples were loaded onto a 12% polyacrylamide gel and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Viral proteins on the gel were visualized using Coomassie blue staining.

4.3.9. Reverse transcription PCR (RT-PCR). TV genomic RNA, in both control and treated samples, were extracted using an Rneasy minikit (Qiagen, Valencia, CA) using the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR kit (Qiagen, Valencia, CA). Two primers targeting the TV VP1 capsid gene were used. The primers used were 5’-CATAGACATGGAAAACAGC-3’ (forward) and 5’-GCCAGCCATTATCTAAAGA-3’ (reverse). The 50µl RT-PCR reaction mixture consisted of 400 µM each deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer, 4 µl of RNA template, 5 units of RNase inhibitor, and 2 µl of RT-PCR enzyme mix. The amplified PCR products were analyzed using 1% agarose gel electrophoresis.
4.3.10. **Statistical analysis.** All experiments were done in triplicate. The surviving viruses were expressed as mean log viral titer ± one standard deviation. Data were analyzed using the General Linear Model function and Tukey’s pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

4.4. **Results and Discussion**

4.4.1. **Effectiveness of sanitizers on the removal of Tulane virus from fresh produce.** It has been reported by several laboratories that commonly used methods of sanitization for fresh produce are ineffective (Gulati et al., 2001; Doyle and Erickson, 2008). Previous research has shown the promise of surfactants as a means to remove norovirus from produce (Predmore and Li, 2011, Zhao et al., 2009). For this reason, we evaluated the use of sanitizers or surfactants to sanitize the newer human norovirus surrogate Tulane virus from both lettuce and strawberries. The results for strawberries are shown in Figure 22. The average amount of viral titer recovered without any treatment was $4.4 \log_{10} \text{PFU/g}$. Treatments with both tap water and chlorine only gave 0.8 and 0.4 log reductions, respectively. Various other treatments were investigated, including multiple concentrations levulinic acid and sodium acid sulfate, SDS in combination with chlorine and levulinic acid, and levulinic acid in combination with chlorine. The most viral reduction was caused by SDS alone, 200 ppm of chlorine in combination with 50 ppm of SDS, and 200 ppm of levulinic acid in combination with chlorine. There was a 1.3 log reduction in TV titer that is significantly greater than the
reduction achieved by chlorine or tap water. The combination of levulinic acid did significantly better than 200 ppm levulinic acid (0.5 log reduction) and 1000 ppm levulinic acid (0.1 log reduction). This 1.3 log reduction is also lower than the previous result achieved for MNV-1 in a study done by Predmore and Li (2011). In their study, they found that MNV-1 was reduced by 3.36 log in strawberries with the same treatment. These results suggest that TV may be more resistant to treatment with SDS than MNV-1.

Figure 22: Effect of sanitizers and surfactants on removal of TV from the strawberry. Fresh strawberries were inoculated with TV, incubated for 1 h, sanitized for 2 min in solutions containing various levels of SDS, sodium acid sulfate, levulinic acid, or a combination of sanitizing agents. Surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
The results for lettuce are shown in Figure 23. Interestingly, these results differed greatly from the results of the strawberries. For lettuce, the viral titer recovered without treatment was $6.2 \log_{10} \text{PFU/g}$, tap water gave a 1.3 log reduction, and chlorine gave a 2.8 log reduction. SDS alone gave a 0.4 log reduction while SDS in combination with chlorine gave a 1.7 log reduction. The combination of levulinic acid and SDS performed well for lettuce as it did with strawberries, giving a 2.6 log reduction. In contrast to positive results that Cannon et al. and Zhao et al. reported for the combination of SDS and levulinic acid, we did not achieve significant reduction for strawberries nor for lettuce (Cannon et al., 2012; Zhao et al., 2009).

![Figure 23](image)

**Figure 23.** Effect of sanitizers and surfactants on removal of TV from romaine lettuce. Romaine lettuce samples were inoculated with TV. After incubation for 1 h, the samples were sanitized for 2 min in washing solutions containing various levels of SDS, sodium acid sulfate, levulinic acid, or a combination of sanitizing agents. Surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
There are many factors that may influence the amount of viral reduction achieved including virus, contact time, sanitizer, sanitizer concentration, and food.

The food matrix of lettuce is much different from that of strawberries, with a greater surface area to allow for a higher rate of virus attachment to the lettuce leaves.

Differences in virus attachment and removal ability may contribute to these results.

