# UNDERSTANDING AND MANAGING RISK OF NOROVIRUS CONTAMINATION ON FROZEN BERRIES FROM FARM TO FORK

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# ABSTRACT OF THE DISSERTATION

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Norovirus is the leading cause of acute gastroenteritis worldwide. Consumption of frozen berries have been identified as a risk factor for norovirus illness. This dissertation studies four scenarios that affect the risk of norovirus on frozen berries: the effect of freezing and frozen storage on the survival of bacteriophage MS2 (a norovirus surrogate) on frozen strawberries and raspberries; thermal inactivation kinetics of MS2 on frozen strawberry and raspberry purées in a water bath; efficacy of microwave heating on the destruction of MS2 in frozen strawberries; and an agent-based model simulating fruit pickers on a farm to quantify the spread of norovirus from an ill worker to coworkers and berries. The results show that bacteriophage MS2 is able to survive frozen storage on frozen berry products over long periods of time; heating of frozen berries following the product's label instructions using the Defrost microwave oven setting resulted in minimal inactivation of MS2; and increased handwashing compliance on farm had a significant effect on the contamination of berries prior to reaching the consumer and an overall decrease in ill consumers.

A quantitative microbial risk assessment was also developed to assess the risk of norovirus associated with consumption of frozen strawberries. A 2012 norovirus outbreak in Germany linked to frozen strawberries was simulated using the model. Model predictions were in good agreement with data from the actual outbreak. The risk assessment model developed may provide useful quantitative data relevant for risk management initiatives, aimed at controlling the risk of norovirus from frozen berry products.

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# Dedication

To the loving memory of my mom, Sondra:

Even though you are gone, it's your belief in me that has made this journey possible. Her beliefs, selflessness, and personality made me who I am now, and I could not have gotten this far without her support. I know she is with me now and will be forever. I am so proud and lucky to be her daughter and I will dedicate my life to make her proud.

"Love as powerful as your mother's
for you leaves its own mark.
To have been loved so deeply,
even though the person who loved us is gone,
will give us some protection forever."

- J.K. Rowling

I also dedicate this to my dad, brothers, sisters, nephews, cousins, aunts, uncles and friends who continue to support and encourage me to keep pushing through the best and worst times.

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# Chapter 1: Literature Review (General)

## 1.1 Introduction

Norovirus (NoV) causes more cases of foodborne illness in the United States (U.S.) than any other pathogen, and with improvements in diagnostic tools and detection methods, it is likely to be attributed to many more outbreaks in the future *(18, 103, 126)*. U.S. NoV outbreaks are strongly associated with the consumption of fresh produce and in the European Union (EU), the virus is strongly associated with consumption of berries *(20)*. A recent U.K. study tested lettuce and fresh and frozen raspberries sold at retail for NoV over a 13-month period. This study found that 5.3% of lettuce, 2.3% of fresh raspberries and 3.6% of frozen raspberries were positive for NoV RNA *(24)*. A recent study in China on retail frozen and fresh berries found the prevalence of NoV to be 9% and 12.11%, respectively *(43)*.

The annual per capita consumption of fresh fruits has increased from 130 to 150 servings from 2004-2015, however with increased consumption has come an increase in the number of foodborne illnesses attributed to fresh produce *(23, 102)*. Since foods can be contaminated with viruses throughout the food chain, prevention remains a challenge. The Food and Drug Administration (FDA) has recently begun surveillance on frozen berries (raspberries, strawberries, blackberries) for NoV and hepatitis A virus (HAV) to better protect consumers *(41)*.

## 1.2 Physical Characteristics of Foodborne Viruses

Foodborne viruses are classified as enteric viruses since they are mostly transmitted through the fecal-oral route and replicate in the gastrointestinal tract of a suitable host. Since foodborne viruses are host-specific, they show a high degree of

tropism for individual cells *(71, 139)*. NoV are unable to grow or multiply in or on food surfaces and require a live host cell for replication, yet foods are still likely vehicles for viral transmission to susceptible hosts. Enteric viruses can withstand many environmental stressors. NoV has been shown to survive for weeks on surfaces, and can survive freezing, heating, extreme pH and common chemical disinfectants *(48, 75)*. Enteric viruses resist most commonly used sanitizers or disinfectants when these compounds are used at manufacturer recommended concentrations *(47)*.

