Enhanced Sanitization of a Human Norovirus Surrogate in Fresh Vegetables and Fruits 
by a Combination of Surfactants and Sanitizers

THESIS

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By

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Abstract

Fruits and vegetables are a major vehicle for transmission of foodborne enteric viruses since they are easily contaminated at pre- and post-harvest stages and undergo little or no processing. Particularly, human norovirus accounts for more than 40% of outbreaks in fresh produce. However, commonly used sanitizers are ineffective for sanitization of foodborne viruses from fresh produce. Therefore, there is an urgent need to develop a more effective sanitizer for removing foodborne viruses from fresh produce. In this study, we systematically evaluated the effectiveness of a panel of sanitizers and surfactants on sanitization of a human norovirus surrogate, murine norovirus 1 (MNV-1), from fresh produce. We showed that organic acids slightly enhanced virus sanitization, and such effect depended on the type of fresh produce. Importantly, we found that surfactants, including sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), Triton X-100, and polysorbates (Tween 20), significantly enhanced the sanitization of viruses from fresh fruits and vegetables. While tap water alone and chlorine solution (200 ppm) only reduced less than 1.2 logs of virus in all fresh produce, 50 ppm of surfactant solution was able to achieve 3 logs of virus reduction in strawberry and approximately 2 logs of virus reduction in lettuce, cabbage and raspberry. Moreover, approximately 3 logs of virus reduction were observed in all the tested fresh produce after sanitization with a combination of 50 ppm of each surfactant and 200 ppm of chlorine solution. These
results demonstrated that the combination of a surfactant with a commonly used sanitizer enhanced the efficiency of removing viruses from fresh produce by approximately 100 times.

We also systemically determined the virucidal activities of surfactants and provided mechanistic insights into how viruses were inactivated by surfactants. Using an enveloped virus vesicular stomatitis virus, VSV) and a non-enveloped virus (MNV-1) as the models. We found that all four tested surfactants (SDS, NP-40, Triton X-100, and Tween 20) were able to show considerable virucidal activity against both VSV and MNV-1 in a concentration-dependent manner. SDS appears to be the most effective surfactant against both viruses. In addition, SDS significantly enhanced the virucidal activity of chlorine solution, the most widely used sanitizer in food industry. We further demonstrated that SDS altered the structure of the capsid of MNV-1 and disrupted the viral envelope of VSV. However, SDS did not degrade the viral capsid protein or viral genomic RNA. Taken together, our results demonstrated that (i) surfactants significantly enhanced virus sanitization in fresh produce, (ii) surfactants possess virucidal activity, and (iii) the combination of surfactants and sanitizers is a more effective strategy to inactivate viruses. Since SDS is an FDA approved food additive and polysorbates are FDA recognized GRAS (Generally Recognized as Safe) products, implementation of these novel sanitization strategies would be a feasible approach to efficiently reduce the virus load in fresh produce. These strategies also have great potential to be used as novel disinfectants for sanitization of other pathogens in the food industry.
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ABBREVIATIONS

ANOVA – Analysis of variance
CaCV – Canine calicivirus
CDC – Centers for Disease Control and Prevention
CPE – Cytopathic effect
DMEM – Dulbecco’s modified eagle medium
ELISA – Enzyme-linked immunosorbant assay
EM – Electron Mircoscopy
FBS – Fetal bovine serum
FCV – Feline calicivirus
EDTA – Ethylenediamine tetraacetic acid
BHK-21 – A baby hamster kidney cell line
FDA – Food and Drug Administration
GRAS – Generally recognized as safe
HBGA - Histo-blood group antigen
HIV-1 – Human immunodeficiency virus 1
HSV-2 – Herpes simplex virus 2
HuNoV – Human norovirus
LLC-PK – A renal epithelial cell line derived from porcine kidneys
MEM – Minimal eagle medium
MNV-1 – Murine norovirus
MOI - Multiplicity of infection
NIAID – National Institute of Allergy and Infectious Diseases
NP-40 – Nonidet P-40
ORF - Open reading frame
PCR – Polymerase chain reaction
PBS – Phosphate buffered saline
PFU – Plaque forming units
Ppm – Parts per million
RAG2 – Recombination-activating gene 2
RAW 264.7 – A mouse macrophage cell line
RdRp - RNA-dependent RNA polymerase
RNA - Ribonucleic acid
RT-PCR - Reverse transcriptase-polymerase chain reaction
SDS- Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
STAT-1 – Signal transducer and activator of transcription 1
TEM - Transmission electron microscopy TMV – Tobacco mosaic virus
TSP – Trisodium phosphate
VAP-A – Vesicle associated membrane protein associated protein A
Vero – An African Green Monkey kidney cell line
VLP - Virus-like particle
VSV – Vesicular stomatitis virus
1.1. Introduction to foodborne viruses

Foodborne illness can be caused by viruses, bacteria, fungi, or parasites, but viruses are the major causative agent of foodborne gastroenteritis. There are approximately 79 million estimated cases of foodborne illness that occur every year in the United States alone, and more than 67% of these cases can be attributed to viruses, a much higher percentage than any other cause (Mead et al., 1999). According to more recent outbreak data from the Centers for Disease Control and Prevention, there are about 48 million cases of foodborne illnesses, with viruses causing about 60% of these (Fox, 2011). Viruses are extremely small microorganisms ranging from 15-400 nm in diameter. They can cause a variety of diseases in humans, animals, and plants (Koopmans and Duzier, 2002). Because viruses are strict intracellular parasites, they can only replicate inside of a host, indicating that the virus is unable to amplify in foods or during storage (Vasikcova et al., 2005). Viruses in general can be transmitted through a number of routes, such as by aerosol, sexual intercourse, contact with blood of an infected person, contact with an infected animal, or by vectors. Specifically, foodborne viruses are most commonly transmitted through the fecal-oral route, i.e. the ingestion of contaminated food or water. A foodborne virus is defined as any virus that may contaminate food or water and is able to cause illness via the fecal-oral route.
Foodborne viruses usually attack and infect the digestive system, and are subsequently dispersed by shedding into stool or by vomiting (Koopmans and Duzier, 2002; Vasicova et al., 2005).

Based on the type of illness they cause, foodborne viruses can be classified into three major groups. The first group includes viruses that cause gastroenteritis. Examples of these viruses include human norovirus, sapovirus, rotavirus, astrovirus, and adenovirus. The second group includes viruses that cause hepatitis in humans. Hepatitis A and hepatitis E viruses are the representatives in this group. The third group consists of viruses that invade human central nervous system. For example, poliovirus and enterovirus 71 cause poliomyelitis and severe neurological diseases in humans, respectively. Among foodborne viruses, human norovirus has long been considered the most prominent cause associated with foodborne illness. Foodborne viruses share some universal features, including: 1) only a few particles are needed to cause infection, 2) high numbers of particles may be shed in stool of an infected person (up to $10^{11}$), 3) living cells are required for viruses to replicate, 4) foodborne viruses are typically very stable in the environment, and 5) they are resistant to low pH and thus can survive in 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 heat, pH, and disinfection than bacteria (Koopmans and Duzier, 2004). As a result, current procedures to prevent bacterial infections in food processing, preservation, and storage may not be fully effective against viral pathogens (Rzezutka and Cook, 2004).

In an effort to meet the urgent demand for novel technologies to inactivate foodborne viruses, we carried out the present study with four objectives: (1) to perform a review of the literature on the subject of norovirus, current sanitization techniques, and surfactants, (2) to investigate the
efficiency of various current sanitizers for removing norovirus from fresh produce, (3) to evaluate the effectiveness of four different surfactants for removing norovirus from fresh produce, and (4) to explore the mechanism underlying the removal or inactivation of the viruses using the selected surfactants and sanitizers.

1.2. The discovery of human norovirus

The first outbreak of human norovirus was reported in Norwalk, Ohio in 1968 (Kapikian et al, 1972). More than 50% of students and teachers at an elementary school there developed a stomach illness that involved nausea and vomiting. When they went home, 32% of household contacts also became sick. It was not until 1972, though, that Kapikian et al. determined the etiology of this disease. Electron microscopy analysis identified small-round structured viral particles, but not bacterial pathogens, in stool samples. This is where norovirus got its former name of Norwalk-like virus (Kapikian, 1996). Some other names used to refer to norovirus are winter vomiting disease, viral gastroenteritis, small round-structured viruses, and ‘stomach flu,’ which is a very broad term describing any inflammation of the stomach caused by either bacteria or viruses (Appleton, 1987). These small round-structured viruses range from 27-40 nm in diameter (Figure 1). Soon after the discovery of human norovirus, many different strains were isolated worldwide. To date, norovirus has been listed as a major causative agent of gastroenteritis in both developed and developing countries where it affects people of all ages.
1.3. Symptoms of human norovirus infection

Symptoms of human norovirus infection include diarrhea, vomiting, nausea, dehydration, and a high fever in some cases. The virus has a 1-3 day incubation period with symptoms normally persisting for 2-3 days (Koopmans et al., 2006). The virus is shed in the stools of infected persons throughout the infection and even after full clinical recovery. The duration of virus shedding is increased in the immunocompromised, the elderly, or in children (Atmar et al., 2008; Rockx et al., 2002). Norovirus has also been shown to be present in the vomit of infected persons (Bednar et al., 1999). Though the virus may cause severe discomfort and embarrassment, the clinical manifestation is normally relatively mild with an average mortality rate of only about 0.003% (CDC, 2011). Out of the 23 million cases of norovirus infection every year, only about 300 of them are fatal, most in the extremely young or elderly, or those who are immunocompromised (Goodgame, 2006). However, the virus is extremely contagious and infectious, with only a few particles (usually less than 10) being sufficient to cause infection (Teunis et al., 2008, Koopmans and Duizer, 2004). Recent human volunteer studies and
mathematical modeling have showed the average probability of infection for a single norovirus particle to be close to 0.5 (Teunis et al., 2008). Outbreaks typically occur in any area where a high number of people are in close contact with each other, such as restaurants, swimming pools, cruise ships, nursing homes, schools, hotels, and military installments (Goodgame, 2006, Rockx et al., 2002).

1.4. Modes of transmission

Fecal-oral transmission is the most important and most common mode of transmission for noroviruses. Transmission through infectious vomit or feces either by direct contamination (through hand and/or mouth contact) or by indirection contamination (such as by aerosolization) may also account for the rapid and excessive transmission of the virus in closed settings (Widdowson et al., 2005). Most commonly, contaminated food or water is the primary source of infection, and person-to-person spread further disseminates the outbreak (Becker et al., 2000). There are many features of norovirus contributing to its transmission. First, norovirus has an extremely low infectious dose (Teunis et al., 2008). Secondly, virus shedding occurs even after viral infection symptoms have diminished. Thirdly, norovirus has high stability in environment. It is resistant to acidic conditions and chlorine, and is also stable at a variety of temperatures (Duizer et al., 2004). Fourthly, norovirus strains are highly diverse. Last but not least, there is no long term immunity to norovirus; therefore, repeated infections may occur throughout life.

1.5. Human norovirus and food safety

According to a recent report from the Centers for Disease Control and Prevention, viruses account for about 58 percent of foodborne illnesses (CDC, 2011). As listed in Table 1, the
number of norovirus cases surpasses all other pathogens by a large margin (CDC, 2011). Among all foodborne viruses, noroviruses are responsible for more than 90% of viral foodborne illnesses. For example, noroviruses cause more than 90% of non-bacterial acute gastroenteritis. Approximately 23 million people suffer from norovirus-induced gastroenteritis each year in the United States (CDC, 2010). In the period of 2006-2007, about 46% of all outbreaks were attributed to noroviruses (Figure 2A). Figure 2B shows the breakdown of norovirus cases by setting. Long-term care facilities had the most outbreaks (33.5%) whereas schools and childcare communities had the fewest at 13.0%. The total number of annual outbreaks may even be underestimated due to underreporting of outbreaks and lack of rapid detection of the virus. In 1982, four criteria were established to confirm a norovirus outbreak: 1) vomiting in more than half of the infected people, 2) a mean incubation period of 24-48 hours, 3) an average duration of illness of 12-60 hours, and 4) the absence of any bacterial pathogen in stool cultures (Kaplan et al., 1982). More recently, these criteria were re-evaluated and they were still found to be accurate with 99% specificity and 68% sensitivity (Turcios et al., 2006).

Table 1. The top 8 pathogens contributing to foodborne illness (adapted from CDC, 2011)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Estimated number of illnesses</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus</td>
<td>5,461,731</td>
<td>58</td>
</tr>
<tr>
<td><em>Salmonella</em>, non-typhoidal</td>
<td>1,027,561</td>
<td>11</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>965,958</td>
<td>10</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.</td>
<td>845,024</td>
<td>9</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>241,148</td>
<td>3</td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>131,254</td>
<td>1.4</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>97,656</td>
<td>1</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>86,686</td>
<td>1</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td><strong>94.4</strong></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Recent foodborne outbreak data from the Centers for Disease Control and Prevention. A) Recent outbreak data from 2006-2007 B) Outbreak data by setting (CDC, 2011)

1.6. Norovirus and high-risk foods

There have been many outbreaks of human norovirus in recent years, most of which occur in high risk foods such as fresh produce and seafood. Fresh produce as a group are considered a major vehicle of norovirus because they normally undergo little to no processing before consumption. This allows for contamination anywhere from pre-harvest to post-harvest stages (Beauchat, 1996; Everis, 2004). It has been reported that norovirus accounted for more
than 40% of outbreaks in fresh produce from 1998-2005 in the United States (CDC, 2006). These outbreaks of norovirus occurred in fresh produce including lettuce, tomatoes, melons, green onions, strawberries, raspberries, blueberries, peppers, fresh cut fruits, and other vegetables (Figure 3) (DeWaal and Bhuiya, 2007). Mixed fruits, mixed vegetables, puree, salad, salsa are also high risk foods for norovirus contamination (CDC, 2010b; Le Guyader et al., 2004). One major route with a high probability of contamination is the use of contaminated water for irrigation or washing. Contamination may also occur by infected workers handling the food during harvesting, processing, or distribution. With an increasing number of people striving to eat healthier by increasing their consumption of fruits and vegetables, this has become a major public health concern.

Continued

Figure 3. Norovirus outbreaks in fresh produce (adapted from DeWaal and Bhuiya, 2007)
A) Pie chart representative of all produce outbreaks between 1990-2005 showing that norovirus accounts for 40% of these outbreaks. B) Norovirus accounts for 50% of all produce dish outbreaks. C) Norovirus accounts for 40% of all fruit related outbreaks. D) Norovirus accounts for 25% of all vegetable related outbreaks
Figure 3 continued

B. Produce Dish Outbreaks

C. Fruit Outbreaks

Continued
Another type of high risk food for norovirus contamination is seafood, particularly shellfish including mussels, oysters, and clams. This is due to the fact that they are filter-feeding animals that sieve liters of water in per day. If viruses or contaminants are present in the water, the seafood can easily become contaminated. Specifically, shellfish and oysters are bio-filters and accumulate the viruses in their tissues. High numbers of norovirus particles can be detected in shellfish and oyster tissues (Metcalf et al., 1979; Rippey, 1994; Burkhardt and Calci, 2000; Croci et al., 2006). The distribution of norovirus in oyster tissues has been experimentally investigated. It was found that norovirus was first accumulated in the stomach of the oyster and then disseminated into muscles. In fact, many norovirus outbreaks were found to be associated with the consumption of uncooked or undercooked shellfish and oysters. In a study monitoring outbreaks of norovirus in England and Wales from 1995-2000, it was found that out of 86 total outbreaks, raw oysters were implicated in 20 of them, and salads or vegetables caused another 17. These findings show the magnitude of impact of these foods on foodborne illness (Lopman et
al., 2003). Outbreaks in shellfish have also been noted in the U.S., China, Japan (Hamano et al., 2005), France (LaGuyader et al., 2006), Germany (Schreier, 2003), Italy (Prato et al., 2004), the Netherlands, and many other countries (Boxman et al., 2006) in recent years.