Clearly the sanitizer and concentration affect the amount of reduction achieved, as shown in both Figure 22 and 23. Chlorine was not neutralized in this experiment; therefore, even though the inoculum used in plaque assays was removed before adding overlay, a minute amount of chlorine may have continued reacting with the virus. SDS with chlorine, and chlorine alone were the most effective treatments. In previous research, it was found that using higher concentrations of SDS above 50 ppm for produce washing did not significantly increase reduction (Predmore and Li, 2011). Therefore we also used a maximum of 50 ppm SDS concentration in this study. From these observations, individual validation studies may be needed for each type of food and treatment to determine which would be most effective.

4.4.2. Viral inactivation by surfactants and sanitizers. Because most of the treatments used in the washing of produce gave some viral reduction, it is hypothesized that these surfactants or sanitizers directly inactivated the virus during treatment. To investigate this premise, we added the surfactants with or without sanitizers directly to TV virus stock and allowed the virus to incubate for a 72 h period. Samples were
collected at various times and the amount of viral survivors was quantified by plaque assay. As shown in Figure 24, each treatment that we tested gave some reduction in viral titer over the 72 h. All of the treatments seemed to remain stable until the 24 h time point, and then the reduction increased greatly. The combination of SDS and chlorine gave a 4.8 log reduction after 72 h of incubation, which was significantly more than any other treatment.

Figure 24. Inactivation of TV by surfactants or sanitizers. TV stock was inoculated with 1,000 ppm SDS, 1,000 ppm SDS plus 1,000 ppm levulinic acid, 1,000 ppm SDS plus 200 ppm chlorine, or 1,000 ppm levulinic acid at 37°C for 72h, respectively. At each time point, 50 µl of virus sample was collected, and the virus survivors were determined by plaque assay. Data are the means of three replicates.
4.4.3. Surfactants and sanitizers damage viral particles. To determine how viruses were inactivated by the surfactants or sanitizers, each treatment was added to a highly purified TV virus stock. After incubation at 37°C for 72 h, viruses were completely inactivated as confirmed by plaque assays. The samples were then negatively stained with ammonium molybdate. The virus particles were visualized by electron microscopy as described previously (Lou et al., 2010). TV is a small round-structured virus of about 30-38 nm in diameter (Fig. 25). After incubation with SDS for 72h, the amount of viral particles visible significantly decreased (Fig. 25B). The shape of the virions remained roughly the same but they did appear smaller in diameter after treatment.

Figure 25: Surfactants and sanitizers damage virus particles. Purified TV was incubated with 1,000 ppm SDS, 1,000 ppm SDS plus 1,000 ppm levulinic acid, 1,000 ppm SDS plus 200 ppm chlorine, or 1,000 ppm levulinic acid at 37°C for 72h, respectively. Virus particles were fixed in copper grids, stained, and visualized by Transmission Electron Microscope. (A) untreated TV; (B) TV treated by SDS; (C) TV treated with SDS and levulinic acid; (D) TV treated by SDS and chlorine, (E) TV treated by levulinic acid.
Chlorine is a well-known strong oxidizer that can react with many food chemicals including proteins, lipids, and nucleic acids. Although virus inactivation by chlorine has been extensively studied (Baert et al., 2008, Baert et al., 2009), the mechanism involved in virus inactivation is less understood and is highly debated. Therefore, we evaluated the damage of the combination of chlorine and SDS on TV. As shown in Fig. 25, we did not observe any suspected virus particles in treated samples. The virus particles physically disappeared with the exception of some minor debris appearing occasionally in the grids. This observation indicated that virus particles were completely disrupted by chlorine. In contrast, SDS only altered the structure of the viral capsid, leaving the overall capsid still visible under electron microscope. (Fig. 25).