## 1.3 Transmission Routes

Transmission of NoV is facilitated because infected individuals shed high concentrations of the virus ($10^5$ to $10^{10}$ particles/g stool) *(4, 5, 123)*, infected individuals continue to shed the virus, the virus has good environmental stability and is quite resistance to disinfection *(75)*. NoV can be transmitted from aerosolized vomit and consuming contaminated food and water, however the fecal-oral route is the most common transmission route. Viruses can be introduced into the food chain from contaminated water or surfaces, or due to poor food handler hygiene. Viruses can be transferred both directly and indirectly to food products in the food chain. Since contamination can occur at many points along the food chain, traceback during epidemiological investigation of a foodborne outbreak remains a challenge.

Fresh produce, including berries, can come into contact with NoV-contaminated surfaces throughout harvest and production. Improperly composted materials containing sewage could contain infectious viral particles and should not be used as fertilizer. Enteric viruses, such as HAV or NoV, can survive for months in sewage, and wastewater used for irrigation has been linked to virus contamination of fresh fruits and vegetables

when inadequately treated *(9, 108, 116, 125)*. Lack of sufficient on-site handwashing facilities and toilets on farms can result in workers defecating in or near the fields or handling fresh produce without proper hand hygiene. Viruses are generally thought to occur on the surface of fresh produce, however some studies suggest that hydroponic or other cultivation methods could allow for the internalization of enteric viruses into edible portions of plants via roots *(31)*.

Viral contamination can also occur during post-harvest through washing or preparation of fresh produce. Viruses can contaminate large volume of product if washed in contaminated water. Contamination can also result from poor personal hygiene of individuals that directly handle produce during harvest, packing or food preparation. Vomit from infected individuals can lead to aerosolization of virus particles that can directly contact the product, or settle on surfaces that then come in contact with the product *(127)*. An understanding of viral contamination routes in needed to develop effective risk management strategies for fresh produce production.

## 1.4 Human Norovirus

The Centers for Disease Control and Prevention (CDC) estimates that there are approximately 21 million symptomatic cases of NoV annually in the U.S.; of which approximately 5.5 million cases are linked to consumption of food, making it the top cause of foodborne illness. Foodborne NoV infections results in more than 15,000 hospitalizations and 150 deaths annually in the U.S. *(111)*. Infections peak during the fall and winter season, due to an apparent increase in person-to-person contact, larger numbers of people inside and increased virus survivability in cold temperatures *(1, 66, 90)*. NoV illness is typically a short, self-limiting disease, with symptoms developing 12

to 48 hours after exposure and lasting 24 to 72 hours *(21)*. Young children, the elderly and immunocompromised individuals are at higher risk when infected and can have prolonged symptoms *(74)*. Common NoV symptoms that can transmit the virus include vomiting and diarrhea. Virus levels in stools may reach $10^{10}$ particles per gram or higher *(105)*.

Human NoV belong to the *Caliciviridae* family and its particles are 27-38 nanometers (nm) in diameter *(142)*. The genus NoV is split into seven genogroups; G1, GII and GIV have been found to infect humans. The epidemiology of NoV is largely influenced by virus evolution through an accumulation of mutations *(112)*. The NoV genus has been shown to have a high mutation rate estimated at $1.21 \times 10^{-2}$ to $1.41 \times 10^{-2}$ point substitutions per site per year, leading to large strain diversity *(98, 109, 131)*. NoV nomenclature is based on two factors that describe the genetic lineage of nucleic acids and capsid proteins *(138)*. Genogroups GI and GII contribute to the majority of outbreaks worldwide, though studies have shown a recent predominance of GII strains, with GII.4 as the primary genotype *(37, 112)*. Studies have determined that the major capsid proteins of GII.4 strains evolve quickly, leading to new epidemic strains *(14, 16, 73, 113)*. The emergence of new strains is likely associated with an increase in outbreaks worldwide *(92)*. Protective immunity to any particular NoV strain following infection is considered temporary, although it is an ongoing area of research *(73, 147)*.

## 1.5 Laboratory Assays for Norovirus Detection and Quantification

There are several detection and quantification methods for viruses. Foodborne virus detection methods for liquid samples are categorized as either traditional or modern. Two traditional methods often used today are the plaque assay and the endpoint dilution

assay, also known as 50% tissue culture infectious dose ($TCID_{50}$). Modern methods include reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Quantification methods continue to be developed and all methods have advantages and disadvantages.