Figure 4. Taxonomy of the Caliciviridae family (adapted from Green et al., 2000). Shows the phylogenetic tree of Caliciviridae and Picornaviridae. There are 4 major branches under the family of Caliciviruses – “Norwalk-like viruses,” “Sapporo-like viruses,” Lagovirus, and Vesivirus.

1.7. Human norovirus genogroups and strain diversity

Noroviruses belong to the family Caliciviridae under the genus of Norovirus. There are four other genera in this family, namely, Vesivirus, Lagovirus, Recovirus and Sapovirus. The classification of Caliciviridae and the representative virus are shown in Figure 4. All of these
Caliciviruses are non-enveloped, single-stranded positive-sense RNA viruses. The outer shell of the virus particle is a highly stable protein capsid which exhibits icosahedral symmetry. These caliciviruses are known to infect many types of organisms, such as humans, pigs, swine, cattle, cats, and chickens (Hansmann, 2010). Many viruses in genus *Norovirus*, *Sapovirus*, *Recovirus* within *Caliciviridae* cause acute gastroenteritis. However, caliciviruses also cause other diseases. For example, murine norovirus in genus *Norovirus* causes systemic infection in mice (Karst et al., 2003). Feline calicivirus within the genus *Vesivir* causes respiratory tract infections (Doultree et al., 1999). Rabbit haemorrhagic disease virus within genus *Lagovirus* causes haemorrhagic disease in rabbit (Belz, 2004).

Since the discovery of norovirus, thousands of norovirus strains have been identified worldwide. Noroviruses are very diverse both antigenically and genetically (Ando et al., 2000). Previously, norovirus was classified based on cross-challenge studies and serum cross-reactivity analysis, but this method was problematic because it had poor reproducibility and accuracy (Green et al., 1995; Ando et al., 2005). Therefore, reverse-transcription polymerase chain reaction (RT-PCR) and sequencing of the viral genome have become more prevalent for determining the differences between strains of norovirus (Ando et al., 2000; Jiang et al., 1990; Katayama et al., 2000). Based on the phylogenetic analysis of viral capsid gene (VP1), five genogroups of noroviruses, GI, GII, GIII, GIV, GV, have been discovered and assigned. Genogroup II contains the majority of human noroviruses, while genogroups I and IV also infect humans (Green et al., 2001; Zheng et al., 2006). In contrast, genogroup III is found in cows, and genogroup V includes murine norovirus which infects mice. Additionally, genogroups I and II also include some porcine noroviruses. These genogroups can be further divided into different subtypes. For example, there are 19 genotypes of GII noroviruses (Gospodarek and Zalas-
Wiecek, 2009). Currently, the most prevalent human norovirus belongs to genogroup II, genotype 4 (GII.4). The evolution of GII.4 noroviruses has resulted in many new variants since 2002, such as the Laurens and Minerva variants, which resulted from outbreaks during 2006-2007 (Siebenga et al., 2008). In the past ten years, more than three global pandemics have occurred, all of which were strains of GII.4 (Hansman, 2010). Up to date, GII.4 is the most studied genotype of norovirus.

1.8. Molecular biology of human norovirus

Norovirus is a single-stranded positive-sense RNA virus and its genome is approximately 7.7 kb and encodes 3 open reading frames (ORFs) (Jiang et al., 1993). Figure 5 represents the genome organization of norovirus. 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 while ORF 3 encodes the minor structural capsid protein VP2 (Hardy, 2006).

![Genome organization of human norovirus](image)

**Figure 5. Genome organization of human norovirus.** The norovirus genome consists of three open reading frames (ORFs). ORF 1 encodes nonstructural proteins. ORF2 and ORF3 encode the major capsid protein VP1 and the minor capsid VP2, respectively.
1.8.1. The major capsid protein VP1

The first structural protein of norovirus is VP1, which is the major capsid protein encoded by ORF2. VP1 normally ranges from 530-555 amino acids in length, and has an approximate molecular weight of 58-60 kDa (Figure 6). VP1 forms the outer shell of virus particle and plays many essential roles in the viral life cycle. First, VP1 protein binds to its functional receptor, the histo-blood group antigen (HBGA) on the host cell that mediates virus entry. Secondly, VP1 protein determines the antigenicity and strain specificity (Prasad et al., 1999). As mentioned earlier, five genogroups of norovirus have been classified according to the diversity of VP1 protein. Thirdly, VP1 protein is the host protective antigen that is responsible for eliciting neutralizing antibody, T cell and mucosal immunities (Bertolotti-Ciarlet et al., 2003). Lastly, VP1 play many other roles in virus life cycle such as uncoating, assembly and exit (Hardy, 2006).

The ORF2 of human norovirus was first expressed using recombinant techniques by Jiang et al. (1992). It was found that expression of VP1 gene alone in E.coli resulted in the formation of virus-like particles (VLPs) that are antigenically and morphologically similar to native human norovirus virions. Subsequently, VP1 has been expressed in many expression systems such as yeast, insect cells, and mammalian cells (Ma and Li, 2011). Indeed, the VLP has been an important tool to study the epidemiological, immunological, structural, and biochemical properties of human norovirus. Cryo-electron microscopy (cryo-EM) and X-ray crystallography analyses of human norovirus VLPs showed that caliciviruses exhibit a T= 3 icosahedral symmetry with 180 molecules of the capsid protein organized into 90 dimers (Figure 7) (Prasad et al., 1999). The structure of the capsid protein can be divided into two principal domains, shell
(S) and protruding (P), linked by a flexible hinge region (Figure 7). The S domain is involved in the formation of the icosahedral shell, and the P domain forms the prominent protrusion emanating from the shell (Prasad et al., 1999). The P domain is further divided into two sub-domains, P1 and P2. P2 is an insertion into the P1 domain of about 127 amino acids (Figure 6). The P domain is the primary site of receptor interaction and receptor binding, which plays an essential role in norovirus infection and determines the host susceptibility (Tan and Jiang, 2003). Furthermore, this histo-blood group antigen (HBGA) binding site and specific amino acids involved in receptor binding have been identified in the P dimer interface (Figure 8) (Tan and Jiang, 2003).

Figure 6. Diagram of the primary sequence of human norovirus capsid protein (adapted from Tan and Jiang, 2003). The capsid protein is composed of an N terminal section, a shell (S) domain, and a protruding (P) domain.
Figure 7. Tertiary structure of human norovirus VLPs (adapted from Hutson et al., 2004)

Human norovirus VLPs are composed of a P domain, S domain, and N terminal arm. Their structure is composed of 90 dimers of capsid protein exhibiting T=3 icosahedral symmetry.

Figure 8. Receptor binding region in norovirus P dimer interface (adapted from Tang and Jiang, 2010). There are four levels of norovirus structure shown. The furthest left image is of norovirus under electron microscopy. The middle left image is a single virus like particle (VLP). The middle right image is the P dimer specifically showing the HBGA binding site in color, and the furthest right image is the crystal structure of the HBGA binding area.
1.8.2. The minor capsid protein VP2

Norovirus and other caliciviruses also encode a minor capsid protein VP2, which is composed of 208-268 amino acids with a molecular weight of about 29kD. Like VP1, VP2 also exhibits high sequence variability among different strains (Seah et al., 1999).

VP2 is a very basic protein which has an isoelectric point (pI) of more than ten. The highly basic nature of VP2 has led to the speculation that it may be involved in RNA binding (Glass et al., 2000). Indeed, even though the exact role of VP2 in the replication cycle remains to be clarified, there is evidence indicating that VP2 plays a role in RNA packaging (Hardy, 2006). It has been reported that the presence of VP2 resulted in an increased VP1 expression levels, suggesting that VP2 regulates the synthesis of VP1 protein (Bertolotti-Ciarlet et al., 2003). Furthermore, VP2 increased VP1 stability and protected VP1 from disassembly and protease degradation (Bertolotti-Ciarlet et al., 2003). The possible mechanism of the VP2-mediated stabilization of VP1 capsid protein is shown in Figure 9.
Figure 9. Mechanism of VP1/VP2 interaction  (adapted from Bertolotti-Ciarlet et al., 2003)
Panel A shows VP1-VLP’s sensitivity to proteases. Panel B shows that VP2 helps to stabilize VP1’s structure.

1.8.3. Non-structural proteins

There are six non-structural proteins encoded by ORF1 of norovirus. The first protein is p48, which gets its name for its molecular weight. P48 interacts with a protein called vesicle associated-membrane protein-associated protein A (VAP-A) which regulates docking and fusion of SNARE-mediated vesicle fusion (Weir et al., 1998). Alternately, p48 may collaborate with VAP-A’s localization characteristics to help anchor membrane-bound complexes for replication (Weir et al., 1998).

The second non-structural protein is the NTPase, also called p41. Pfister and Wimmer found that this p41 has NTPase activity but not helicase activity (Pfister and Wimmer, 2000).

The third structural protein is p22. It is present in a p22-VPg-3CL^pro sequence, a precursor in the proteolytic processing pathway, while its function is yet to be identified (Belliot et al., 2003).

The fourth non-structural protein is VPg of about 15kDa. VPg is believed to bind to genomic
and subgenomic RNAs (Burroughs and Brown, 1978) and thus stabilize the genomic RNA. VPg also plays a role in recruiting translational machinery to mRNA. This hypothesis was further supported by the finding that VPg interacts directly with eIF3 and 40S subunits (Daughebauch et al., 2003).

The fifth nonstructural protein is 3CL\textsuperscript{pro}, which represents ‘3C-like’ because of its similarity to picornavirus 3C protein. 3CL\textsuperscript{pro} is a protease that proteolytically cleaves the polyprotein. Someya et al. (2002) suggested that norovirus 3CL\textsuperscript{pro} consisted of a catalytic dyad of His 30 and Cys 139. Many substrate specificities have been identified in norovirus 3CL\textsuperscript{pro}. It was proposed that 3CL\textsuperscript{pro} plays a role in inhibiting synthesis of cellular proteins in infected cells, as evidenced by the fact that 3CL\textsuperscript{pro} was able to cleave polymerase A (polyA) binding protein in vitro (Kuyumcu-Martinez et al., 2004).

The last nonstructural protein is the RNA-dependent RNA polymerase (RdRp). RdRp is located at the C-terminal end of ORF1. RdRp plays an essential role in viral genome replication and RNA synthesis. It was found that the norovirus RdRp contained RNA polymerase catalytic and structural elements that were homologous to other positive RNA viruses (Ng et al. 2002; Ng et al., 2004). In addition, it has been reported that a protein, the RdRp-3CL\textsuperscript{pro} precursor with dual function as a protease and polymerase accumulates both \textit{in vitro} and in infected cells (Ng et al., 2002).
1.9. Human norovirus receptors

Surface receptors are required for virus attachment to host cells. The histo-blood group antigens (HBGAs) have been identified as receptors that interact with human norovirus and mediate virus attachment and entry. HBGAs are carbohydrate complexes that are present on the surface of erythrocytes as well as the intestinal, genitourinary, and respiratory epithelia (Ravn and Dabelsteen, 2000). They also exist as free oligosaccharides in saliva, blood, milk, and contents of the intestine (Tan and Jiang, 2005). There are three major families of HBGAs- Lewis, ABO, and secretor, which are specifically recognized by different norovirus strains. Since virus-like particles (VLPs) are structurally and antigenically similar to the authentic viruses, the mechanism of the interaction between VLPs and HBGAs receptors has been extensively studied. It has been demonstrated that, and many amino acid residues in P domain of the VP1 protein are responsible for the specificity of receptor binding (Tan and Jiang, 2005; Tan et al., 2008).

The discovery of human HBGAs as norovirus receptors also resolved many historical puzzles about the infectivity of norovirus. In a human volunteer study, it was found that individuals with an O blood type were easily infected by some norovirus strains, while those with a B blood type had the lowest risk of infection (Hutson et al., 2002). Presumably, volunteers with a B blood type never became infected with certain norovirus strains following challenge simply because they lacked the matched viral receptors on their intestinal epithelium. The specificity in virus-receptor interaction also contributed to the observation that some individuals with a high level of antibodies against norovirus were even more susceptible to norovirus challenge than those with no or lower levels of antibodies (Parrino, et al., 1977; Greenberg et al., 1981). Clearly, receptors play an important role in host susceptibility.
1.10. Challenges of working with human norovirus

Although human norovirus causes significant problems in food safety and public health, research on this biodefense agent has been severely hampered. Currently, many aspects of human norovirus such as general biology, molecular biology, replication, gene expression, pathogenesis, and immunology are poorly understood. In the food safety aspect, the stability and sensitivity of human norovirus to food processing technologies is not understood. Two major challenges have been encountered in norovirus research. First, human norovirus cannot propagate in cell culture, and thus research relying on cell culture cannot be conducted. Secondly, there are no small animal models for human norovirus. All questions that must rely on animal models, such as pathogenesis and immunology, cannot be studied. To date, there is limited information about the survival of human norovirus, which came from human volunteer subjects (Parrino et al., 1977; Greenberg et al, 1981; Hutson et al., 2002). However, studies on volunteers not only require human subject protocol, but it is also very uncommon and impractical to have people voluntarily become infected with a highly infectious and contagious agent that causes gastroenteritis. Therefore, under current situations, we must rely on human norovirus surrogates to study the properties of human norovirus.

1.11. Human norovirus surrogates

Many studies have been done to identify proper surrogates for human norovirus. To date, canine calicivirus (CaCV), feline calicivirus (FCV), murine norovirus (MNV), bacteriophage MS2, and poliovirus has been used as human norovirus surrogates (Bae and Schwab, 2006). However, FCV and MNV are by far the most frequently tested and most commonly used surrogates. Ideally, a good surrogate for food safety research should include the following
properties. First, it should be genetically related to human norovirus. Secondly, the virion should be structurally similar to human norovirus. Thirdly, the surrogate should have biophysical similarities to human norovirus. Fourthly, the virus should cause similar disease as human norovirus does.

Figure 10. Murine norovirus (MNV-1) under electron microscopy (adapted from Lou et al., 2011)

1.11.1. Murine norovirus

Murine norovirus (MNV-1) was first isolated from severely immunocompromised mice that were deficient in recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT-1) (RAG2/STAT1−/− mice) (Karst et al., 2003). MNV causes systemic infection and lethal disease in STAT1−/− mice (Karst et al., 2003). MNV can be found in lung, liver, spleen, intestine, brain, and blood in infected mice. Since its discovery, many new MNV strains have been isolated from the feces of laboratory mice (GenBank accession no. DQ269192 and DQ269205). Interestingly, MNV does not cause typical symptom of human norovirus infection such as vomiting and diarrhea. There are many advantages of using MNV-1 as a surrogate for human norovirus. First, MNV-1 shares many biochemical features with human
norovirus. Figures 1 and 10 show the EM pictures of human norovirus and MNV. MNV-1 and human norovirus are similar in size (28-35nm), shape (icosahedral), and also buoyant density (Green et al., 2001; Karst et al., 2003). Secondly, the structure of MNV-1 is similar to human norovirus. Both viruses contain a highly stable outer shell capsid which is composed of VP1 and VP2. Thirdly, MNV-1 is genetically closely related to human norovirus. Both MNV-1 and human norovirus belong to genus Norovirus within the family of Caliciviridae. At the molecular biology level, MNV shares many genetic characteristics with human norovirus. MNV-1 has a similar genome size and gene organization (Figure 11). Similar to human norovirus, the genome of MNV-1 contains three open reading frames, ORF1, ORF2 and ORF3. ORF1 encodes nonstructural proteins including NTPase, 3A-like protein, protease, and RNA dependent RNA polymerase. ORF2 and ORF3 encode the major capsid protein VP1, and the minor capsid protein VP2, respectively. Finally, MNV is resistant to acid and heat, and highly stable and persistent in environment (Cannon et al., 2006; Wobus et al., 2006; Taube et al., 2009).
Figure 11. Genome comparison of human norovirus and murine norovirus (adapted from Wobus et al., 2006). This figure shows the similarities in genome structure. MNV-1 is a good surrogate for norovirus based on its genetic similarity to human norovirus.