Levulinic acid is an organic acid that is GRAS by the FDA (FDA CFR 172.515). The use of levulinic acid has been suggested to extend shelf life of fresh produce because of its ability to arrest chloroplast development during greening, and because it can be removed from the leaves of produce without causing any toxic effects (Jilani et al., 1996). In Figure 25 the viral particles treated by levulinic acid (E) and levulinic acid in combination with SDS (C) are shown. In the case of SDS and levulinic acid, only one potential viral particle is distinguishable and it is highly disrupted and misshapen. In the micrograph of virions treated by levulinic acid alone, no virus is detected at all. Only debris is visible. Thus this visual evidence shows SDS, levulinic acid, and chlorine all have the ability to severely disrupt TV viral particles.
4.4.4. Effect of sanitizers and surfactants on viral proteins. TV is a single-stranded positive-sense RNA virus which is surrounded by highly stable capsid proteins. Its capsid is composed of two proteins- the major capsid protein VP1 and the minor protein VP2 (Farkas et al., 2008). The VP1 protein plays critical roles in not only the viral attachment and entry into the host cell, but also in packaging and release of the mature virion (Jiang et al., 1992; Prasad et al., 1999). Although SDS, levulinic acid, SDS with chlorine, and levulinic acid with SDS have the ability to alter the structure of the viral capsid (Fig. 26), it remains to be clarified whether surfactants directly degrade the viral capsid protein. To detect direct action on the viral capsid, we incubated highly purified TV virus stock with each surfactant or sanitizer until the virus was completely inactivated (72 h), and then analyzed the samples by SDS-PAGE. The TV VP1 protein is approximately 58 kDa (Farkas et al., 2008). In Figure 26, as expected, there was a band for the untreated control. Bands were also visible for all other treatments with the exception of SDS in combination with chlorine. Because there was visible damage to the viral particles, but the VP1 capsid protein was still intact as established by SDS-PAGE, these results suggest that SDS and levulinic acid may alter the tertiary and quaternary structures, but not the primary structure of the viral capsid protein. SDS in combination with chlorine was the only treatment able to effectively degrade the viral capsid protein.
Figure 26: Effect of sanitizers and surfactants on viral protein. Highly purified TV was treated with 1,000 ppm SDS, 1,000 ppm SDS plus 1,000 ppm levulinic acid, 1,000 ppm SDS plus 200 ppm chlorine, or 1,000 ppm levulinic acid at 37°C for 72h. After the complete inactivation of virus, total viral proteins were analyzed by 12% SDS-PAGE followed by Coomassie blue staining.

4.4.5. Effect of sanitizers and surfactants on viral RNA. If viral genomic RNA is damaged or degraded, it is likely lethal to the virus. We used RT-PCR to determine whether surfactants and sanitizers can degrade TV viral RNA. Two primers were designed to amplify the VP1 gene from the viral genomic RNA. The treatments used were the untreated control, 1000 ppm of SDS, 1000 ppm SDS + 1000 ppm levulinic acid, 1000 ppm SDS + 200 ppm sodium hypochlorite, and 1000 ppm levulinic acid. Samples were incubated for 72 h and
then RNA extraction was done immediately following incubation. Experiments were done with extreme caution to ensure that no RNase was introduced into the samples because RNA can be easily degraded by environmental RNases. As expected, a band for the VP1 gene (with a size of approximately 1.5 kb (Farkas et al., 2008)) was present in the untreated control (Figure 27). Similar amounts of the VP1 gene were amplified from MNV-1 samples treated with each of the other treatments, with the exception of the SDS in combination with chlorine. As mentioned previously, chlorine has been proposed to interact with nucleic acids (Gerba, 2009; Sanglay, 2011). Thus, these results further suggest that chlorine does in fact disrupt viral RNA.
Figure 27. Effects of surfactants and sanitizers on viral RNA. TV was treated with 1,000 ppm SDS, 1,000 ppm SDS plus 1,000 ppm levulinic acid, 1,000 ppm SDS plus 200 ppm chlorine, or 1,000 ppm levulinic acid at 37°C for 72h. After complete inactivation of virus, viral genomic RNA was extracted from the samples. The VP1 gene was amplified by a one-step RT-PCR, and PCR products were visualized by 1% agarose gel electrophoresis.

4.5. Conclusion

The effectiveness of chlorine, SDS, levulinic acid, and combinations of these treatments for the removal or inactivation of Tulane virus in fresh produce and in virus stock over time was investigated in this study. Modest concentration of SDS in combination with chlorine was the most effective sanitizer associated with strawberries and virus removal. For lettuce, traditional chlorine solution performed the best, followed
by levulinic acid in combination with chlorine. Over a 72 h period incubation with the treatments, SDS with chlorine showed the most virucidal activity, giving 4.8 logs of viral reduction. None of the other treatments tested were able to successfully degrade viral protein or viral RNA, except for SDS in combination with chlorine. Ultimately, chlorine remains the most effective antiviral treatment under these conditions.
Chapter 5: Effectiveness of pulsed electric field processing on two human norovirus surrogates