Plaque assays are used to determine the number of plaque forming units (pfu) within a virus sample. This microbiological assay may be conducted in petri dishes or well plates and are used in virology to determine viral titer. A susceptible host monolayer of cells is infected with appropriate dilutions of a virus sample in the plaque assay *(8)*. When a virus particle infects a cell, the cell lyses producing a clear zone (or plaque) in the cell monolayer. The lysed cell spreads the infection to adjacent cells, repeating the infection-to-lysis cycle. Eventually the plaques become visible to the naked eye and is then manually counted. The counts are used together with the dilution value to calculate the original number of plaque forming units *(8)*.

$TCID_{50}$ assay determines when 50% of cells in a culture have been infected. This assay provides a qualitative measurement of uninfected and infected cells *(114)*. It is more often used in clinical research where the lethal dose of the virus must be determined or if the virus is unable to form plaques. Since there are distinct differences among $TCID_{50}$ and plaque assays, the results are not used interchangeably. However, there is a theoretical relationship between $TCID_{50}$ and PFU where 0.69 PFU = 1 $TCID_{50}$ *(141)*.

ELISA is considered an accurate and highly sensitive test when compared to other immunoassay methods. ELISA uses a specific antibody linked to an enzyme to detect and measure the presence of an unknown amount of antigen (e.g. virus), in a sample. This method has high specificity because of the selectivity of the antigen-antibody binding.

This binding is quantified as an enzyme converts a reagent into a detectable signal, which is then used to calculate the concentration of the antigen in the sample *(64, 79)*.

Real-time RT-PCR is a sensitive rapid method with improved reproducibility when compared to traditional methods *(80)*. RT-PCR involves extraction of the viral nucleic acids from the sample, conversion from RNA to DNA via reverse transcriptase (if needed), amplification by PCR, and detection of the amplified DNA. Disadvantages of RT-PCR is that it *(60)* can result in false positive results due to potential contamination and (2) the specificity of primers may lead to false negative results for genetically diverse viruses *(132)*. Since most foodborne viruses contain RNA, the requirements to be suitable for an RT-PCR assay must also be carefully considered *(17)*.

## 1.6 Immunity to Norovirus

Immunity to NoV is believed to be temporary and is genotype- or strain-specific with little protection or none at all across the genogroups. Human susceptibility to NoV is thought to depend on an individual's fucosyltransferase 2 (FUT2) genotype. People with a functional FUT2 gene, termed secretor positive, have increased sensitivity to common viruses because FUT2 controls the secretion of ABO histo-blood group antigens (HBGAs) at the surface of the gut. HBGAs are needed for most NoV genotypes to bind, attach and infect cells in the gut. People who lack a functional FUT2 gene, termed non-secretors or secretor negative, have shown protection from infections with several NoV genotypes *(27, 76, 107)*. The relationship between secretor status and susceptibility to NoV has been determined by Thorven et al. (2005), who looked at susceptibility to gastroenteritis resulting from hospital outbreaks in patients and medical staff in Sweden *(124)*. The results showed that patients homozygous for non-secretor status were

protected from viral infection. Larsson et al. (2006) also found that there were significantly higher antibody titers to NoV GII in secretors compared to non-secretors *(68)*. European descendants are believed to be 80% secretor positive and 20% non-secretors (secretor negative), although in other populations such as Mesoamerican, there can be as many as 95% secretor positive individuals *(93)*.

## 1.7 Norovirus Vaccine Development

No vaccine to prevent NoV currently exists *(49)*. A vaccine to minimize overall NoV disease burden would be beneficial given the NoV burden on the general population. Though everyone may benefit from receiving a NoV vaccine, the populations that are at the highest risk, including young children, the elderly and the immunocompromised would be the primary candidates. Many challenges exist in NoV vaccine development, including the continuously evolving genetic diversity of NoV, difficulty in culturing NoV in a laboratory, few human studies and short-term immunity following NoV infection *(77)*.

Virus like particles (VLPs) are antigenically and morphologically similar to NoV but non-replicating since they lack genetic material. VLPs are used as an alternative when developing diagnostic assays for NoV *(56, 121)*. When recombinant NoV capsid proteins are expressed as (VLPs), they are thought to be antigens for NoV vaccines, and a majority of vaccination studies to date utilize VLPs. Gardasil and Cervarix, the two most successful human papillomavirus (HPV) vaccines, are VLP-based vaccines and have been highly effective in preventing HPV infections for strains included in the vaccine *(29, 100)*. NoV vaccines will likely face complications similar to those of the seasonal influenza vaccine due to emerging variants in genogroups and genotypes *(25)*.