Nonetheless, there are differences between murine and human noroviruses. MNV-1 does not cause the clinical manifestation of gastroenteritis that the human counterpart does. In addition, MNV-1 uses a different cellular receptor for virus entry and infection. The functional receptor for MNV-1 is sialic acid (Wobus et al., 2006). MNV-1 has tissue tropism in macrophage and dendritic cells. In comparison, human norovirus utilizes histo-blood group antigens as cellular receptors for infection (Tan and Jiang, 2005; Tan et al., 2008).

To date, MNV-1 is generally considered to be the best surrogate for human norovirus (Bae and Schwab, 2006; Cannon et al., 2006). Cannon et al. (2006) directly compared the viabilities of murine norovirus and feline calicivirus as surrogates for human norovirus. Specifically, they compared the stability of these two viruses to pH extremes, organic solvents, thermal inactivation, and surface persistence under wet and dry conditions. It was found that MNV-1 was stable across the entire pH range of 2-10 while feline calicivirus was rapidly
inactivated at a pH less than 3 and greater than 9. In a temperature stability experiment, FCV was more stable than MNV-1 at 56°C but they both exhibited similar stability at both 63 and 72°C. Long-term persistence studies found that MNV-1 was more stable than FCV at room temperature when both viruses suspended in a fecal matrix and inoculated onto stainless steel coupons (Cannon et al., 2006). Taken together, these studies show that MNV-1 is currently the most relevant and promising surrogate for human norovirus due to its genetic similarity and environmental stability.

1.11.2. Feline calicivirus

Feline calicivirus (FCV) was first discovered in the 1950s. It is one of the leading causative agents of infectious upper respiratory tract disease in cats, but it does not infect humans. Clinical signs of FCV infection include pneumonia, oral ulceration, sneezing, discharge from the eyes or nose, fever, lethargy and loss of appetite (Doultree et al., 1999). FCV does not cause gastroenteritis as human norovirus does. At taxonomy level, FCV belongs to genus Vesivirus within the family of Caliciviridae. Because of its genetic relatedness to human norovirus, FCV has been widely used as a surrogate for human norovirus for many years. However, FCV has distinct biochemical properties as compared to human norovirus. As aforementioned, FCV is less stable and persistent in environment. It can be rapidly inactivated under acidic condition (Cannon et al., 2006). Therefore, its ability to serve as an excellent human norovirus surrogate was compromised. With the discovery of MNV in 2003, MNV-1 has been used as a human norovirus surrogate in most laboratories.
1.11.3. Other potential surrogates

(1) Porcine sapovirus. Porcine sapovirus is a member of the genus Sapovirus within the family of Caliciviridae (Wang et al., 2005). Porcine sapovirus strain Cowden was first detected by Saif and her colleagues in 1980 (Saif et al., 1980). Subsequently, the Cowden strain was adapted to cell culture by serial passage in a continuous cell line (LLC-PK) (Chang et al., 2005). The growth of this virus depended on the presence of bile acid or intestinal content fluid filtrate obtained from uninfected gnotobiotic pigs. A distinct advantage of using porcine sapovirus as a surrogate is that this virus is enteric and cause gastroenteritis in pigs (Martella et al., 2008).

(2) Tulane virus. The Tulane virus, also known as monkey calicivirus, was isolated in stools of rhesus macaques captivated in the Tulane National Primate Research Center (Farkas et al., 2008). The Tulane virus replicates in vitro of rhesus monkey kidney cells and causes typical cytopathic effect (CPE). Another advantage is that Tulane virus recognizes the histo-blood group antigens similar to human norovirus. The complete genome of Tulane virus has been cloned and is genetically closely related with human norovirus compared with other caliciviruses (L’Homme et al., 2008). Thus the Tulane virus could serve as a useful surrogate for human norovirus although it has not been reported as a surrogate in food safety research.

1.12. Detection of human norovirus

Cell-based assays cannot be used for human norovirus detection since it cannot grow in cell culture. Therefore, detection of human norovirus must rely on electron microscopy, antigen-based assays, and molecular biology techniques. (1) Transmission electron microscopy (TEM) has been used for detection of human norovirus. In fact, the virus was first identified by TEM in 1972 (Kapikian et al., 1972). However, TEM requires a high number of virus particles (more
than $10^4$) and is more applicable for specimens (such as stool and vomitus) from clinical outbreaks. It may not be feasible to detect human norovirus from foods by TEM. (2) Enzyme linked immunosorbant assays (ELISAs) are often used for human norovirus detection. This method is based on the reaction between the norovirus capsid protein and a norovirus-specific antibody. ELISA is a quick and cost-effective way to screen for norovirus, but it needs to be improved with respect to sensitivity (Rabenau et al., 2003). (3) Reverse transcription polymerase chain reaction (RT-PCR) is probably the best method for human norovirus detection in clinical specimens and food samples because of its high sensitivity and specificity. This method is based on the detection of viral RNA genome. Real-time RT-PCR has been widely used for quantifying genome copies of the virus. The major disadvantage of a PCR-based method is that it could not distinguish the viable from the inactivated viruses. In addition, the food matrix may bring challenges in the extraction of viral RNA. Thus, a combination of antigen and PCR-based methods is recommended for human norovirus detection (Rabenau et al., 2003).

### 1.13. The interaction of norovirus with fresh produce

It is well established that bacterial pathogens can be internalized and disseminated in fresh produce via stomata or wounds in the cuticula. Viruses are approximately 1000 times smaller than bacteria, yet their interactions with plant organs, tissues, and cells are still poorly understood. It is known that norovirus are highly stable in the environment. However, the ecology of norovirus in fresh produce is poorly understood. Specifically, the penetration, uptake, internalization, dissemination, and persistence of viruses in plants are not understood.
1.13.1 Attachment of norovirus to fresh produce

The attachment of virus is dependent on the pathogen itself, the type of fresh produce, and the surfaces investigated. For leafy greens, the surface of leaf is easily contaminated by norovirus, although uptake by the roots of plants can potentially occur. Fruits may be directly contaminated by norovirus since there is no direct evidence support that pathogens in roots can be transported into fruits. There are six main pathways by which contamination may occur: 1) direct contact of produce with the ground, 2) direct contact of produce with human waste and animal contaminants, 3) dislodgment of contaminated soil through irrigation water splashing or through rain onto produce, 4) aerosolization of pathogens onto produce, 5) direct application of contamination through spraying irrigation water onto the produce, and 6) contamination of fresh produce by farmers, processors, and food handlers (Doyle and Erickson, 2008).

It is known that viruses and other pathogens attach to the surface of produce, but the mechanism of how they attach is still unclear. There have been a number of proposed mechanisms including extracellular polymeric substances (Junkins and Doyle, 1992), whether fimbriae are present or not (Fratamico and others, 1996), cell surface hydrophobicity (Dickson and Koohmaraie, 1989), divalent cationic bridges (Hassan and Frank, 2003), and surface charge of the pathogens (Ukuku and Fett, 2002).

One possible mechanism for the attachment of human norovirus to produce is the presence of carbohydrate moieties which resemble the human blood group antigens (HBGAs), the functional receptors for human norovirus attachment in host tissues. This hypothesis is supported by recent evidence that HGBA-like molecules are present in the gastrointestinal tissues of clams, mussels and oysters, and that these molecules are responsible for the tenacity with which molluscan shellfish hold on to viruses (Le Guyader et al., 2006). The HGBA-like
moieties may also be present in plants although they have not yet been identified. Carbohydrate moieties, the analogues of the human norovirus receptors, such as glucose and glycan, are highly abundant in vegetables and fruits. One leaf of lettuce and one strawberry possesses 0.3 g and 0.6 g of carbohydrate, respectively. In addition, plants produce a number of lectins and polysaccharides, such as phytohemaglutin, which interact with some human viruses and also activate the human immune system (Johnson, 1964). For example, a polysaccharide isolated from rooibos tea leaves inhibited binding of HIV to human cells (Nakano et al., 1997). Extracts from *Stevia rebaudiana* (sweet leaf) has been shown to bind to human rotavirus (Takahashi, et al., 2001). Using human norovirus virus-like particles (VLPs) as a model, Tian et al. (2011) recently found that these VLPs attached to the surface of romaine lettuce and were localized primarily on the leaf veins. An extract of romaine lettuce leaves bound to VLPs in a dose-dependent manner. However, this extract did not bind VLPs by histo-blood group antigens (HBGA), nor was it competitive with VLPs in binding to porcine gastric mucin. These results suggested that non-HBGA molecules in extract bind to rNVLP by a binding site(s) different from the defined binding pocket on the virion. Further research needs to be done in order to gain more knowledge whether receptors are indeed involved in attaching of human norovirus to fresh produce.

**1.13.2. Internalization and dissemination**

There have been many studies done with bacterial pathogens such as *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*. Studies have shown that the amount of pathogen entry into the tissue is dependent on the type of plant (Solomon and Matthews, 2005) and the strength of plant defense mechanisms which help keep out foreign pathogens from invading cells (Iniquiez et al.,
2005). There are other conditions which also may affect the internalization of pathogens such as the surrounding environment. If there are soil and other microflora present, these organisms may compete with the foodborne pathogens (Warriner et al., 2003). No linear relationship has been detected between the amount of microbial load present and the amount of internalization (Dong et al., 2003).

It has been shown by some studies that movement of pathogens through the plant vasculature is possible (Guo et al., 2002; Solomon et al., 2005; Solomon and Matthews, 2005; Bernstein et al., 2007). It has also been shown, though, that even though pathogens are known to internalize, such as E. coli O157:H7, there are instances where they cannot be recovered in the tissues of mature plants (Jablonske et al., 2005). It should be noted that most studies are done under very simplistic conditions, and therefore, these results may or may not represent what would actually happen in the field.

The feasibility of internalization of human enteric viruses by plants is supported by the ability of plants to internalize their own viral pathogens, which can be taken up from soil and water. It is likely that human enteric viruses may also be taken into the plant through the roots and/or leaves since they may be present in sewage-contaminated soil or water. Recently, the internalization of viruses by green onion has been reported using irrigation water inoculated with fluorescent microspheres (Chancellor et al., 2006). It was found that fluorescence associated with the spheres reached a peak at day 7 and persisted until day 21. This evidence suggests that the plants can transport and perhaps even concentrate viral particles from the soil. Of further concern is the fact that once contaminated, soils and produce items remain contaminated with enteric viruses for weeks to months since they are highly stable. It is now generally accepted that bacterial pathogens become internalized in plant tissue via a passive process (Doyle and
Erickson, 2008). For example, bacteria present in irrigation water can be taken up by root systems and enter the edible portion of the plants. Bacteria may also gain entry via wounds or structure such as lenticels and stomata. The size of virus is approximately 1000 times smaller than bacteria. Therefore, it would be easier for a smaller pathogen to enter and disseminate in plants (Chancellor et al., 2006).

There have been very few studies that have actually investigated norovirus internalization in produce. Urbanucci et al. (2010) showed the first norovirus internalization in plants. The roots of lettuce, growing either in hydroponic culture or in soil, were exposed to canine calicivirus (CaCV) and a human genogroup 2 norovirus (HuNoV). For CaCV, it was found that internalization occurred in both seedling and mature plants, in both hydroponic and soil cultures, and occurred whether the roots were intact or damaged. However, the frequency of positive results was low. Interestingly, internalization of HuNoV was not detected although the plants were exposed to a high concentration of HuNoV. These results suggested that viral contamination of lettuce plants via roots cannot be excluded, but may not be an important transmission route for viruses in plants. Wei et al. (2011) reported that murine norovirus can be internalized via roots in romaine lettuce (Lactuca sativa). MNV genomic RNA was detected in some lettuce leaf samples when the roots of lettuce inoculated with both high and low doses of MNV. However, infectious MNV was found in lettuce samples challenged with high virus inoculums grown hydroponically and in soil but not in lettuce grown with low virus inoculums. This evidence suggests that murine norovirus can become internalized via plant roots and disseminated into the leaf portion (Wei et al., 2011). Taken together, these results suggest that norovirus can be potentially internalized and disseminated although the detailed mechanism is still unclear.
1.14. Current processing practices for fresh produce companies

Fresh produce consumption has increased in recent years with consumers’ increased desire to live a healthier lifestyle. Fresh produce undergoes little or no processing, and does not contain any additives or preserving agents, thus being deemed as natural and healthy food. The 2010 Dietary Guidelines for Americans recommended a combined 4.5 servings of fruits and vegetables per day (Department of Health and Human Services, 2010). In addition, fruits and vegetables are able to be prepared quickly and easily. Indeed, some grocery stores now offer fresh-cut produce already packaged and ready for consumption. It is also worthwhile to note that improved transportation and importing of produce from other countries makes a variety of produce available throughout the year regardless of the current season. Nevertheless, this large increase in consumption of fruits and vegetables brings about an increase in foodborne illnesses related to fresh produce.

Due to the large amount of foodborne outbreaks caused by norovirus, there is an urgent need to develop novel strategies to remove the noroviruses from foods, especially fresh produce. To better understand such need, it is essential to know current processing practices in the food industry. Figure 12 represents a typical processing chain for fresh produce (leafy greens). Apparently, fresh produce is at a high risk for contamination by norovirus because it normally undergoes little to no processing, and can be contaminated at almost any step from pre-harvest to post-harvest (Fröder et al., 2007).
Figure 12. A representative processing chain for fresh produce (leafy greens) in the food industry. The square boxes show the supply chain flow for leafy greens (such as lettuce) in fresh produce industry.

First, produce may be contaminated by irrigation runoff, soil, human waste, and organic fertilizers in the field (Beuchat, 1996). Irrigation water containing raw sewage or particles from a raw sewage treatment plant may carry norovirus, hepatitis A, and other enteric viruses (Beuchat and Ryu, 1997), whereas the types of these microorganisms which thrive may vary with the season.

Secondly, contamination may occur during harvesting via field workers, soil, water, air, or unclean harvesting equipments (Brady, 2009). Any worker who picks, processes, or packages produce may transfer contaminants to the produce via cuts or sores. With regard to the fact that the primary route of transmission for norovirus is fecal-oral, transmission from a sick worker to the produce could be a major source of contamination (Beuchat and Ryu, 1997).

Thirdly, contamination can occur during any post-harvest step, such as transporting, processing, and storage. After harvesting, the produce (leafy greens) is usually sprayed or rinsed with a chlorinated solution to remove or eliminate some microorganisms before the produce is transported en route to its destination. Microorganisms on vegetables or from other resources
may accumulate in this solution and then be spread to other produce that comes into contact with the water afterward. In fact, these washing solutions are frequently re-used in the fresh produce industry. Subsequently, the produce is typically transferred to a processing facility and cleaned/sanitized to further reduce possible microbial contamination before sending it to stores and consumers. This involves a two-step process in which the produce is first dipped into a water solution, and then dipped into a sanitizing solution. The sanitizing solution contains FDA-approved sanitizers, one of which is chlorine (up to 200 ppm), the most commonly used sanitizer in the produce industry. The efficacy of a sanitizer is dependent on a number of factors, such as the sanitizer concentration, treatment temperature, pH, contact time, and food matrix. For example, if chlorine is used, the concentration of free chlorine should be monitored at least every hour to ensure its effectiveness (da Cruz et al., 2006).

Lastly, during the packaging and transport of the produce, all pieces of equipment and their operators are possible sources of contamination. Taken together, potential threats to food safety exist in every step of the processing chain for fresh produce. If every step in the process is executed correctly with no contamination issues, the produce is healthy and safe to consumers, but unfortunately, this is not always the case. Therefore, improving the efficacy of the sanitizing step in virus reduction could be an easier and economical approach to enhance the safety of fresh produce.