5.1 Abstract

Fresh fruits and vegetables are a major vehicle for the transmission of foodborne enteric viruses such as norovirus. Both fresh produce and raw juice consumption are increasing to achieve a healthy diet. Multiple businesses have advertised the benefits of raw juice, claiming the nutrients, minerals, and desirable flavors remain intact. These are otherwise lost when juices are thermally treated. These non-processed juices typically have a shelf life of only 1-2 days and may not be microbiologically safe. Thermal processing provides a microbiologically safe product with a longer shelf life, but these juices are less desirable from both a sensory and functional food aspect. Pulsed electric field (PEF) processing may be an alternative non-thermal method of processing to solve the safety issue of fresh or raw juices. PEF processing allows the inactivation of many enzymes and microorganisms with minimal heat, therefore keeping nutrients intact and potentially extending product shelf life. In this study, the reduction of two human norovirus surrogates (MNV-1, TV) by PEF was evaluated. We found that under specific conditions, 1.2 and 2.4 log reductions were achieved for MNV-1 and TV, respectively. This is the first account of foodborne viral reduction achieved by PEF.
5.2. Introduction

Viruses are the most common source of foodborne outbreaks globally. Of these viruses, norovirus is the most prevalent, causing an estimated 19-21 million illnesses per year just in the United States (Hall et al., 2013). These disease incidence estimates are now much more reliable due to increased population-based surveillance, and more sophisticated modeling in recent studies. It is well known that fresh foods such as fruits and vegetables are one of the most common vehicles for norovirus gastroenteritis infection. In a meta-analysis study done by Hall et al., (2014), it was found that of 324 norovirus outbreaks that occurred during 2009-2012 with an implicated food, 75% of these outbreaks were attributed to food that was consumed raw. Furthermore, of the outbreaks where the source of contamination was narrowed down to a pinpointed single food source, 30% were from leafy vegetables and 21% were from fruits (Hall et al., 2014).

Many consumers have increased their fruit and vegetable consumption in order to fulfill a healthier lifestyle. This includes the consumption of not only store bought juices, but also the juicing of fresh fruits and vegetables at home for personal use. There have been many new boutique type juice stores advertising customizable “cold pressed” juice varieties, meaning juice that has not been processed and therefore retains all of the nutrients and minerals of the fresh foods going into these juice products. In the past two years in Columbus, Ohio alone, five cold pressed juice businesses have been established, all pushing the same benefits of raw juice with no thermal processing treatment to appeal to customers.
Traditionally, juices are most commonly thermally processed. Conversely, when at home or in these new juice businesses, consumers choose and may customize their own combinations of fruits and vegetables. These typically have undergone little to no processing beforehand. For commercial juice products, processors have been able to provide microbiologically safe products with a modest shelf life, but these products may have also lost some of the nutritional and sensory properties that are desired by consumers. Eliminating the thermal processing step and juicing fresh fruits and vegetables for consumption may keep the nutrients and sensory quality intact, while sacrificing the microbiological safety of the product and its shelf life. For the purposes of both preserving sensory and nutritional aspects and extending shelf life, the use of pulsed electric fields is an alternative non-thermal means of processing beverages such as juices, smoothies, or milk products.

Pulsed electric field (PEF) processing is a novel non-thermal technology that may be used to pasteurize or sterilize liquid food products. PEF processing applies high voltage rapid pulse electricity to a fluid food or beverage. It has successfully inactivated both harmful microorganisms and deleterious enzymes with minimal increases in temperature during treatment in fruit juices and milk (Mosqueda Melgar et al., 2008; Ahyan et al., 2012; Bendicho et al., 2012). It gained regulatory approval and was successfully used by the Genesis Juice Corporation, Eugene Oregon, in 2003 to pasteurize their entire inventory of fresh juices after receiving a warning letter from the FDA (FDA, 2003). The lethal effects of PEF have been reported to cause irreversible
damage to cell membranes. The most important factors in PEF processing are field strength, pulse duration, and treatment temperature (Shilling et al., 2008). Consumer awareness of both food processing and their desire for minimally processed fresh-like foods has increased. While electricity is more acceptable to consumers than irradiation, the idea of using electricity in foods has met some challenges with consumers. Processing with PEFs may serve as a way to achieve safe, fresh like food qualities and extend shelf life of food products while maintaining the quality, sensory, and nutritional aspects desired by consumers (Jia et al., 1999; Bendicho et al., 2002). It was suggested to be more efficient than thermal processing in certain instances (Hoogland and de Haan, 2007; Sampedro et al., 2009; Sanchez-Moreno et al., 2005; Charles-Rodriguez et al., 2007).