A majority of NoV vaccine trials thus far have used a baculovirus expression system (BEVS) to produce capsid proteins of NoV and proteins from VLPs *(121)*. The BEVS uses a helper-independent virus that can be grown to high titers in insect cells that are able to grow in suspension cultures allowing for large amounts of recombinant protein to be produced *(56, 57)*. Vaccine candidates in pre-clinical stages include a *(60)* trivalent vaccine including GII.4 and GI.3 NoVs and rotavirus, (2) bivalent GII.4 NoV and enterovirus 71 vaccine, (3) P particle-based vaccines, and (4) viral vectors incorporating NoV capsid genes (virus replicon particles, VRPs). VLP-based vaccine candidates in clinical stages include *(60)* intranasal monovalent vaccine containing GI.I VLPs, (2) intranasal bivalent vaccine containing GI.1 and GII.4 VLPs, and (3) intramuscular bivalent vaccine containing GI.1 and GII.4 VLPs *(77, 104)*.

## 1.8 Surrogate organisms in virus research

A major problem in the study of NoV was (until recently) the lack of lab-based methods to cultivate NoV in cell lines or animal models. NoV surrogates have been selected and used for experimental studies based on their similarity in behavior, genetic makeup, size and shape to NoV. The virus surrogates used for persistence, survival, inactivation and internalization studies can have very different characteristics from the enteric viruses they intend to represent, therefore data from those studies must be used carefully in quantitative microbial risk assessments (QMRA). Viruses from the *Caliciviridae* family, which contains NoV, are the most logical surrogate option *(26)*. Surrogates that are commonly used for NoV include Feline calicivirus (FCV), Murine norovirus (MNV), Tulane virus (TV), bacteriophage MS2 (MS2) and Poliovirus (PV).

Each surrogate has advantages and disadvantages and its characteristics are shown in Table 1.

FCV is easily propagated but it is known to be less stable at a low pH. Survivability at a low pH is an important resistance mechanism for NoV, allowing it to survive the environment of the stomach *(45)*. FCV is less resistant than MNV-1 to pH and organic solvents *(101)*, however MNV-1 has been shown to be less resistant than NoV to certain disinfectants, showing a 1-2 log greater reduction in viral titer by bleach than that shown with NoV *(45)*. TV is also an important NoV surrogate since cell cultured TV binds similarly to HBGAs as NoV *(97)*.

Another surrogate includes the male-specific bacteriophage MS2 (MS2). MS2 is similar in shape and size to NoV and like NoV, is a single-stranded RNA virus *(53)*. MS2 has been shown to be an effective surrogate in resistance and survival studies for NoV in experimental research involving food products such as oysters and fresh produce *(33)*. MS2 has also been used as an internal control to validate extraction, recovery and detection methods for NoV *(89)*. Bacteriophages are particularly useful surrogates since they are easy to propagate and have a shorter incubation period requirement for detection relative to virus-plaque assays. Male-specific, also called F-specific (F1) bacteriophages, are good indicators for monitoring the viral quality of water and food since they superficially resemble enteroviruses, caliciviruses, and HAV *(19, 35, 51, 61)*. They are also regularly present in wastewaters, resistant to treatment processes considered suitable for enteric virus indicators and are persistent in the environment *(7, 91)*.

## 1.9 Internalization of viruses in foods

NoV outbreaks have most often been linked to a variety of fruits and vegetables consumed raw including fruits (strawberries, raspberries, blueberries and melons) and vegetables such as lettuce *(34, 38, 50, 70, 78, 110)*. Internalization of pathogens (including viruses) in fruits and vegetables is poorly understood. Several studies have investigated internalization using NoV surrogates and showed that fruits and vegetables are susceptible to viral internalization to some degree during production *(30-32, 36, 54, 134, 135, 143)*.

Yang et al. (2018) researched the internalization and dissemination rates of NoV and TV in romaine lettuce, green onions and radishes. They observed a difference in internalization based on the growth substrate of hydroponically grown produce versus soil systems. In a soil growth system, infectious TV was not detected in radishes or green onions, though romaine lettuce plants grown in soil had recoverable TV from plant tissues after 14 days. Hydroponically grown green onions inoculated with a higher TV titer saw increased rates of internalization and dissemination as compared to green onions inoculated with a lower titer *(143)*. DiCaprio et al. (2012) investigated internalization of NoV and its surrogates through the roots and the edible portions of the plant of romaine lettuce over a 14-day period. They found that NoV, MNV and TV can be internalized through the roots and spread to the shoots and leaves of the lettuce *(31)*. This same group *(32)* looked at the effect of abiotic and biotic stress on the internalization and spread of NoV surrogates in romaine lettuce and found that drought stress decreased internalization and dissemination rates of MNV and TV, flood stress and biotic stress did not have a significant impact on the ability for the virus to internalize or disseminate. A study using porcine sapovirus (SaV) as a NoV surrogate found that when SaV was inoculated through