1.15. Current sanitizers

Many federal agencies do recognize the importance of controlling foodborne viruses in food and enhancing public health, yet an overwhelming number of food safety research examines bacterial contamination only, with little focus given to the most common cause of foodborne
illness: viruses. The survival and inactivation of these viruses, especially in fresh fruits and vegetables, is still poorly understood.

Chlorine, the most commonly used sanitizer for washing fresh produce, has been studied extensively. According to the FDA List of Approved Food Additives List, sodium hypochlorite at the concentration of less than 200 ppm may be used for sanitization purposes (FDA CFR 178.1010, 2011). In spite of its wide application in food industry, chlorine is used mainly to limit cross contamination of produce during washing procedures (Doyle and Erickson, 2007). Moreover, 200 ppm of chlorine solution typically only gives about a moderate virus reduction of 1-1.2 logs in fresh produce (Baert et al., 2009; Dawson et al., 2005). Under certain conditions, the efficacy of chlorine in reducing virus contamination is even lower. For example, it was found that tap water washing only gave an average of 0.94 logs of reduction of murine norovirus in shredded lettuce, while the addition of 200 mg/liter of sodium hypochlorite only brought about an additional reduction of 0.48 logs (Baert et al., 2009). This observation is consistent with previous studies showing that chlorine water washing gave an average reduction of 0.89 logs in viral titer across all fruits and vegetable samples in comparison with tap water washing (Belliot et al., 2005).

Since chlorine is not particularly effective at removing noroviruses from fresh produce (Dawson et al., 2005), the effectiveness of many other similar sanitizers in removing norovirus surrogates has been evaluated. In the aforementioned study conducted by Baert and his colleagues, 80mg/liter of peroxyacetic acid in the washing solution gave an additional 0.77 logs in virus reduction in comparison with tap water (0.94 logs). In a similar study using sodium hypochlorite, 70% ethanol, and trisodium phosphate (TSP, a household cleaner known to be effective against \textit{E. coli} O157:H7) to test against high titers (7 \text{log}_{10} PFU) of murine norovirus
(MNV-1), 70% ethanol gave no reduction whatsoever. 10% sodium hypochlorite solution led to 2.73 logs of reduction, but this concentration is much higher than allowed by the FDA. 1% and 2% TSP solutions brought about 0.28 and 1.05 logs of reduction, respectively (D’ Souza and Su, 2010). Many other sanitizers, such as chlorine dioxide and hydrogen peroxide (Belliot et al., 2008; Thurston-Enriquez et al., 2005), have been studied previously, however, results from these studies are not very meaningful for the safety of food produce since the effective concentrations of these sanitizers are much higher than allowed by the FDA.

Overall, none of those commonly used sanitizers is effective in removing virus contaminants from fresh produce at levels that are allowed by food administration agencies, indicative of a need to find “novel” sanitizers for produce processing.

1.16. Surfactants

Surfactants are “surface-acting” compounds that can essentially reduce the surface tension of a liquid. Surfactants contain both a hydrophilic and hydrophobic group which interacts with the substance they are mixed with to alter the surface properties (normally lowering the interfacial free energy) of the water either at the water and air or water and solid interface.

Surfactants are widely used in our daily lives. In the last decade alone, the demand for surfactant use in the United States has increased more than 300% (Greek, 1990). Surfactants are currently used in many products ranging from toothpastes, soaps, shampoos, and detergents, to laxatives, cosmetics, herbicides and insecticides, as detergents, wetting agents, emulsifiers, foaming agents, and dispersants. Moreover, some biosurfactants have been used recently as a means to emulsify hydrocarbon-water mixtures in oil spills in order to help degrade hydrocarbons in the environment (Harvey et al., 1990). Furthermore, surfactants have even been
used as a therapy for infants with respiratory distress syndrome (Ghaemi et al., 2009) as well as a potential spermicide for use in condoms (Vieira et al., 2008).

Based on their chemical structure, surfactants can be classified as either ionic or nonionic compounds, where the ionic surfactants can be either anionic or cationic. Out of the surfactants chosen for use in this study, Triton X-100, NP-40 and Tween 20 are examples of non-ionic surfactants, while SDS is an anionic surfactant.

SDS is an FDA approved food additive and, but the use of SDS as a sanitizing agent for fresh produce has not been regulated. The FDA Code of Federal Regulations mentions SDS as a means for sanitizing food processing equipment but not the food itself. (FDA CFR 178.1010, 2011). SDS is used in products such as toothpastes, where it is suspected to have an inhibitory effect on plaque formation (Jenkins and others, 2005), as well as various cosmetics, and shampoos and soaps for detergent activity. Concentrations of SDS may be up to 40% in some shampoos (Ostroumov, 2006).

The polysorbates such as Tween 20 or Tween 80 are generally recognized as safe (GRAS) by the FDA. The ‘Tweens’ are used in various food products, particularly in ice cream where they serve as emulsifiers. They are also used as wetting agents in products such as mouth drops. Like SDS, there is no regulation on using polysorbates sanitizers in food processing (FDA CFR, 2011).

Triton X-100 and NP-40 were also chosen for our current study since they may have similar effects in enhancing sanitization, although currently there is no record about the safety of these two surfactants in the FDA Code of Federal Regulations. Triton X-100 and NP-40 are both routinely used in biological research settings.
1.17. Virucidal activity of surfactants

It has been documented that surfactants can interact with viral proteins (Banat et al., 2000; Hartman et al., 2006; Howett et al., 2009; Koopmans and Duzier, 2004; Krebs et al., 1999). This interaction can influence protein folding/refolding, denaturation, and aggregation, thus inhibiting or even inactivating viruses. Also, surfactants can directly disrupt the viral envelope. The virucidal activity of certain surfactants for sexually transmitted viruses has been reported. For example, Howett et al., (1998) found that sodium dodecyl sulfate (SDS) possessed a virucidal activity against papillomaviruses, herpes simplex virus-2 (HSV-2), and human immunodeficiency virus-1 (HIV-1) (Howett at al., 1999). It has been also reported that HIV-1 was inactivated by SDS in breast milk to avoid transfer of the virus to infants when formula feeding is not practicable. Moreover, SDS has been used to prevent the transmission of HIV during sexual intercourse (Howett and Kuhl, 2005). In addition, it has been shown that SDS, NP-40, and Triton X-100 exhibit the potentials to reduce the infectivity of hepatitis C virus (Song et al., 2010), whereas Triton X-100 is able to partially denature the coat protein of tobacco mosaic virus (TMV) and induce its aggregation (Panyukov et al., 2008). These results show that surfactants are able to successfully inactivate various enveloped viruses. However, the effectiveness of surfactants inactivating foodborne viruses, which are typically non-enveloped, is less understood. To date, the potentials of surfactants to inactivate foodborne viruses have not been reported.
1.18. Surfactants may serve as novel sanitizers that enhance virus removal from fresh produce.

Addition of surfactants in a washing procedure for fresh produce could make the liquid spread more easily and lower the interfacial tension between the two liquids, or between the liquid and the solid, thus facilitating the release of tightly bound contaminations such as foodborne pathogens from the surface. To address this premise, we evaluated the effectiveness of chosen surfactants at removing and inactivating a human norovirus surrogate in our current study.

![Diagram showing the potential application of surfactants in improving safety of fresh produce (leafy greens) during processing.](image)

**Figure 13. Potential application of surfactants in improving safety of fresh produce (leafy greens) during processing.** The square boxes show the supply chain flow for leafy greens (such as lettuce) in fresh produce industry. Proposed interventions to minimize the virus contamination are shown as ovals.
In this study, we systematically evaluated the effectiveness of surfactants in sanitization of viruses in fresh produce using murine norovirus (MNV-1) as a surrogate for human norovirus. We found that surfactants alone or a combination of a low concentration of surfactants with chlorine solution significantly enhanced the removal of MNV-1 from fresh produce. Using this strategy, more than 3 logs of virus reduction was achieved in either fruits (strawberry and raspberry) or leafy greens (cabbage and lettuce). Therefore, the combination of a surfactant and chlorine solution is a novel approach to enhance the safety of fresh produce.

Our findings could be easily included in the current processing chain for fresh produce. Figure 13 shows the potential applications of surfactants during produce processing. After being harvested from the field, the leafy greens are usually subjected to a spray of chlorinated water. To keep the leafy greens fresh, the produce is then transported for vacuum cooling. After the cooling step, the produce continue to be transported to processing plants for cutting, washing by chlorinated water, and packaging, followed by retail distribution. In this processing chain of events, the use of surfactants could be applied during the sanitization step by simply adding 50 ppm of SDS, for example, to the chlorine solution already used currently. Such a strategy would yield more virus reduction than what the industry is currently achieving, and would not be much of a change for food processing companies. One alternative could be to spray a solution containing surfactants on the fresh produce before the transport to vacuum cooling. Consequently, this spray solution would help with any contamination acquired during pre-harvest or harvesting, and then the sanitization step with chlorine would be another hurdle for harmful microorganisms. Of note, a spray solution containing both SDS and chlorine would likely enhance the virucidal activity. Another possible way to use surfactants for enhancing
safety would be to coat packages of produce with SDS before they are sent to the stores for consumers to purchase. Since it is known that viruses can survive in foods with high stability for many days to weeks, SDS could kill the viruses on the produce while they are stored. Evidently, this approach would only work for types of produce that are packaged instead of free, unpackaged types of produce, but it is still another hurdle technology that could be used in the future. Another way to enhance the food safety is by antiviral packaging. Once fresh cut fruits and vegetables are packaged, if surfactants are lining the package, this may be another way to decrease the amount of pathogens before the products reach consumers. Lastly, a sanitizing solution of chlorine and surfactant could serve as an effective way to clean surfaces where infected persons have vomited, surfaces that may have contacted fecal material, or surfaces where contaminated food has touched. Any or all of these applications could be implemented in the food industry to further enhance the safety of fresh produce and hopefully reduce the incidences of produce-associated outbreaks of norovirus and other types of foodborne viruses or bacteria as well. The possibilities are endless for the impact that such a small change could have on the food industry.
CHAPTER 2

ENHANCED SANITIZATION OF A HUMAN NOROVIRUS SURROGATE IN FRESH VEGETABLES AND FRUITS BY A COMBINATION OF SURFACTANTS AND SANITIZERS

2.1. Abstract

Fruits and vegetables are a major vehicle for transmission of foodborne enteric viruses since they are easily contaminated at pre- and post-harvest stages and undergo little or no processing. However, commonly used sanitizers are relatively ineffective for removing human norovirus surrogates from fresh produce. In this study, we systematically evaluated the effectiveness of a panel of sanitizers and surfactants on sanitization of a human norovirus surrogate, murine norovirus 1 (MNV-1), from fresh produce. We showed that organic acids slightly enhanced virus sanitization, and such effect depended on the type of fresh produce. Strikingly, we found that surfactants, including sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), Triton X-100, and polysorbates (Tween 20), significantly enhanced the sanitization of viruses from fresh fruits and vegetables. While tap water alone and chlorine solution (200 ppm) only reduced less than 1.2 logs of virus in all fresh produce, 50 ppm of surfactant solution was able to achieve 3 logs of virus reduction in strawberry and approximately 2 logs of virus reduction in lettuce, cabbage and raspberry. Moreover, approximately 3 logs of virus reduction
were observed in all the tested fresh produce after sanitization with a combination of 50 ppm of each aforementioned surfactant and 200 ppm of chlorine solution. Taken together, our results demonstrated that the combination of a surfactant with a commonly used sanitizer enhanced the efficiency in removing viruses from fresh produce by approximately 100 times. Since SDS is an FDA approved food additive and polysorbates are FDA recognized GRAS (Generally Recognized As Safe) products, implementation of this novel sanitization strategy would be a feasible approach to efficiently reduce the virus load in fresh produce.

2.2. Introduction

Viruses cause more than 67% of all foodborne illnesses worldwide (Estes et al., 2006; Mead et al., 1999). Human norovirus is a major enteric foodborne virus that is a significant problem in foods due to its small infectious dose (<10 particles) and its high stability in the environment (Duizer et al., 2004; Rzezutka and Cook, 2004; Teunia et al., 2008). It is estimated that at least 90% of acute non-bacterial gastroenteritis outbreaks can be attributed to norovirus infection, but this number may even be underestimated due to the large number of unreported infections and lack of methods for rapid detection of the virus (de Wit et al., 2007; Estes et al., 2006; Katpally et al., 2008). According to a recent report from the Centers for Disease Control and Prevention, approximately 48 million people suffer from norovirus-induced gastroenteritis each year in the US, there are 128,000 hospitalizations, and 3,000 people die from norovirus each year (CDC, 2010). Outbreaks of human norovirus are common in any environment where people are in close contact such as cruise ships, restaurants, hotels, schools, the military, nursing homes, and hospitals (Adler and Zickl, 1969; Howett et al., 1999; Mead et al., 1999). Transmission of norovirus is primarily by the fecal-oral route, either by person to person spread
or ingesting contaminated food or water (Cliver, 1997; Estes et al., 2006; Patel et al., 2009; Rockx et al., 2002). The primary symptoms of human norovirus infection include diarrhea, vomiting, fever, chills, and extreme dehydration. It has been a challenge to work with human norovirus since it does not propagate in cell culture and there is no suitable animal model for the virus (Duizer et al., 2004, Estes et al., 2006). For this reason, studies of human norovirus must rely on surrogates such as murine norovirus 1 (MNV-1) or feline calicivirus (FCV) (Bae and Schwab, 2008; Belliot et al., 2008; Cannon et al., 2006; Wobus et al., 2006). Because of these challenges, human norovirus and other caliciviruses are classified as category B priority bio-defense agents according to the National Institute of Allergy and Infectious Diseases (NIAID).

Fresh produce is at a high risk for contamination by norovirus because it normally undergoes little or no processing, and can be contaminated at any step from pre-harvest to post-harvest. According to recent outbreak data, fruits and vegetables are a major vehicle in the transmission of foodborne illness (Berger et al., 2010; Beuchat, 1996; Heaton and Jones, 2008; Lynch et al., 2009). It has been reported that norovirus accounted for more than 40% of outbreaks in fresh produce from 1998-2005 in the US (Doyle and Erickson, 2008). These outbreaks of norovirus have occurred in lettuce, tomatoes, melons, strawberries, raspberries, fresh cut fruits, and other vegetables (Butot et al., 2009; Doyle and Erickson, 2008; Pönkä et al., 1999; Vandekinderen et al., 2009). One major route with a high probability of contamination is the use of contaminated water for irrigation or washing. Contamination may also occur by infected workers handling the food during harvesting, processing, or distribution (Butot et al., 2009; De Giusti et al., 2010; Doyle and Erickson, 2008; Lynch et al., 2009). With an increasing number of people striving to eat healthier by increasing their consumption of fruits and vegetables, this has become a major public health concern (Baert et al., 2009; Baert et al., 2008;
Sivapalasingam et al., 2004). However, while numerous studies have been reported on bacterial contamination of fresh produce to date, knowledge about viral contamination of fresh produce remains limited.

In current industry, fresh produce usually undergoes a brief sanitization step after harvest from field. Unfortunately, current commonly used sanitizers are relatively not effective in removing viral contaminants from fresh produce (Bae and Schwab, 2008; Baert et al., 2009; Baert et al., 2008; Baldev et al., 2001; Dawson et al., 2005). The most common sanitizer, 200 ppm of chlorine solution, typically only gives less than 1.2 logs virus reduction in fresh produce (Baert et al., 2009; Baldev et al., 2001; Doyle and Erickson, 2008; Rzezutka and Cook, 2004). Recently, Baert et al. (2009) found that tap water washing only gave an 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 (Baert et al., 2009). Therefore, there is urgent need to develop a more effective sanitizer to remove noroviruses from fresh produce.