The effects of PEF on spoilage and pathogenic bacteria, molds, and yeasts have been studied extensively (references). Unfortunately, there have been limited studies investigating the effects of PEF on viruses, and only one previous study that focused on a foodborne virus, specifically. In 2002, Khadre and Yousef found that varied titers of rotavirus were resistant to PEF treatment conditions of 20-29 kV/cm, 3 µsec pulses, and a total duration of 145.6 µsec per treatment. The researchers noted that experiments were carried out at 25°C, and noted previously that at electric field intensities above 20 kV, the temperature after treatment was higher than 35°C (Unal et al., 2001).

In this study, we attempted to optimize the conditions of PEF treatment in order to generate inactivation of two different human norovirus surrogates- murine norovirus and the newer human norovirus surrogate Tulane virus.
5.3. Materials and Methods

5.3.1. Cell culture and virus stock. Murine norovirus strain MNV-1 was provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine. MNV-1 was propagated in the mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA) as follows. RAW 264.7 cells were cultured and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37˚C and a 5% CO2 atmosphere. To prepare MNV-1 stock, confluent cells were infected with MNV-1 at a multiplicity of infection (MOI) of 10. After 1 h of incubation at 37˚C, 15 mL of serum free DMEM were added. Two days after infection, the virus was harvested. The flasks were subjected to freeze-thaw three times, and the supernatant was collected after centrifugation at 5,000 rpm for 20 min at 4°C. The titer of the virus stock was $10^8$ PFU/mL.

TV was kindly provided by Dr. Xi Jiang (Cincinnati Children’s Hospital, Cincinnati, OH). TV was propagated in the LLC-MK2 cell line (ATCC, Manassas, VA). Cells were cultured in 150 cm² tissue culture flasks (Corning Life Sciences, Wilkes-Barre, PA) containing Opti-MEM (minimum essential media) supplemented with 2% fetal bovine serum (Invitrogen, Carlsbad, CA) and 2mM GlutaMAX-1 (Invitrogen, Carlsbad, CA). The growth medium was removed from confluent flasks of LLC-MK2 cells and infected with TV at a MOI of 10. Flasks were incubated at 37°C and 5% CO2 for 1 h, with agitation every 15 min. Opti-MEM was added to the flasks and they were
incubated at 37℃ and 5% CO2 for 48h. When CPEs were observed, flasks were subjected to freeze-thaw cycles, centrifugation, and re-suspension as described above. The titer of the virus stock was $10^6$ PFU/mL.

5.3.2. Pulsed electric field. A bench scale PEF processor model OSU 4H (The Ohio State University, Columbus, OH) was used for treatments. The parameters used during experiments are listed in Table 15. An operating voltage of 29.5 kV and treatment times of 2.2 and 3 μsec were used.

Table 15. Summary of PEF operating conditions.

<table>
<thead>
<tr>
<th>Description</th>
<th>Operating Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pulse generator</strong></td>
<td></td>
</tr>
<tr>
<td>Operating voltage</td>
<td>29.5 kV</td>
</tr>
<tr>
<td>Peak current</td>
<td>8.8 A</td>
</tr>
<tr>
<td>Polarity</td>
<td>Bipolar</td>
</tr>
<tr>
<td>Wave shape</td>
<td>Square wave</td>
</tr>
<tr>
<td>Pulse duration</td>
<td>0, 2.2 μs, 3 μs</td>
</tr>
<tr>
<td>Repetition rate</td>
<td>2 kHz</td>
</tr>
<tr>
<td><strong>Treatment chamber</strong></td>
<td></td>
</tr>
<tr>
<td>Number of chambers</td>
<td>4</td>
</tr>
<tr>
<td>Cooling capacity</td>
<td>Circulating water from ice bath</td>
</tr>
<tr>
<td>System flow rate</td>
<td>121.1 ml/min</td>
</tr>
<tr>
<td>Number of pulses per chamber</td>
<td>11.9</td>
</tr>
<tr>
<td><strong>Total treatment time</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0, 104 μs, 143 μs</td>
</tr>
<tr>
<td><strong>Fluid handling</strong></td>
<td></td>
</tr>
<tr>
<td>Batch size</td>
<td>1 L</td>
</tr>
<tr>
<td>Type of fluid</td>
<td>Liquid, viscosity of 1.0 cP</td>
</tr>
<tr>
<td>Conductivity of fluid</td>
<td>0.18 S/M</td>
</tr>
<tr>
<td>Back pressure</td>
<td>0 PSI</td>
</tr>
</tbody>
</table>
5.3.3. MNV-1 and TV plaque assay. MNV-1 plaque assay was performed in RAW 264.7 cells as described previously. In short, RAW 264.7 cells were seeded in 6 well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of $2 \times 10^5$ cells per well. After 24 h of incubation, cells were infected with 400 µL from a 10-fold dilution scheme of the virus. After 1 h of incubation at 37°C with agitation every 15 min, overlay containing 2.5 mL of minimal eagle medium (MEM) containing 2% FBS, 1% sodium bicarbonate, 0.1 mg/mL of kanamycin, 0.05 mg/mL of gentamicin, 15 mM HEPES (pH 7.7), 2mM L-glutamine, and 1% agarose was added to the plates. After incubation at 37°C for two days, the plates were fixed with 10% formaldehyde, and the plaques were then visualized by staining with crystal violet. TV plaque assay was performed in the same way except that LLC-MK2 cells were used in the assays and the plaques were fixed 72 h post-inoculation.