soil, viral RNA could be detected on the roots of the plant for up to 14 days. Viral RNA persisted on the roots and leaves for up to 28 days post-inoculation when SaV was inoculated through the plant roots *(36)*. Wei et al. (2011) observed the internalization of MNV in romaine lettuce during irrigation and found that at high inoculum levels, MNV was detected in lettuce leaves after 5 days *(136)*.

Though most studies have been performed using romaine lettuce, Dicaprio et al. (2015) observed the internalization of MNV and TV in strawberry plants. The virus was introduced to the strawberry plant via the roots in a soil system and found that 31.6% of strawberries had internalized MNV and 37.5% of strawberries were positive for infectious TV *(30)*. Factors that have been shown to affect internalization from published studies include the plant species, inoculum level, plant developmental stage, pathogen type and growth substrate *(30, 32, 36, 135, 143)*.

## 1.10 NoV outbreaks

NoV was first identified in stool specimens that were collected from a gastroenteritis outbreak in Norwalk, OH in 1968, and the organism was first named Norwalk virus *(63)*. The illness was initially described as the "winter vomiting disease" in 1929 due to its seasonal patterns and frequent vomiting symptom *(145)*. Outbreaks of NoV can occur anywhere and at any time but have often been associated with restaurants, schools, catered events and cruise ships *(22)*.

Consuming raw or improperly cooked shellfish *(137)* and fresh produce items, such as lettuce *(81, 133)* and raspberries *(38, 70, 83)*, has been linked to NoV illness. Contamination can sometimes be traced to infected food handlers but can occur at any step in the food chain. Contamination of shellfish could occur through pollution of human

feces in and around the growing areas and fresh produce can be contaminated as a result of pre- and post-harvest practices. Pre-harvest contamination of fresh produce may occur through contaminated irrigation water and improperly treated manure for fertilization *(136)*.

The frozen fruit market has continued to thrive due to its ability to be consumed year-round *(102)*. Though not well documented in the published literature, potential risk factors of viral contamination of berries at primary production include *(60)* environmental factors such as heavy rainfall (2) sewage-contaminated agricultural water intended for use as irrigation water or pesticide application (3) food handlers health and hygiene (4) and the cleanliness of equipment at harvest or post-harvest *(122)*.

From late September to early October of 2012, Germany experienced the largest documented foodborne outbreak in history *(13)*. This outbreak involved more than 390 foodservice institutions with approximately 11,000 cases; NoV was identified as the causative agent of this outbreak. Most of those affected by this outbreak included children and teenagers, all with symptoms of acute gastroenteritis, though staff members at the institutions also fell ill. Following interviews with victims and the catering supplier, an epidemiological analysis led investigators to believe meals containing strawberries (strawberry compote and strawberry tart) were likely vehicles for transmission *(13)*. Frozen strawberries produced by a regional catering kitchen were used as a component of the meals involved. The strawberries were traced to a 22-ton lot that was distributed to several catering agencies throughout Germany and imported from China. Once the product was identified, these strawberries were quickly withdrawn from

the market, preventing additional illnesses. More than 11 tons of contaminated product did not reach consumers due to the recall *(13)*.

FDA reports one U.S. NoV outbreak linked to frozen berries between 1997-2016, resulting in 136 illnesses *(41)*. Many more outbreaks of NoV associated with frozen berries have occurred in Europe in recent years. There were 29 European alerts involving NoV and frozen berries from 2014-2017 *(42)*. Two large outbreaks in Denmark in 2005 and Finland in 2009 occurred from frozen raspberries imported from Poland, resulting in over 900 NoV cases in each outbreak *(38, 110)*.