Organic acids, such as citric acid, acetic acid, lactic acid, and peroxyacetic acid, are important to the food industry because they are considered natural antimicrobials. It has been well documented that these organic acids can inhibit and/or kill some types of bacteria, such as *Listeria monocytogenes* and *E. coli* O157:H7 (Sofos, 2003; Doyle, 2005; Theron and Lues, 2007), thus are widely used as additives to fresh produce and/or meat to inactivate bacterial pathogens. In regard to their antimicrobial potentials, one may assume that these organic acids provide alternative strategies to sanitize the fresh produce. However, the effectiveness of organic acids in sanitizing viruses is much less studied except that studies showing that only a moderate MNV-1
reduction of 1.5 logs was achieved in shredded lettuce using 250 mg/liter of peroxyacetic acid (Baert et al., 2008; Baert et al., 2009; Baert et al. 2011).

Surfactants are surface-active compounds that can reduce the surface tension of a liquid. The addition of surfactants in a washing procedure will make the liquid spread more easily and lower the interfacial tension between the two liquids, or between a liquid and a solid. In addition, they may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants (Banat et al., 2000; Mukherjee and Das, 2010). Surfactants contain both a hydrophilic and hydrophobic group which interacts with the substance they are mixed with in order to alter the surface properties of the water either at the water and air or water and solid interface (Banat et al., 2000; Mukherjee and Das, 2010). Because of these properties, surfactants allow the release of tightly bound contaminants such as foodborne pathogens from the surface, which leads us to hypothesize that surfactants may enhance the sanitization of foodborne pathogens in fresh produce. Out of numerous ionic (anionic or cationic) and nonionic surfactants, we chose sodium dodecyl sulfate (SDS), polysorbates (such as Tween 20), Triton X-100, and NP-40 for the following reasons: (i) SDS is an anionic surfactant and an FDA approved food additive (FDA 21 CFR 172.822); (ii) polysorbates are a class of non-ionic surfactants and GRAS (generally recognized as safe) substances recognized by the FDA (21 CFR 172.840, 172.836, and 172.838); (iii) Triton X-100 and NP-40 are another two widely used non-ionic surfactants that may have similar effects in enhancing sanitization, although there is no record about the safety of these two surfactants in the FDA Code of Federal Regulations to date.

Here we report a systematic evaluation of the effectiveness of a panel of organic acids and surfactants in sanitization of viruses in fresh produce using murine norovirus 1 (MNV-1) as a surrogate. We showed that organic acids did give slightly more virus reduction than chlorine
solution in fresh produce. Remarkably, we found that surfactants alone or a combination of surfactants (at low concentrations) with chlorine solution significantly enhanced the removal of MNV-1 from fresh produce. Using this strategy, more than 3 logs of virus reduction was achieved in either fruits (strawberry and raspberry) or leafy greens (cabbage and lettuce). These results strongly support that the combination of a surfactant and chlorine solution is a novel and feasible approach to enhance the safety of fresh produce.

2.3. Materials and Methods

2.3.1. Cell culture and virus stock

Murine norovirus strain MNV-1 was a generous gift from Dr. Herbert W. Virgin IV, Washington University School of Medicine (Karst et al, 2003). MNV-1 was propagated in murine 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 (Invitrogen, Carlsbad, CA) with the addition of 10% fetal bovine serum (Invitrogen) at 37°C under a 5% CO₂ atmosphere. To prepare MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 20. After 1 h of incubation at 37°C, 15 mL DMEM supplemented with 2% fetal bovine serum (FBS) were added. After two days post-infection, the virus was harvested by freeze-thawing three times, and the supernatant was collected after centrifugation at 5,000 g for 20 min at 4 °C.

2.3.2. MNV-1 plaque assay

MNV-1 plaque assay was performed in RAW 264.7 cells as described previously (Karst et al., 2003). In brief, 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 of the virus from a 10-fold dilution scheme. After 1 h of incubation at 37°C with agitation every 15 min, the cells were overlaid with 2.5 mL of minimum 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. 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.

### 2.3.3. Inoculation of MNV-1 to fresh produce

Fresh produce samples (strawberries, raspberries, cabbage, and romaine lettuce) were purchased from a local supermarket. A sample contained 50g of fresh produce and was placed in a sterile plastic bag. First, MNV-1 stock ($5.0 \times 10^8$ PFU/ml) was added to each sample to reach an inoculation level of $3.0 \times 10^6$ PFU/g. Subsequently, 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.

### 2.3.4. Preparation of sanitizers

Various sanitizing solutions (citric acid, acetic acid, peroxyacetic acid, and hydrogen peroxide) were purchased from Sigma (St. Louis, MO). Chlorine bleach containing 6% sodium hypochlorite was purchased from a local supermarket. Citric acid and acetic acid were diluted into sterile water to the final concentration of 200 ppm. Peroxyacetic acid and hydrogen peroxide were prepared at the concentration of 80 ppm and 59 ppm respectively, which are the maximum
concentrations approved by the FDA. SDS (powder), and NP-40, Triton X-100, and Tween 20 (liquid) were purchased from Sigma (St. Louis, MO). Surfactant solutions at the desired concentrations were prepared accordingly.

2.3.5. Sanitization procedure

The MNV-1 inoculated fresh produce was sanitized by tap water, chlorine solution (200 ppm), citric acid (200 ppm), acetic acid (200ppm), peroxycetic acid (80 ppm), hydrogen peroxide (59 ppm), surfactant alone, and solutions containing both surfactants and chlorine. For strawberries and raspberries (50g), the amount of washing solution was 2 L. For lettuce and cabbage (50g), 4 L of washing solution were used. Freshly-prepared washing solution was used for each replicate, and the washing container was cleaned and rinsed out between replications. Each produce sample was washed by each sanitizer with gentle agitation for 2 min. After sanitization, the fresh produce was drained and placed into a stomach bag. The remaining viruses were eluted by addition of 20 mL of phosphate buffered saline (PBS, pH 7.0) and stomached for 3 min. To neutralize any potential residual chlorine, 200 μl of 0.25M sodium thiosulfate was added to each sample. The residual detergents were removed by Detergent-OUT Micro Kit (Millipore, Billerica, MA). The viral survivors were evaluated by plaque assays.

2.3.6. Statistical analysis

All experiments were done in triplicate. The surviving viruses were expressed as mean log viral titer ± standard deviation. Statistical analysis was done using one-way ANOVA, with a value of p<0.05 being statistically significant. Post-hoc Tukey’s test was done to determine the where the differences between the treatments.
2.4. Results

1. Organic acids slightly increase the effectiveness of sanitization of a human norovirus surrogate in strawberries. In the current fresh produce industry, chlorine solution (up to 200 ppm) is widely used in sanitization of fresh produce. However, it has been well documented that 200 ppm of chlorine solution is not effective at removing human norovirus surrogates and other viral contaminants (such as hepatitis A virus and bacteriophage) from fresh produce, therefore, alternate strategies are needed in sanitization of fresh produce to reduce virus load. Previously, it was found that 250 mg/liter of peroxyacetic acid only gave 1.5 logs MNV-1 reduction in shredded lettuce (Baert et al., 2009), implying that organic acids, such as peroxyacetic acid, citric acid, and acetic acid, may have potentials in reducing the virus load in fresh produce. Nonetheless, the effectiveness of organic acids other than peroxyacetic acid in sanitizing viruses has not been investigated to date. Therefore, we systemically evaluated the efficacies of six sanitizers (tap water, chlorine, citric acid, acetic acid, peroxyacetic acid, and hydrogen peroxide) in sanitization of MNV-1, a surrogate for human norovirus, in fresh produce. As described in Materials and Methods, MNV-1-contaminated samples (50 g of strawberries) were washed with 2 L of each sanitizer solution for 2 min at room temperature. The amount of surviving viruses after treatment was quantified by plaque assay. Figure 14 shows the amount of the surviving MNV-1 in strawberries after each treatment. Consistent with previous observations, tap water washing only gave a 0.5 log reduction in virus titer. 200 ppm of chlorine solution brought about 0.9 log virus reduction, which had a slight increase in virus reduction in comparison with tap water alone, but such increase was not statistically significant (P>0.05). We also found that washing with 80 ppm of peroxyacetic acid or 59 ppm of hydrogen peroxide did not lead to more reduction in MNV-1 titer (P>0.05). However, citric acid (200 ppm) and acetic
acid (200 ppm) gave 2.5 and 2.1 log virus reduction, respectively. These two organic acids significantly improved virus sanitization in strawberries as compared to tap water and the chlorine solution (P<0.05). Taken together, our data demonstrated that (1) commonly used sanitizers (chlorine, peroxycetic acid and hydrogen peroxide) are not effective in removing MNV-1 from strawberries; (2) at the concentration of 200 ppm, citric acid and acetic acid increased sanitization of MNV-1 by approximately 1 log in comparison with chlorine solution. Citric acid appeared to be the most effective of all the sanitizers tested.

Figure 14. Effectiveness of various sanitizers at removing MNV-1 from strawberries. Fresh strawberries were inoculated with MNV-1 to a final concentration of approximately 3 \times 10^6 PFU/g. After incubation for 1 h, the samples were sanitized with gentle agitation for 2 min in each sanitizer. After sanitization, the strawberries were stomached, and the surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
2. All the sanitizers, including organic acids, were not effective in removing MNV-1 in lettuce. The type of fresh produce may affect the effectiveness of a sanitizer because each type of fresh produce has a strikingly different surface structure. In addition, the virus may have different affinities for each individual type of fresh produce. Therefore, we evaluated the effectiveness of six selected sanitizers in removing virus from romaine lettuce, a leafy green. The experimental design and washing procedure was essentially identical to that described above. As shown in Figure 15, all the sanitizers were not effective in removing MNV-1 from lettuce. Tap water only gave 0.4 log virus reduction. Chlorine and hydrogen peroxide only brought about approximately 1.1-1.2 log virus reduction. Citric acid, acetic acid, and peroxycetic acid achieved 1.5-1.7 log virus reduction. None of the sanitizers tested were significantly more effective than any other treatment for lettuce (P>0.05). Taken together, these results indicate that commonly used sanitizers including organic acids are not effective in removing MNV-1 from lettuce. These results also suggest that the type of fresh produce affects the washing efficiency. For example, 200 ppm of citric acid achieved 2.5 log virus reductions in strawberries, which is significantly higher than in lettuce (1.5 log virus reduction).
Figure 15. Effectiveness of various sanitizers at removing MNV-1 from lettuce. Fresh lettuce was inoculated with MNV-1 to a final concentration of approximately $3 \times 10^6$ PFU/g. After incubation for 1 h, the samples were sanitized with gentle agitation for 2 min in each sanitizer. After sanitization, the lettuce was stomached, and the surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.

3. The residual sanitizer in fresh produce after washing had a negligible impact on viral plaque assay. In our sanitization procedure, we soaked 50g of fresh produce in 2 or 4 L of sanitizers. After washing for 2 min, we drained the liquid from the fresh produce and stomached the fresh produce for another 2 min. Immediately, we performed a 10-time serial dilution for viral plaque assay to determine the virus survivors. There may be a minimal amount of sanitizers remaining in fresh produce after sanitization, which may interfere with the viral plaque assay. To rule out this possibility, we neutralized the possible residual chlorine by sodium thiosulfate prior to viral plaque assay. Briefly, 200 μl of 0.25M sodium thiosulfate was added to each sample after...
the stomaching step, followed by viral plaque assays. Presumably, the residual chlorine should be neutralized by sodium thiosulfate. As shown in Figure 16, there is no significant difference in virus titer between samples treated or not treated by sodium thiosulfate (P>0.05). This data suggests that (i) residual sanitizer in fresh produce after washing procedure is minimal; and (ii) residual sanitizer had a negligible impact on viral plaque assays.

**Figure 16.** The effect of residual sanitizer in fresh produce on viral plaque assay. Fresh strawberries were inoculated with MNV-1 to a final concentration of approximately \(3 \times 10^6\) PFU/g. After incubation for 1 h, the samples were sanitized with gentle agitation for 2 min in 200 ppm of chlorine solution. The strawberries were stomached. After 5,000 rpm centrifugation for 10 min, 200 μl of 0.25M sodium thiosulfate was added to each sample to neutralize the possible residual chlorine. After 10 min incubation, the surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
4. Enhanced sanitization of murine norovirus in fresh strawberries by SDS alone or by combination of SDS with chlorine solution. Since commonly used sanitizers are not effective in sanitization of viruses from fresh produce, we attempt to develop new sanitizers that may enhance the virus sanitization. Based on the fact that surfactants can reduce the surface tension of liquids and possibly possess virucidal activities, we hypothesized that surfactants may enhance the removal of virus from fresh produce. To address this premise, we first evaluated the reduction of MNV-1 on strawberries using either SDS alone or a combination of SDS with 200 ppm of a chlorine solution. As described in Materials and Methods, MNV-1-contaminated samples (50 g of strawberries) were washed with either SDS solution alone or combination of SDS with chlorine solution for 2 min at room temperature. The amount of surviving viruses after treatment was quantified by plaque assays. Figure 17 shows the viral survivors after each treatment. Consistent with previous observations, tap water washing only gave a 0.8 log reduction in virus titer. Two hundred ppm of chlorine solution brought about a slight increase in virus reduction (1.0 log) in comparison with tap water alone, but the increase was not statistically significant (P>0.05). Interestingly, the virus removal was significantly improved upon sanitization with a SDS solution. Moreover, the amount of reduction in MNV-1 titer gradually increased as the concentration of SDS increased. For example, a 3.14 log virus reduction was achieved when 50 ppm of SDS solution was used, indicating that 50 ppm of SDS is significantly more efficient than that of tap water and 200 ppm of chlorine solution (P<0.05). However, 1 and 10 ppm of SDS gave about 1.25 and 2.60 log virus reductions, respectively. 100 ppm of SDS slightly increased virus reduction (3.41 log) compared to 50 ppm of SDS. However, the washing efficiency (log virus reduction) of SDS did not continuously increase after its concentration reached 200 ppm. For example, 1,000 ppm of SDS gave a 3.51 log virus reduction, which was
only slightly higher than that of 200 ppm concentration (3.12 log reduction) (P>0.05). Overall, these results demonstrated that SDS solution alone significantly increased the removal of virus from strawberries even at a very low concentration (20-100 ppm).

Figure 17. Effect of SDS concentration on removal of MNV-1 from strawberry. Fresh strawberries were inoculated with MNV-1 to a final concentration of approximately 3 ×10⁶ PFU/g. After incubation for 1 h, the samples were sanitized with gentle agitation for 2 min in washing solutions containing various levels of SDS. After sanitization, the fresh produce was stomached, and the surviving viruses were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.

Because chlorine solution is the most commonly used sanitizer in fresh produce, we then attempted to achieve improved virus reduction by combining SDS and chlorine solution. Using identical washing procedure, strawberries were washed with chlorine solution containing increasing amounts of SDS ranging from 10 to 1000 ppm. As shown in Fig. 18, SDS enhanced
the efficiency of virus removal in a concentration-dependent manner. While chlorine solution alone only gave a 0.96 log virus reduction, a 2.94 log virus reduction was observed when a very low amount of SDS (10 ppm) was added to chlorine solution. Notably, a 3.36 log virus reduction was achieved using chlorine solution containing 50 ppm of SDS. Similar to SDS solution alone, the washing efficiency was not further enhanced by addition of SDS into chlorine solution at 200 ppm or higher concentrations. Apparently, these observations indicate that virus removal was significantly enhanced by combination of SDS with chlorine solution.

Figure 18. Enhanced removal of MNV-1 in strawberry by combination of SDS and chlorine solution. Fresh strawberries were inoculated with MNV-1 to a final concentration of approximately 3 ×10^6 PFU/g. After incubation for 1 h, the samples were sanitized in washing solutions containing various levels of SDS in combination of 200 ppm of sodium hypochlorite.
The surviving viruses after washing were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.