5.3.4. Statistical analysis. All experiments were done in triplicate. The surviving viruses were expressed as mean log viral titer ± one standard deviation. Data were analyzed using the General Linear Model function and Tukey’s pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

5.4. Results and Discussion

The only published attempt of PEF inactivation of foodborne viruses was by Khadre and Yousef (2002). These authors did not find any significant reduction for both
high and low titers of rotavirus. In this study, the sensitivities of two human norovirus surrogates to PEF were evaluated. The results for MNV-1 are listed in Table 16.

Table 16. Effect of PEF on MNV-1 viral titer. Values are listed as log_{10} viral titer ± one standard deviation. All experiments were carried out in triplicate. Letters are representative of being significantly different.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MNV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>11.0 ± 0.7 a</td>
</tr>
<tr>
<td>2.2 µsec</td>
<td>10.7 ± 0.3 a</td>
</tr>
<tr>
<td>3.0 µsec</td>
<td>9.8 ± 0.3 b</td>
</tr>
</tbody>
</table>

When MNV-1 was treated with 2.2 µsec pulses, only a 0.3 log reduction was observed, but after treatment with 3.0 µsec pulses, a 1.2 log reduction was observed which was statistically significant. There was a slight increase in temperature corresponding to each treatment for both viruses tested. All experiments were conducted at ambient temperature (25ºC), and the temperature of the virus stock after treatment was 32ºC and 42ºC for the 2.2 and 3 µsec treatments, respectively. The values for PEF inactivation of TV are shown in Table 17. Showing similar results to MNV-1 after the 2.2 µsec treatment, only a 0.3 log reduction was observed. Interestingly, for the 3µsec treatment a 2.4 log reduction was demonstrated, which was also statistically significant. From these experiments, we find that at higher pulse frequency and pulse durations of PEF, MNV-1 is likely more resistant to treatment.
Table 17. Effect of PEF on TV viral titer. Values are listed as $\log_{10}$ viral titer ± one standard deviation. All experiments were carried out in triplicate. Letters are representative of being significantly different.

<table>
<thead>
<tr>
<th></th>
<th>TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>$10.1 \pm 0.5$ a</td>
</tr>
<tr>
<td>2.2 μsec</td>
<td>$9.8 \pm 0.6$ a</td>
</tr>
<tr>
<td>3.0 μsec</td>
<td>$7.7 \pm 0.5$ b</td>
</tr>
</tbody>
</table>

These results are promising because they are the first account showing significant reduction of a foodborne virus by PEF treatment. This is promising as PEF treatment is superior to thermal processing with regard to nutritional content and sensory quality, and is equivalent to thermal processing in shelf-life of these types of juice or milk products. It has been reported that PEF requires an optimal conductivity range (most foods without the addition of salt range from 0.1-0.5 S/M) and requires the product in liquid state that is easily pumped through the system (Buckhow et al., 2013; FDA, 2011). If the conductivity in the solution is higher, the flow of electrical current will be increased, reducing the resistance of the chamber, thus requiring more energy to achieve a specific pulse electric field (FDA, 2011). These criteria limit PEF applications, but this technology may be ideal for wide range of liquid food products that can be successfully pasteurized in this manner.
5.5. Conclusion

These results show that under specific treatment condition we used (3.0 μsec pulses) we were able to effectively reduce the viral titer of two human norovirus surrogates in liquid virus stock. This is the first account of PEF causing a significant reduction in viral titer of a foodborne virus.
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