## 1.11 Berry Production Practices

Strawberries are produced in the U.S. on more than 55,000 acres, mainly in Florida and California. The U.S. ranks first in production of strawberries, followed by Turkey and Spain *(140)*. Strawberry plants have a shallow root system, so irrigation systems are recommended for efficient production. Supplemental nutrients are often applied to the soil of these products through a drip irrigation system *(144)*. Most of the water taken up by the plant evaporates through the stomata of the leaves and stems; strawberry plants must be properly irrigated to replenish the water taken up by the plant. The majority of strawberries are harvested by hand *(40)*. Berries such as raspberries and strawberries are often hand harvested since they are fragile and more susceptible to bruising. Berries may be sold as fresh, frozen or heated for products such as jams or purées.

## 1.12 Labeling Requirements for Frozen Fruit

There are several labeling requirements by regulatory agencies for frozen food products. Some of these requirements include the common name of the ingredients and

the style of the product such as whole and sliced. If the product is visible through the package, the style does not need to be specified. Additional required information includes nutritional information, expiration date, storage requirements, the packer and/or distributors name and place of business and heating instructions. Labels may also include the quality or grade of the product and serving suggestions *(39)*. The FDA also has a set of labeling and regulations for frozen fruit which also includes a "keep frozen" statement, allergen declaration if the product contains a major allergen, and a code for traceability for recall purposes. Distributors must also meet specifications for frozen fruit including grade, style, net weight and fruit to sugar ratio allowance *(128)*.

## 1.13 Freezing Process

Freezing products has been used for decades to extend the shelf life of foods. Freezing may also cause structural changes to the fruit, affecting texture. As food cools, a nucleus (or seed) is needed for an ice crystal to form. Once this first crystal forms, the phase change from liquid to solid progresses, leading to more crystal growth *(10, 129)*. Fruits (like strawberries) are made up of ~ 90% of water which is held within rigid cell walls that gives structure and texture to the fruit. As the water in fruit freezes, the ice crystals expand, rupturing cell walls. When fruit thaws, those ruptured cell walls mean the fruit is a much softer product. When berries are quick frozen, they retain better cell integrity due to the formation of smaller ice crystals. Slow freezing forms large ice crystals which have been shown to increase drip loss after the product is thawed *(55)*.

Commercial freezing processes are able to freeze foods within minutes. Individual quick freezing (IQF) is often used since for fruit since it is more suited to smaller pieces of food products. Taste, color, texture and nutritional value of the products is improved in

quicker freezing processes. Several quick-freezing processes can be used in commercial freezing such as air-blast tunnel freezers, belt freezers, cryogenic freezers, fluidized-bed freezers and plate freezers. Air-blast and belt freezing uses precooled (-40°C) air blown over the food. In air blast freezing, packaged products are placed on a tray and sent through a freezing tunnel. In belt freezing, food products are placed on a conveyor belt and sent through the freezing temperature zone. Fluidized-bed freezers use conveyor belts and high velocity cold air to tumble and float food pieces in a stream of cold air, ensuring all sides of the food are exposed to the cold air. Plate freezers are often used to freeze products that are flat, and the food is pressed between metal plates that have been cooled to subfreezing temperatures *(65)*. Cryogenic freezing immerses the food in liquid nitrogen at low temperatures allowing it to freeze rapidly. The food is continuously moved to avoid the product lumping together. Though this method is very efficient, costs can be higher due to the use of liquid nitrogen *(99)*.

## 1.14 Microwave heating

Organizations such as the American Frozen Food Institute (AFFI) and the Grocery Manufacturers Association (GMA) have issued guidelines for food processors to develop heating instructions for microwaveable food products *(3, 46)*. These recommendations are not specific to all products and vary significantly for not ready to eat (NRTE) versus ready-to-eat (RTE) frozen foods. Food processors should develop instructions for the products quality and safety based on its volume, size, composition and the microwave capability. Validation of cooking and heating instructions by the manufacturer is essential to ensure that products are safe if consumers follow the proper manufacturer preparation instructions *(46)*. The variability in microwave power levels

and wattage can have a significant effect on the survival of pathogens that may be present on the food.

European Union (EU) regulation now requires 5% of consignments of frozen strawberries imported to the EU from China to be tested for NoV because of the Germany NoV outbreak. The EU also recommends that the catering sector heat-treat berries prior to consumption *(12)*. Though many consumers purchase frozen fruit as a RTE product with the intention of using it in its frozen state, packages do provide microwave instructions. Table 2 shows the variability of microwave package instructions for whole frozen strawberries available in U.S. supermarkets.