We noticed that there is no significant difference in virus reduction between SDS solution and SDS-chlorine combined solution. For example, the combination of 50 ppm of SDS and 200 ppm of chlorine solution led to a virus reduction of 3.36 logs, which is just slightly higher than the reduction caused by 50 ppm of SDS alone (3.14 logs). Further comparisons of the washing efficiency between SDS and SDS-chlorine solutions showed that SDS and SDS-chlorine solutions have comparable efficacies in removing MNV-1 from strawberries. Taken together, these results clearly demonstrated that virus removal from strawberries is significantly improved by using SDS alone or combination of SDS and chlorine solution. Under our experimental conditions, we also concluded that 50 ppm of SDS is an optimal working concentration since it is cost effective, highly efficient in virus removal, and safe to consumers. To our knowledge, this is the first report showing that a sanitizer is able to achieve a viral reduction of more than 3 logs in fresh produce. Apparently, SDS solution alone or the combination of SDS and chlorine enhanced the efficiency of virus sanitization by over 100 times in comparison with traditional sanitizer only (such as chlorine).

5. Enhanced sanitization of a human norovirus surrogate in other fruits and vegetables by SDS solution or by combination of SDS with chlorine solution. Our ultimate goal is to develop a novel sanitizer that can be used to enhance the safety of fruits and vegetables. Hence, after it was observed that SDS was able to give a significantly higher virus reduction in strawberries, we expanded our studies to other fruits and vegetables. We selected
two leafy greens (cabbage and romaine lettuce) and one other fruit (raspberries) since these commodities are often contaminated by norovirus and the surface of these products is strikingly different than that of strawberries. Like strawberries, MNV-1-contaminated cabbage, lettuce, and raspberries samples were washed with tap water, chlorine, SDS (50 ppm), and SDS (50 ppm)-chlorine solutions, respectively, and the surviving viruses were quantified by plaque assay.

Similar to our previous observation for strawberries, the tap water and 200 ppm of chlorine solution only brought about 1.23 and 1.48 log virus reductions in raspberries, respectively (Fig.19). Surprisingly, 50 ppm of SDS alone caused a 2.63 log virus reduction in raspberry (Fig.19) in comparison with a 3.14 log virus reduction in strawberry (Fig.17). Moreover, a 3.05 log virus reduction was achieved when SDS (50 ppm) was combined with chlorine solution (Fig.19). In cabbage, tap water and chlorine solution gave 0.61 and 1.31 log virus reduction, respectively (Fig.19), whereas SDS alone (50 ppm) exhibited an efficiency of virus reduction virtually equivalent to that of chlorine solution. Importantly, a 2.56 log virus reduction was obtained when SDS (50 ppm) was combined with chlorine solution (Fig. 19). For lettuce samples, tap water and chlorine solution only led to 0.23 and 1.12 log virus reduction, respectively (Fig.19). In contrast, SDS alone (50 ppm) gave a 2.26 log virus reduction, which is significantly higher than that of chlorine solution (P<0.05). The combination of SDS and chlorine further enhanced the virus removal (2.90 log virus reduction). Therefore, our results demonstrated that the combination of SDS (50 ppm) and chlorine (200 ppm) brought about the biggest reduction in virus titer, at about 3 logs, for all four tested fruits or vegetables. In addition, while SDS solution alone generally improved the sanitization efficiency compared to chlorine solution, there were notable differences in virus reduction among different types of produce. For example, SDS alone at the concentration of 50 ppm was able to efficiently remove the viruses
from strawberries with a 3.14 log virus reduction, whereas the corresponding virus reductions in raspberries, cabbage, and lettuce were 2.63, 1.80 and 2.26 log, respectively. Taken together, the addition of SDS in chlorine solution significantly enhances the sanitization of virus in fruits and vegetables in general.

Figure 19. Enhanced removal of MNV-1 in lettuce, cabbage, and raspberry by SDS solution or by combination of SDS with chlorine solution. Fresh lettuce, cabbage or raspberries were inoculated with MNV-1 to a final concentration of approximately $3 \times 10^6$ PFU/g. After 1 h incubation, the samples were sanitized in washing solutions containing 50 ppm of SDS alone, and also in combination with 200 ppm of sodium hypochlorite. Data are the means of three replicates. Error bars represent ±1 standard deviations.

6. Enhanced sanitization of viruses in fruits and vegetables by other surfactants.

Since SDS exhibited significant potential in enhancing the virus removal in fresh produce, we continued to determine whether other surfactants retain similar potentials in enhancing the
sanitization of norovirus in produce. These commonly used surfactants include NP-40, Triton X-100, and polysorbates (such as Tween 20). Experimental design and sanitization procedure for each surfactant were essentially identical to that with SDS. Four types of MNV-1-contaminated fresh produce (strawberries, raspberries, cabbage, and lettuce) were washed by tap water, chlorine (200 ppm), surfactant (50 ppm), and surfactant (50 ppm)-chlorine (200 ppm) solutions for 2 min at room temperature. Tap water and chlorine solutions did not give virus reductions of more than 1 log in any tested fruit or vegetable (Fig. 20). While NP-40 alone (50 ppm) gave more virus reduction (1.2-2.7 log) than tap water or chlorine solution, the combination of NP-40 (50 ppm) and chlorine (200 ppm) was the most efficient at removing norovirus in all tested samples as evidenced by the fact that it gave virus reductions of about 3.0 logs in raspberries, lettuce, and cabbage, and up to 3.5 logs in strawberries (Fig. 20). Similar results were observed when Tween-20 was used for sanitization (Fig. 21), which showed that the combination of Tween-20 and chlorine was the most effective strategy in removing viruses (approximately 3 logs reduction). Interestingly, results from Triton X-100 (as well as SDS) are somehow different. For raspberries and cabbage, Triton X-100 (50 ppm) gave similar results as chlorine (200 ppm), but for strawberries and romaine lettuce, Triton X-100 (50 ppm) caused almost 1 log of additional reduction compared to the chlorine solution (Fig. 22). In any case, the combination of Triton X-100 and chlorine remained to be the most efficient at removing MNV-1 fresh produce (approximately 3 log virus reduction). Taken together, these results demonstrated that surfactants other than SDS also significantly enhanced virus removal in fresh produce and the combination of a surfactant and chlorine was the most effective sanitizer.
Figure 20. Enhanced removal of MNV-1 in fruits and vegetables by NP-40. Fresh produce samples were inoculated with MNV-1 to a final concentration of approximately $3 \times 10^6$ PFU/g. After 1 h incubation, the samples were washed with tap water, 200 ppm of chlorine water, 50 ppm NP-40, or 50 ppm of NP-40 in combination with 200 ppm of sodium hypochlorite. The surviving viruses after washing were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
Figure 21. Enhanced removal of MNV-1 in fruits and vegetables by Triton X-100. Fresh produce samples were inoculated with MNV-1 to a final concentration of approximately $3 \times 10^6$ PFU/g. After 1 h incubation, the samples were washed with tap water, 200 ppm of chlorine water, 50 ppm of Triton X-100, or 50 ppm of Triton X-100 in combination with 200 ppm of sodium hypochlorite. The surviving viruses after washing were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.
Figure 22. Enhanced removal of MNV-1 in fruits and vegetables by Tween 20. Fresh produce samples were inoculated with MNV-1 to a final concentration of approximately $3 \times 10^6$ PFU/g. After 1 h incubation, the samples were washed with tap water, 200 ppm of chlorine water, 50 ppm of Tween 20, or 50 ppm of Tween 20 in combination with 200 ppm of sodium hypochlorite. The surviving viruses after washing were quantified by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviations.

2.5. Discussion

Fresh fruits and vegetables are foods at a high risk for norovirus contamination. Currently, no effective methods have been established in reducing virus contaminants in fresh produce. To improve the sanitization, we investigated the ability of a panel of sanitizers and
surfactants to remove a human norovirus surrogate, MNV-1, in a number of fruits and vegetables. We found that organic acids slightly enhanced virus sanitization in fresh produce. We found that virus sanitization was significantly enhanced by addition of surfactants. Our results indicate that surfactants may be a novel and feasible sanitizer for removing viral contaminants from fresh produce thus reducing the number of produce-related foodborne viral outbreaks.

**Effect of organic acids on virus sanitization in fresh produce.** It has been a challenge to remove viruses from fresh produce. Foodborne viruses such as human norovirus are non-enveloped RNA viruses, and their lack of envelope makes them very resistant to agents such as acids, pH, environmental stresses, and disinfectants. The typical washing solution used in food industry currently is sodium hypochlorite, but this usually gives only about 1 log of virus reduction (Baert et al., 2009; Baert et al., 2009; Baldev et al., 2001; Doyle and Erickson, 2008). In this study, we evaluated a total of six sanitizers including four organic acids in sanitization of virus in fresh produce. These organic acids were chosen because all of them are FDA approved food additives. To date, there is no mandatory limitation on the amount of citric acid and acetic acid used as a washing solution, whereas peroxyacetic acid cannot exceed 80 ppm in a solution of washing water. Consistent with many previous studies, tap water, chlorine, peroxyacetic acid, and hydrogen peroxide were not effective in sanitization of MNV from fresh produce. Approximately 1 log virus reduction was achieved for these sanitizers. We did observe an enhanced virus sanitization using two organic acids, citric acid and acetic acid. However, the washing efficiency varied with the type of fresh produce. For example, both citric acid and acetic acid achieved 2.1-2.5 log virus reductions in strawberries, but only gave 1.3-1.5 log virus
reductions in lettuce. Given the fact that less than ten virus particles can cause illness, there is an urgent need to explore other novel sanitization methods.

**Surfactants enhance the virus sanitization in fresh produce: factors affecting viral removal in fresh produce.** Surfactants such as SDS, polysorbates, Triton X-100, and NP-40 can reduce the surface tension of water by adsorbing at the liquid-gas or liquid-liquid interface, thus can potentially enhance the removal of viruses from fresh produce. Another alternative is that the surfactants are able to directly denature the virus, resulting in inactivation during sanitization. In the present study, we tested surfactants alone, and then in combination with 200 ppm of a sodium hypochlorite solution. In all cases, the combination of a tested surfactant and the sanitizer was the most effective way to remove MNV-1 from fresh produce. Also, SDS was the most effective surfactant used, followed by NP-40, Triton X-100, and Tween 20. Using the combination of SDS and the chlorine solution, more than 3 log virus reductions were achieved in all fresh produce samples. Surfactants could be a novel method to be used for enhancing virus removal in fresh produce when combined with a chlorine sanitizer.

There are many factors that can influence the efficiency of virus removal, including choice of a sanitizer, concentration used, washing or contact time, and the nature of the food the virus has attached to. Clearly, the concentration of surfactants plays an important role in virus removal. We found that as the concentration of surfactants increased, the amount of log reduction increased. However, once the concentration of surfactants was over 50 ppm, the virus reduction did not significantly increase. Hence, we decided to use 50 ppm as the primary concentration for all the experiments. While the use of more surfactant led to slightly more reduction in viral titer, it was not enough to outweigh the fact that it would be less cost effective,
and potentially cause more health concerns for consumers. 50 ppm of a surfactant still brought about a significant increase in virus reduction compared to either tap water or chlorine water alone, and was most effective in combination with 200 ppm of chlorine.

As for washing contact time and the type of food the virus is attached to, we only tested one time point (2 min) and four types of fresh produce. We expect that there would be a greater log reduction in virus titer if the length of contact time increases. We also expect there should be changes in the amount of reduction when the amount of wash solution is dramatically scaled up as in industry, or if the produce is agitated more aggressively than gently agitating by hand as we did in our study. Foods such as strawberries and raspberries typically showed more reduction than foods such as cabbage and lettuce. This is most likely attributed to the larger surface area that the virus can attach to in the case of cabbage and lettuce. The texture of a strawberry is also much different from that of a piece of lettuce, which may also have an effect on virus attachment and removal ability. In addition, there are many structures in leafy greens such as wrinkles, which may provide shielding effect and thus increase removal difficulty. It has been found that bacterial pathogens become internalized in leafy green via stomata where CO₂ and O₂ exchange occurs (Doyle and Erickson, 2008; Lou et al., 2010). Recent evidence has suggested that viral pathogens can also be internalized although it is unknown whether the internalization occurs at the stomata (Rawsthorne et al., 2009; Urdaneta et al., 2005; Wei et al., 2010). Thus, we cannot exclude the possibility that some viruses already became internalized because we mixed MNV-1 with leafy greens (lettuce and cabbage) for 1 h prior to sanitization. Presumably, fewer viruses can be removed if virus internalization occurs in leafy greens.
Surfactants are a novel intervention to enhance the safety of fresh produce. In this study, we were able to show that surfactants are effective in enhancing the removal of human norovirus surrogate in various types of fresh produce. SDS, the most effective surfactant against MNV-1, appears in many daily used products such as dish soaps, toothpastes, and shampoos, and is an FDA approved food additive (FDA, 21 CFR 172.822). Shampoos and soaps contain dodecyl sulfate derivatives (sodium or ammonium dodecyl sulfate) at concentrations exceeding 10%. Toothpaste that is routinely used in the oral cavity also has very high concentrations (5 to 8%) of SDS and its derivatives. In foods, SDS is approved for use at concentrations of 25 to 1,000 ppm, depending on the type of products (FDA, 21 CFR 172.822. On the other hand, all of the polysorbates similar in structure to Tween 20 have either GRAS status or are FDA approved food additives as well (FDA, 21 CFR 172.840, 172.836, and 172.838). For example, Tween 80 has been used as an emulsifier in ice cream and custard products, as a dispersing agent in pickle products and gelatin products, as an emulsier in shortenings and whipped toppings, and as a defoaming agent in the production of cottage cheese (FDA 21 CFR 172.840). Tween 80 is typically used at levels not exceeding 0.1% of the finished product (FDA, 21 CFR 172.840). In addition, even though Triton X-100 and NP-40 are not currently FDA approved, they are similar in function to SDS and Tween 20. Hence, they may be feasible alternatives in the future once more research is conducted on their safety.
Figure 23. Potential application of surfactants in minimizing virus contamination in fresh produce. The square boxes show the supply chain flow for leafy greens (such as lettuce) in fresh produce industry. Proposed interventions to minimize the virus contamination are shown as ovals.

Figure 23 represents the flow chart of current practice for processing of leafy greens (such as lettuce) in fresh produce industry. After being harvested from the field, the lettuce is usually subjected to a spray of chlorinated water. To keep fresh, the produce is then transported for vacuum cooling. After the cooling step, the produce continues to be transported to processing plants for cutting, washing by chlorinated water, and packaging, followed by retail distribution. Other produce such as berries and fruits may not exactly follow the flow chart shown in Fig.23. However, they are usually washed before after retail for human consumption. In the chain of this processing event, the use of surfactants could be especially applied during the sanitization step.
by simply adding 50 ppm of SDS, for example, to the chlorine solution already used currently. Such strategy would yield more virus reduction than what the industry is currently achieving, and would not be much of a change for food processing companies. To avoid cross contamination, we used fresh washing solution for each sanitization. In industry, the washing solution may be frequently re-used. Indeed, we found that viruses were inactivated in chlorine solution or chlorine plus SDS (please see data in Chapter 3). One alternative could be to spray a solution containing surfactants on the fresh produce before the latter is transported to vacuum cooling. Consequently, this spray solution would help with any contamination acquired during pre-harvest or harvesting, and then the sanitization step with chlorine would be another hurdle for harmful microorganisms. Of note, a spray solution containing both SDS and chlorine would likely enhance the virucidal activity (please see data in Chapter 3). Another possible way to use surfactants for enhancing safety would be to coat packages of produce with SDS before they are sent to the stores for consumers to purchase. Since it is known that viruses can survive in foods with high stability for many days to weeks, SDS could kill the viruses on the produce while they are stored. Evidently, this approach would only work for types of produce that are packaged instead of free, unpackaged types of produce, but it is still another hurdle technology that could be used in the future. Any or all of these applications could be implemented in the food industry to further enhance the safety of fresh produce and hopefully reduce the incidences of produce-associated outbreaks of norovirus and other types of foodborne viruses or bacteria as well. Further research is needed in this area, but with more studies done on foodborne viruses, this problem can be combated.
Rationales that surfactants can also enhance sanitization of human norovirus and other pathogens. Since human norovirus is noncultivable, we are unable to directly address the removal of this biodefense agent in fresh produce. Indeed, most of our understanding the survival and biology of human norovirus comes from the studies of proper cultivable surrogates such as MNV-1, feline calicivirus (FCV) and canine calicivirus (CaCV) (Cannon et al., 2006; Cromeans et al., 2010; Dawson et al., 2005; Tree et al., 2005; Wobus et al., 2006). In fact, MNV-1 appears to be the most suitable surrogate because of its stability and genetic relatedness to human norovirus (Cannon et al., 2006; Wobus et al., 2006). Although it is possible that a different virus may have different binding affinity to fresh produce, it is reasonable to propose that the removal of any pathogens from a surface should be increased due to the fact that surfactants can reduce the surface tension. Apparently, this concept has been used in routine products such as soap and toothpaste. Therefore, our study highlights a new notion that combining surfactants and sanitizers may function more efficiently in virus removal in fresh produce. Ongoing studies in our laboratory are to evaluate other types of commonly-used surfactants, such as cationic or zwitterionic detergents or combination of surfactants and organic acids, in removing virus particles in fresh produce.

In conclusion, we found that surfactants can significantly enhance the sanitization of virus contaminant from fresh produce. To our knowledge, this is the first report of a novel sanitization approach that resulted in 3 log virus reduction in fresh produce. Implementation of this novel strategy would likely reduce the virus load in fresh produce and improve the safety of fresh produce.
CHAPTER 3

VIRUS INACTIVATION BY SURFACTANTS: SENSITIVITY AND MECHANISM

3.1. Abstract

Surfactants are surface-acting agents which are known to interact with proteins, enzymes, lipid, and cell membrane. However, the effectiveness of surfactants on virus inactivation and mechanisms underlying virus inactivation are less understood. In this study, we systemically determined the virucidal activity of surfactants on both enveloped and non-enveloped viruses using vesicular stomatitis virus (VSV) and murine norovirus (MNV-1) as the models. We found that all four tested surfactants, including sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), Triton X-100, and polysorbates (Tween-20) were able to show considerable virucidal activity against both enveloped and non-enveloped viruses in a concentration-dependent manner. SDS appears to be the most effective surfactant against both MNV-1 and VSV. In addition, SDS significantly enhanced the virucidal activity of chlorine solution, the most widely used sanitizer in food industry. We further demonstrated that SDS altered the structure of the capsid of MNV-1 and disrupted the viral envelope of VSV. However, SDS did not degrade the viral capsid protein or viral genomic RNA. Taken together, our results suggest that combination of surfactants and sanitizers is a feasible and more effective strategy to inactivate viruses and perhaps other pathogens in food industry.
3.2. Introduction

In Chapter 2, we demonstrated that surfactants (SDS, NP-40, Triton X-100, and Tween 20) significantly enhanced the sanitization of murine norovirus (MNV-1) in fresh produce. However, the mechanism by which surfactants enhance virus sanitization remains to be further explored. On one hand, surfactants generally reduce the surface tension thus resulting in enhanced removal of pathogens from any surface. On the other hand, surfactants may specifically inactivate the virus during the washing procedure, which in turn leads to the reduction of infectious virus particles. The latter notion is supported by the fact that surfactants may interact with viruses, more specifically viral proteins and enzymes (Gloxhuber and Kunstler, 1992; Banat et al., 2000). Theoretically, the interactions of surfactants with viruses may result in (i) disruption of the viral capsid and envelope; (ii) alteration of viral proteins such as protein folding/refolding, denaturation, stabilization, and/or aggregation; and (iii) damage to viral genetic materials. Nonetheless, the mechanisms underlying surfactants-mediated viral inactivation may vary with viruses and surfactants.

Based on the structure of virion, viruses can be classified into two distinct categories, namely, non-enveloped and enveloped viruses. For non-enveloped viruses, the outer shell is the highly stable capsid that protects viral genomic materials. Of note, all foodborne viruses including human norovirus are non-enveloped viruses. Enveloped viruses have viral envelopes covering their protein capsid. The envelopes typically are derived from the host cell membranes (phospholipids and proteins) with viral spike proteins or glycoproteins anchored into the surface of the envelope.

The virucidal activities of surfactants on enveloped viruses have been studied. It has been shown that SDS can effectively inactivate sexually transmitted viruses including papilloma
viruses, herpes simplex virus-2 (HSV-2), and human immunodeficiency virus-1 (HIV-1) (Howett et al., 1998; Howett et al., 1999; Howett and Kuhl, 2005). Moreover, Song et al. (2010) demonstrated that SDS, NP-40, and Triton X-100 significantly reduced the infectivity of hepatitis C virus, another enveloped virus (Song et al., 2010). In addition, it has been reported that Triton X-100 is able to denature the coat protein of tobacco mosaic virus thus drive the latter into aggregation (Panyukov et al., 2008). Yet the effectiveness of surfactants on foodborne viruses such as norovirus has not been reported to date.

In the present study, we intended to evaluate the effectiveness of surfactants in virus inactivation and explore the underlying mechanism(s). The enveloped and non-enveloped viruses used in this study were vesicular stomatitis virus (VSV) and murine norovirus (MNV-1), respectively. We chose VSV in our study since the virion structure of VSV is significantly different compared to MNV-1. VSV is the prototypic member of the Vesiculovirus genera of the Rhabdoviridae family (Li et al., 2005). VSV is a nonsegmented negative-sense RNA virus that encodes five major proteins: glycoprotein (G), large protein (L), phosphoprotein (P), matrix protein (M) and nucleoprotein (N). The G protein is located in the surface of envelope that mediates virus attachment and entry. Our results show that all four chosen surfactants exhibited virucidal activities against both enveloped (VSV) and non-enveloped viruses (MNV-1). Apparently, VSV is more sensitive to surfactants than MNV-1. We also found that SDS disrupted the viral capsid and envelope thus leading to virus inactivation. Interestingly, SDS did not degrade viral proteins or genomic RNA, indicating that SDS (surfactants in general) alters tertiary and quaternary structures of the viral capsid, but not the primary structure. Our findings also suggest that surfactants may have important applications in the fresh produce industry other than enhancing virus removal during sanitization. For example, surfactants may be used as a
strategy for antiviral packaging to reduce the viral load in packaged produce, or as an additive to the currently used chlorine spray solution to pre-treat the produce as it is transported to processing plants.

3.3. Materials and Methods

3.3.1. Cell culture and virus stock

Murine norovirus strain MNV-1 was a generous gift from Dr. Herbert W. Virgin IV, Washington University School of Medicine (Karst et al., 2003). MNV-1 was propagated in murine 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 (Invitrogen, Carlsbad, CA) with the addition of 10% fetal bovine serum (Invitrogen) at 37°C under a 5% CO₂ atmosphere. To prepare MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 20. After incubation at 37°C for 1 h, 15 mL DMEM supplemented with 2% fetal bovine serum (FBS) were added. After two days post-infection, the virus was harvested by freeze-thawing three times, and the supernatant was collected after centrifugation at 4°C, 5,000 g for 20 min.

Vesicular stomatitis virus (VSV) Indiana strain was generously provided by Dr. Sean Whelan at Harvard Medical School (Li et al., 2005). VSV was grown in baby hamster kidney (BHK-21) cells (ATCC, Manassas, VA). VSV stock was prepared as previously described (Li et al., 2005). Briefly, confluent BHK-21 cells were infected with VSV at a MOI of 3. After incubation at 37°C for 1 h, 15 ml of DMEM supplemented with 2% FBS were added. Virus was harvested after 18 h post inoculation by centrifugation at 4°C, 5,000 g for 10 min. The virus suspension was stored at -80°C in aliquots.
3.3.2. Virucidal assay

A non-enveloped virus (MNV-1) and an enveloped virus (VSV) were used to test whether surfactants can directly inactivate the viruses. 1 ml of MNV-1 (10^8 PFU/ml) and VSV (10^10 PFU/ml) stocks were incubated with each surfactant at 37 °C. At each time point, 50 µl of the virus sample was collected, and the virus survivors were determined by plaque assays. To avoid any cytotoxic effect that may be caused by surfactants, the inoculum solutions were removed after 1 h of incubation before the overlay was added. For VSV inactivation, only one concentration (200 ppm) of each surfactant was used. The virus samples were collected after 1, 4, 8, 12, 24, 36, and 48 h of incubation. For MNV-1 inactivation, four three concentrations (50 ppm, 200 ppm, 1,000 ppm, and 10,000 ppm) of each surfactant were used. The time points were 1, 4, 8, 12, 24, 36, 48, 60, and 72 h. The kinetics of viral inactivation were generated for each surfactant.

3.3.3. MNV-1 and VSV plaque assay

MNV-1 plaque assay was performed in RAW 264.7 cells as described previously (Karst et al., 2003). In brief, RAW 264.7 cells were seeded in 6 well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2 × 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, 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), 2mM L-glutamine, and 1% agarose. 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. VSV plaque assay was performed in the same way.
except that Vero cells were used in the assays and the plaques were fixed 24 h post-inoculation (Li et al., 2005; Ma and Li, 2010).

3.3.4. Purification of MNV-1 and VSV

To grow a large stock of MNV-1, 18 confluent T150 flasks of RAW 267.1 cells were infected with MNV-1 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 MNV-1 was performed using the method described by Katpally et al. (2008) with minor modifications (29). Briefly, virus suspension was centrifuged at 10,000 × g for 15 min to remove cellular debris. The supernatant was digested with DNase I (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 concentrated by centrifugation at 82,000×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 × g for 6 h at 4°C through a sucrose gradient (7.5 to 45%) in an SW55 Ti rotor (Beckman). The final virus-containing pellets were re-suspended in 100 µl of PBS. The virus titer was determined by plaque assay on RAW 264.7 cells. The amount of total viral proteins was measured using Bradford reagent (Sigma Chemical Co., St. Louis, MO).

Purification of VSV was performed using the method described in our previous publication (Li et al., 2005; Ma and Li, 2010). Briefly, 10 confluent T150 flask BHK-21 cells were infected by VSV at a MOI of 0.01. At 1 h post-absorption, 15 ml of DMEM (supplemented with 2% FBS) was added to the cultures, and infected cells were incubated at 37°C. After 24 h
post-infection, cell culture fluid was harvested by centrifugation at 3,000 \times g for 5 min. Virus was concentrated by centrifugation at 40,000 \times g for 90 min at 4°C in a Ty 50.2 rotor. The pellet was re-suspended in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) and further purified through 10% sucrose NTE by centrifugation at 4°C, 150,000 \times g for 1 h in an SW50.1 rotor. The final pellet was re-suspended in 0.3 ml of NTE buffer. The virus titer was determined by plaque assay on Vero cells, and the protein content was measured using Bradford reagent (Sigma Chemical Co., St. Louis, MO).

### 3.3.5. Transmission electron microscopy

Negative staining electron microscopy of purified virions was performed to determine whether surfactants damage the virus particles. Twenty μl of highly purified MNV-1 and VSV suspension was incubated with 1,000 and 200 ppm of SDS at 37°C for 48 h, respectively. Further experiments were also done to test the damage of 200 ppm of chlorine solution, a combination of 200 ppm of chlorine and 200 ppm of SDS, and a combination of 200 ppm of chlorine and 1,000 ppm of SDS on MNV-1. Viral plaque assays were 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) and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).
3.3.6. Analysis of viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

20 μl of highly purified MNV-1 was incubated with 10,000 ppm of SDS, NP-40, Triton X-100, and Tween 20. After 48 h of incubation, viral plaque assays were conducted to confirm the inactivation of viruses. The samples were boiled for 5 min in a loading buffer containing 1% SDS, 2.5% mercaptoethanol, 6.25 mM Tris-HCl (pH 6.8), and 5% glycerol and loaded onto a 12% polyacrylamide gel. Viral proteins were visualized by Coomassie blue staining.

3.3.7. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to evaluate whether a detergent damaged viral genomic RNA. Briefly, viral genomic RNA was extracted from a MNV-1 control (untreated) and the four samples of MNV-1 treated by each surfactant using an RNeasy kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. RT-PCR was performed using a One Step RT-PCR kit (Qiagen, Valencia, CA) using the following primers to amplify the viral capsid gene VP1: 5’-ATGAGGATGAGTGATGGCGC-3’ (forward) and 5’- TTATTGTTTGAGCATTCGGCC-3’ (reverse). Each reaction mixture contained 400 μM of each dNTP, 0.6 μM of each primer, 4 μl of RNA template, 5 unit of RNase inhibitor, and 2 μl of RT-PCR Enzyme Mix in a total volume of 50 μl. The amplified products were analyzed by 1% agarose gel electrophoresis. The gel was stained with ethidium bromide, and visualized by UV light.
3.3.8. Statistical analysis

All experiments were carried out in triplicate. Virus survival was expressed as mean log titer ± standard deviation. Statistical analysis was done using one-way ANOVA, with a value of p<0.05 being statistically significant.

3.4. Results

Viral inactivation by surfactants. Since all tested surfactants enhanced the removal of MNV-1, a non-enveloped virus, from fresh produce, one may assume that surfactants can directly inactivate the virus during sanitization. To address this premise, we investigated the virucidal activities of surfactants by directly adding the surfactants to virus stocks. Briefly, after incubation of MNV-1 with each surfactant, virus samples were collected after certain time points of incubation, and virus survivors were determined by plaque assay. As shown in Fig.24, all four surfactants showed considerable virucidal activities against MNV-1. Viral titer gradually reduced when incubation time increased. There was no significant difference in virus reduction among these four surfactants at the concentrations of 50 and 200 ppm (p>0.05) (Fig.4A and 24B). At 72 h incubation time, approximately 2.0-2.5 log virus reduction was observed for all four surfactants. At 1,000 ppm, SDS was the most effective surfactant, giving the highest reduction in MNV-1 titer after 72 h incubation (Fig. 24C). The virucidal activity of SDS dramatically increased when the concentration was increased to 10,000 ppm (Fig. 24D). Interestingly, for NP40, Triton X-100, and Tween 20, there was no significant increase in the virucidal activity at 10,000 ppm as compared to other three concentrations (50, 200 and 1,000 ppm) (P>0.05). At 72 h incubation time, 6.1, 2.4, 2.5, and 2.6 log virus reductions were observed for SDS, NP40, Triton X-100, and Tween 20, respectively. Figure 25 represents the correlation between SDS
concentration and virus inactivation. The virucidal activity of SDS significantly increases as its concentration increases to 10,000 ppm.

It has also been expressed in previous literature that MNV-1 is very stable under various conditions. A control of untreated MNV-1 was incubated for 72 h to test its stability, and the results are shown in figure 3. The viral titer does not significantly change over a 72 h period.

**Figure 24. Inactivation of MNV-1 by surfactants.** MNV-1 stock was inoculated with each surfactant (SDS, NP-40, Triton X-100, or Tween 20) and was incubated at 37°C for 72 h. 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. (A) 50 ppm, (B) 200 ppm, (C) 1,000 ppm, and (D) 10,000 ppm.
Figure 24 Continued

(B) 200 ppm

(C) 1,000 ppm

Continued
Figure 25. Effect of SDS concentration on MNV-1 inactivation. MNV-1 stock was inoculated with different concentrations of SDS (50, 200, 1000, 10,000 ppm) and was incubated at 37°C for 72 h. At each time point 50µl of virus sample was collected and the virus survivors were determined by plaque assay. Data are the means of three replicates.
Figure 26. Stability of MNV-1 during incubation. Untreated MNV-1 stock was incubated at 37°C for 72 h to determine its stability over time. At each time point 50µl of virus sample was collected and the titer of the virus was determined by plaque assay. Data are the means of three replicates.