Microwave ovens are useful for heating products quickly versus conventional heating *(52)*. Microwaves are defined as wavelengths along the electromagnetic spectrum ranging from 300 MHz (1 m) to 300 GHz (1 mm) *(62, 67)*. The U.S. Federal Communications Commission has designated two microwave frequencies used for food processing and industrial microwave heating. The 915 MHz band is for industrial heating and the 2,450 MHz band is for industrial and domestic microwave ovens *(28)*. The application of microwaves for thawing of frozen foods *(72, 120)*, pasteurization *(69, 115)*, baking foods *(2, 119)*, frying of foods such as oils, bacon and potatoes *(44, 59, 96)* and microwave sterilization of foods *(6)* have all been reviewed.

Size and shape of the food product affect microwave heating; the larger the food mass, the longer time required to heat the product compared to smaller products *(52, 95)*. Foods with unique geometries are a major drawback for microwave heating leading to non-uniform temperature distribution *(130)*. The non-uniformity of microwave heating

results in cold and hot spots in the product, potentially affecting the final quality and safety. Pathogenic microorganisms may survive in cold spots of microwaved foods *(146)*.

## 1.15 Quantitative Microbial Risk Assessment

Quantitative microbial risk assessment (QMRA) is used to better assess and manage the food safety risks that pathogenic microorganisms pose to human health. A risk assessment is the science-based element of risk analysis. Models can be developed to determine the risk posed by specific pathogens for a specific food production chain. QMRA models help to identify data gaps in the literature, estimate consumer exposure to the pathogen in food and evaluate alternate control scenarios. Risk assessments showing the influence of NoV-contaminated water on the spread of the virus on crops consumed raw, such as leafy greens *(11, 82, 87)*, have been conducted. Other risk assessments have also focused on the spread of NoV by ill food handlers, highlighting the importance of hand hygiene *(58, 84, 88, 117, 118)*. A quantitative farm-to-fork exposure model was developed that described the spread of HAV and NoV during harvesting and processing of berries and leafy greens *(15)*. We recently published a QMRA of the NoV outbreak linked to consumption of Chinese-grown strawberries consumed in Germany *(86)*, which is included as a chapter in the dissertation. We also recently published a short review on virus risk in the food supply chain *(85)* which is also included as a chapter in this dissertation.

Table 1.1: Comparison of human norovirus surrogates (Adapted from Hoelzer 2013 and Kniel 2014)

| | **Human norovirus *(411)*** | Feline calicivirus (FCV) | Murine norovirus (MNV-1) | Tulane virus (TV) | Coliphage (MS2) | Poliovirus (PV) |
|---|---|---|---|---|---|---|
| Family | ***Caliciviridae*** | *Caliciviridae* | *Caliciviridae* | *Caliciviridae* | *Leviviridae* | *Picornaviridae* |
| Genus | **Norovirus** | Vesivirus | Norovirus | Recovirus | Levivirus | Enterovirus |
| Envelope | **No** | No | No | No | No | No |
| Virion diameter | **28-35 nm** | 27-35 nm | 28-35 nm | 35-37 nm | 27 nm | 28-30 nm |
| Host receptor, coreceptors | **Histo blood group antigens (HBGA), heparan sulfate** | JAM-1, sialic acid | Sialic acid, glycoproteins | HBGA | F-pilus | Immunoglobulin-like receptor, CD155 |
| Genome composition | **(+) ss RNA** | (+) ss RNA | (+) ss RNA | (+) ss RNA | (+) ss RNA | (+) ss RNA |
| Genome size and organization | **7.5 kb, 3 ORF** | 7.5 kb, 3 ORF | 7.5 kb, 3 ORF | 6.7 kb, 3 ORF | 3.5 kb, 4 ORF | 7.2-7.5 kb, 1 ORF |