As mentioned earlier, the structure of a virion may affect the virucidal activity of a surfactant. To further address this issue, we compared the effectiveness of the aforementioned four surfactants (200 ppm) in the inactivation of VSV, an enveloped virus. As shown in Fig. 27, VSV is much more sensitive to SDS, NP40 and Triton X-100 than MNV-1 (compare Fig. 25b and Fig. 27). NP40 appears to have the highest virucidal activity against VSV, followed by SDS, Triton X-100, and Tween 20. At 72 h of incubation time, 10.0, 7.5, 6.7, and 2.7 log virus reductions were observed with NP40, SDS, Triton X-100, and Tween 20, respectively. Apparently, these results demonstrate that enveloped virus (VSV) is more sensitive to surfactants than non-enveloped virus (MNV-1). In addition, SDS is considerably effective in inactivating
both MNV-1 and VSV compared to other tested surfactants. For example, 10,000 ppm of SDS dramatically increased the virucidal activity against MNV-1 and almost completely inactivated MNV-1 after 72 h incubation. For VSV, 200 ppm of SDS gave 7.5 log virus reductions after 72 h incubation.

**Figure 27. Inactivation of VSV by surfactants.** VSV stock was inoculated with 200 ppm of each surfactant (SDS, NP-40, Triton X-100, or Tween 20) and was incubated at 37°C for 72 h. 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.

**Combination of SDS and chlorine solution enhanced the virucidal activity.** Since SDS alone, particularly at higher concentrations, has considerable virucidal activity, we expect that combination of SDS and chlorine will further enhance the viral inactivation. To address this question, 200 ppm of chlorine solution was combined with different concentrations of SDS (200, 1,000 and 10,000 ppm), and the virucidal activity was examined. As shown in Figure 28, addition
of SDS in chlorine solution significantly enhances the virucidal activity. For the entire time period, all the combination treatments were more effective than chlorine alone (P<0.05). The combination of chlorine and 10,000 ppm of SDS was the most effective treatment, which is significantly better than the combination of chlorine with 200 ppm or 1,000 ppm of SDS. However, there is no significant difference between 200 ppm and 1,000 ppm of SDS (P>0.05).

For example, after 24 h incubation, chlorine alone gave 3.85 log virus reductions. In contrast, 5.53, 5.00, 6.36 log virus reductions were observed when chlorine was combined with 200, 1,000, and 10,000 ppm of SDS, respectively. Taken together, these results demonstrate that combination of SDS and chlorine solution significantly enhances the virucidal activity.

![Figure 28. Inactivation of MNV-1 by a combination of chlorine and various concentrations of SDS.](image)

MNV-1 stock was inoculated with a combination of 200 of chlorine and various concentrations of SDS (200, 1,000, and 10,000 ppm) and was incubated at 37°C for 72 h. 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 two replicates.
**Surfactants damage virus particles.** To determine how viruses were inactivated by the surfactants, SDS was added to a purified virus stock to the final concentration of 10,000 ppm. After incubation at 37°C for 72 h, viruses were completely inactivated as confirmed by plaque assays. Subsequently, the samples were negatively stained with ammonium molybdate. The virus particles were visualized by electron microscopy as described previously (Lou et al., 2010). MNV-1 is a small round-structured virus of about 30-38 nm in diameter (Fig.29A). After incubation with SDS for 72h, the outer capsid of the MNV-1 was severely damaged and aggregated (Fig.29B). The shape of MNV-1 was also altered and was no longer completely circular (Fig. 29B). The virions appeared smaller than 30 nm.

Chlorine is a well-known strong oxidizer that can react with many materials including proteins, lipids and nucleic acids. Although virus inactivation by chlorine has been extensively studied (Baert et al., 2008, Baert et al., 2009), mechanism involved in virus inactivation is less understood. Therefore, we examined the damage of chlorine and combination of chlorine and SDS on MNV-1. As shown in Fig. 29, we failed to observe any suspected virus particles in samples treated by chlorine or chlorine in combination with SDS (Fig. 29C, 29D). That is, virus particles physically disappeared except that there was some visible debris in the grids occasionally. This observation indicated that virus particles were completely disrupted by chlorine. In contrast, SDS only alters the structure of the viral capsid such as aggregation and distortion whereas the overall capsid is still visible under electronic microscope (Fig. 29B).

As for VSV, it is a bullet-shaped virus of 70 nm in diameter and 140 nm in length, and there are visible spikes anchored in the viral envelope (Fig.20A). After treatment with SDS, the viral envelope was damaged and the shape of VSV was severely distorted (Fig.30B).
Furthermore, some virions were completely disrupted and genetic materials were spilled out from the particles (Fig. 30B). Taken together, these results show that SDS is able to cause significant damages to viral structures of both enveloped and non-enveloped viruses.

**Figure 29. SDS and chlorine disrupt MNV-1 particles.** Purified MNV-1 was incubated with 10,000 ppm of SDS, 200 ppm of chlorine, or a combination of SDS and chlorine at 37°C for 48 h, respectively. Complete virus inactivation was confirmed by plaque assay. The samples were fixed in copper grids, and negatively stained with 1% ammonium molybdate. Virus particles were visualized by Transmission Electron Microscope. (A) untreated MNV-1; (B) MNV-1 treated by SDS; (C) MNV-1 treated by chlorine; (D) MNV-1 treated by SDS and chlorine.
Figure 30. SDS disrupts VSV particles. Purified VSV was incubated 200 ppm of SDS at 37°C for 48 h. Virus inactivation was confirmed by plaque assay. The samples were fixed in copper grids, and negatively stained with 1% ammonium molybdate. Virus particles were visualized by Transmission Electron Microscope. (A) Untreated VSV; (B) VSV treated by SDS.

Surfactants do not degrade viral capsid proteins. MNV-1 is a single-stranded positive-sense RNA virus which is surrounded by highly stable capsid proteins. The MNV-1 capsid is composed of two proteins- the major capsid protein VP1 and the minor protein VP2 (Jiang et al., 1993). VP1 plays critical roles in viral attachment and entry into the host cell, and packaging and release of the mature virion (Jiang et al., 1992; Prasad et al., 1999). Although SDS alters the structure of the viral capsid (Fig. 29B), it remains to be clarified whether surfactants directly degrade the viral capsid protein. To address this question, we incubated highly purified MNV-1 with each surfactant until the completed inactivation of virus, and subsequently analyzed the samples by SDS-PAGE. For untreated MNV-1, the capsid protein VP1 (with an approximately molecular weight of 62 kDa) was present in SDS-PAGE (Fig. 31A, lane 2). Interestingly, comparable amounts of VP1 protein were observed in MNV-1 samples treated with SDS, NP-40,
Triton X-100, and Tween 20 (Fig. 31A lane 3 and 31B lanes 2-4), implying that surfactants do not degrade the viral capsid protein. Together with our results in previous EM studies (Fig.29), these results suggest that surfactant may alter the tertiary and quaternary structures, but not the primary structure of the viral capsid protein.

**Figure 31. Surfactants do not degrade viral capsid proteins.** Highly purified MNV-1 was treated with surfactants. After the complete inactivation of virus, total viral proteins were analyzed by 12% SDS-PAGE followed by Coomassie blue staining.
Surfactants do not degrade viral genomic RNA. If viral genomic RNA is damaged and/or degraded, it would be lethal to the virus. With regard to this perspective, we used RT-PCR to determine whether surfactants can degrade viral RNA. Two primers were designed to amplify the VP1 gene from the viral genomic RNA. 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) was present in the untreated control (Fig. 32, lane 2). Similar amounts of the VP1 gene were amplified from MNV-1 samples treated with each of the four surfactants (Fig. 32, lanes 3-6). Hence, our results indicate that viral genomic RNA is not degraded by surfactants.
Figure 32. Surfactants do not degrade viral RNA. MNV-1 was treated with each surfactant. After complete inactivation of virus, viral genomic RNA was extracted from MNV-1. The VP1 gene was amplified by a one-step RT-PCR, and PCR products were visualized by 1% agarose gel electrophoresis.

3.5. Discussion

In this study, we determined the virucidal activities of surfactants and provided mechanistic insights into how virus was inactivated by surfactants. All chosen surfactants exhibited considerable virucidal activities with SDS as the most effective surfactant in virus inactivation. We further showed that SDS altered the structure of the capsid of MNV-1 and disrupted the viral envelope of VSV. However, SDS did not degrade the viral capsid protein or
viral genomic RNA. Our results strongly support that combination of surfactants and sanitizers is a more effective strategy to inactivate viruses and perhaps other pathogens.

**Virucidal activity of surfactant and potential applications.** It is well known that surfactants can interact with viral proteins (Banat et al., 2000; Hartmann et al., 2006; Howett and Kuhl, 2005; Krebs et al., 1999). Such interaction can influence protein folding/refolding, denaturation, and aggregation. In this study, we found that all four surfactants were able to inactivate a human norovirus surrogate in a concentration-dependent manner. SDS appears to be the most effective surfactant against MNV-1. Incubation of MNV-1 with 200 ppm of SDS solution at 37°C for 4 h resulted in a 3 log virus reduction. VSV, an enveloped virus, is much more sensitive to surfactants than MNV-1 as evidenced by a 5 log reduction of VSV upon incubation with 200 ppm of SDS at 37°C for 4 h. Taken together, these results suggest that SDS as well as other surfactants can be useful in the inactivation of many viruses, both enveloped and non-enveloped.

We also found that virucidal activity was significantly increased when SDS was combined with chlorine solution. After 24 h incubation, chlorine alone gave 3.85 log virus reductions. However, 5.53, 5.00, and 6.36 log virus reductions were observed when chlorine was combined with 200, 1,000, and 10,000 ppm of SDS, respectively. By comparing Fig. 25 and Fig. 27, it can be observed that any combination treatment of SDS and chlorine showed significantly more virucidal activity than SDS alone. After 24 h incubation, 200 ppm of SDS alone (Fig. 25) gave a total reduction of 2.38 logs while a combination of 200 ppm of chlorine and 200 ppm SDS (Fig. 27) gave a total reduction of 5.53 logs.
In Chapter 2, we showed that surfactants significantly enhanced the sanitization of MNV-1 from fresh produce and the combination of surfactants and sanitizers was the most effective washing method. There are two major mechanisms that contribute to the increased virus sanitization. First, surfactants can reduce surface tension. Second, surfactants also enhanced the virucidal effect. The finding of the virucidal effect of surfactants further supports the notion that SDS may be a good reagent for antiviral packaging in food industry. As shown in Fig. 23 in Chapter 2, leafy greens are usually subjected to a spray of chlorinated water after harvesting from field. One important application is to use a pray solution containing both chlorine and SDS, which may result in enhanced inactivation of viruses as well as other pathogens. Finally, the combination of chlorine and SDS can always be used as a novel disinfectant for any purposes such as surface decontamination, sanitization of food service utensils, and equipment.

**Mechanism(s) underlying inactivation of MNV-1 by surfactants.** Our current study also provided mechanistic insights into surfactants-mediated virus inactivation. It is known that surfactants can interact with proteins, but unfortunately, none of previous studies have investigated how virus was inactivated by surfactants. Here we demonstrated that surfactants were able to alter and disrupt the surface structure of virus particles. For MNV-1, the non-enveloped virus, the outer shell capsid became aggregated after incubation with SDS and the structure of MNV-1 capsid was severely altered. However, SDS did not completely disrupt the structure of virion. It is known that the capsid protein VP1 plays many crucial roles in virus life cycle (Hardy, 2006). First of all, VP1 possesses receptor binding activity, which mediates virus attachment to host cells, the first step of initiation of a virus infection (Jiang et al., 2003). Alterations of the capsid structure likely affect the receptor binding activity which in turn blocks
virus entry and may be lethal to virus. The capsid is also involved in virus replication, assembly and release (Jiang et al., 1992). Presumably, any structure alteration may impaire these functions and result in inefficiency in virus replication, assembly and release. We also investigated whether SDS degraded the viral capsid protein and viral genomic RNA. Our results demonstrate that the viral major capsid protein and viral genomic RNA were not degraded by SDS. This is consistent with results from previous EM studies showing that the round structure of capsid was still visible although the surface of capsid was aggregated and distorted. Together, these findings suggest that SDS may impair the tertiary and quaternary structures, but not the primary structure of viral capsid protein. Since the viral capsid is not completely disrupted, SDS may not have access to viral genomic which is located inside of the capsid. Our results also suggest that mechanisms underlying virus inactivation by SDS and chlorine may be different. Chlorine is a strong oxidizer that completely breaks down the viral capsid. In fact, it has been reported that proteins physically disappear upon chlorine treatment (Predmore and Li, 2011). Therefore, chlorine may not only disrupt the tertiary and quaternary structures, but also degrade the viral capsid protein itself.

For VSV, SDS disrupted the viral envelope and distorted the shape of virions. Unlike non-enveloped viruses, enveloped viruses enter host cells via receptor-mediated fusion in which the viral envelope fuses with the host cell membrane. Thus, it is lethal to an enveloped virus if its envelope is damaged. Generally, envelope virus is less stable than non-enveloped virus because the viral envelope is considerably susceptible to environment stresses. Consistent with this notion, we found that VSV was more easily inactivated by surfactant than MNV-1. Additionally, envelope virus usually possesses spike proteins which anchor in the envelope and mediate receptor binding and virus entry. Apparently, our results do not rule out the possibility that SDS
can alter the structure of the spike proteins (VSV G protein) thus blocking virus attachment and entry.

In conclusion, we found that all tested surfactants possess virucidal activities and SDS is the most effective surfactant. SDS is able to damage viral capsid and envelope which results in virus inactivation. SDS also significantly enhances the virucidal activity of chlorine, a sanitizer widely used in food industry. Therefore, combination of SDS and chlorine would be a feasible and more effective strategy to inactivate viruses and other pathogens. This strategy has a great potential to be used in fresh produce industry and other food industry.
CHAPTER 4

CONCLUSION

Firstly, this study developed a novel strategy that significantly enhanced virus sanitization in fresh fruits and vegetables. Specifically, we found that surfactants, including sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), Triton X-100, and polysorbates (Tween 20), significantly enhanced the sanitization of viruses from fresh fruits and vegetables. The most commonly used sanitizer (200 ppm of chlorine solution) only reduced less than 1.2 logs of virus in fresh produce. Notably, approximately 3 logs of virus reduction were observed in all the tested fresh produce after sanitization with a combination of 50 ppm of each surfactant and 200 ppm of chlorine solution. To our knowledge, this is the first report of a novel sanitization approach that enhanced the efficiency of removing virus from fresh produce by approximately 100 times.

Secondly, this study also demonstrated that surfactants show considerable virucidal activity against both enveloped and non-enveloped viruses in a concentration-dependent manner. SDS appears to be the most effective surfactant in virus inactivation. Moreover, we demonstrated that SDS significantly enhanced the virucidal activity of chlorine solution, the most widely used sanitizer in food industry. Therefore, the combination of surfactants and sanitizers is a more effective strategy to inactivate viruses in fresh produce.
Thirdly, this study provided insight into the mechanism of viral inactivation by surfactants. We demonstrated that SDS altered the structure of the capsid of murine norovirus and disrupted the viral envelope of vesicular stomatitis virus. However, SDS did not degrade the viral capsid protein or viral genomic RNA. These results suggest SDS (surfactants in general) alters the tertiary and quaternary structures, but not the primary structure of the viral capsid protein.

Finally, the combination of surfactants and sanitizers will have important applications in industry. SDS is an FDA approved food additive and polysorbates are FDA recognized GRAS (Generally Recognized as Safe) products. Thus, implementation of these novel sanitization strategies would be a feasible approach to efficiently reduce the virus load in fresh produce. These strategies also have great potential to be used as novel disinfectants for sanitization of other pathogens in the food industry.
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