Table 1.2: Microwave package instructions for heating whole frozen strawberries

| **Brand** | **Power level** | **Time** | **Serving Size (1 cup = 140g)** | **Heating Instructions** |
|---|---|---|---|---|
| Great Value | 30% power | 1 min | 1 cup | "Thaw desired amount at room temperature for approximately 30 minutes, or thaw in microwave on defrost setting (30% power) for 1 minute. " |
| Nature's Promise | Defrost | 1-2 min | 1 cup | * |
| Cascadian Farm | Defrost | 4 min | 1.25 cup | "Do not microwave in bag. For a 1200 watt microwave, heat quarter bag for 2 minutes on defrost or half bag for 6 minutes on defrost. For a 1000 watt microwave, heat quarter bag for 4 minutes on defrost or half bag for 8 minutes on defrost. For a 700 watt microwave, heat quarter bag for 6 minutes on defrost or half bag for 10 minutes on defrost. Microwave wattages vary; adjust defrost time for the wattage of your microwave. Do not leave microwave unattended." |
| Dole | Defrost | 1 min | 1 cup | "…place in microwave dish and thaw on Defrost setting for 1 minute. Serve slightly frozen. Do not refreeze." |
| Stop & Shop | Defrost | 1-2 min | 1 cup | "Place desired amount of frozen fruit into a 1-1/2 quart microwave-safe dish and cover. Defrost on defrost setting 1 to 2 minutes, or until thawed. Do not thaw completely." |
| Shoprite | Defrost or low setting | 2-3min | 1 cup | * |
| Wegmans | Defrost (30% power) | 2-5min | 1 cup | "Microwave (1100 Watt): Place ½ package or less of fruit in microwave safe dish and cover. Set to defrost (30% power) 2-5 minutes, stirring halfway through or until desired softness." |
| Woodstock | Defrost | 1 min | 1 cup | "…thaw in a microwave on defrost setting for 1 minute. Serve while there are a few ice crystals on the fruit for a firmer texture." |

*no heating instructions available

## 1.16 Bibliography

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# Chapter 2: Literature Review (Mechanisms of Viral Inactivation)

## 2.1 Background Information

There are two major characteristics that differentiate viruses (1) the presence or absence of a viral envelope and (2) the genetic core of the virus (composed of DNA or RNA) *(130)*. All viruses contain a protein coat, or capsid. The capsid is formed by units known as capsomeres and the capsid contains the viral genetic material. The capsid of enveloped viruses is enclosed by a lipid membrane or envelope. Non-enveloped viruses do not have a lipid membrane and are known to be more resistant to environmental stressors such as freezing, drying, heating and acidic conditions *(113, 294)*. Viruses have evolved using different strategies for packaging their DNA or RNA genomes within the viral capsid *(32, 250)*. Understanding the structure and functions of the viral protein capsid is essential in developing effective strategies to inactivate viruses.

The basic steps in viral infection start with the virus injecting its genome into the host cell. This genome is translated into viral proteins, and viral DNA or RNA by the hosts genetic machinery, and these components spontaneously reassemble into new viruses that go on and infect other cells. The virus depends on the host ribosome to make its protein, and ribosomes only read mRNA so any virus genome (DNA or RNA) must first be translated into mRNA so the ribosome can make viral proteins. The genome of RNA viruses can be classified as positive-sense or negative-sense RNA. Positive-sense (5’ to 3’) RNA means that the specific viral RNA sequence can be directly translated into viral proteins. Viral RNA genome functions as mRNA, allowing it to be translated directly by the host cell in positive-sense RNA viruses *(293)*. Negative-sense (3’ to 5’) RNA is not readable for the ribosome and must first be converted into positive-sense

RNA, so all negative-sense RNA viruses must carry an enzyme inside the virion that can transfer negative RNA into positive RNA. This enzyme is RNA-dependent RNA polymerase, also called RNA replicase *(257, 330, 335)*. Most enteric viruses are non-enveloped with a single-stranded, positive-sense RNA genome and have a protein capsid that protects the nucleic acid. Some exceptions include Rotavirus (RV) that are double-stranded RNA viruses, Human parvovirus B19 and Adenovirus (AdV) which have a DNA genome and Coronavirus (CoV) which are enveloped *(85)*.

Our knowledge of the different mechanisms for inactivation of viruses continues to develop. Virus inactivation can be categorized as biological, chemical or physical. Inactivation is considered biological when the attack on the capsid is by enzymes or other microbial-related products. Chemical inactivation involves products such as strong acids, strong oxidizing agents and alcohols that affect the capsid. Physical inactivation includes methods such as heat and drying. Factors such as heat, pH and chemical treatments are known to primarily affect the capsid structure, which then affects the ability for the capsid to bind to viral receptors *(317)*. It is generally accepted that for ozone and UV light processes, genomic damage was the primary cause of inactivation and for heat exposure and chemical treatments such as chlorine and chlorine dioxide, inactivation was associated with damage to the viral capsid *(53)*. Though not all viruses have been researched equally, available data on these inactivation methods and how they affect the viral capsids is discussed below. Characteristics of foodborne viruses are shown in Table 1.

## 2.2 Foodborne Viruses

### Norovirus

NoV's are in the *Caliciviridae* family, belonging to the Norovirus genus. They are non-enveloped, positive-sense single-stranded RNA viruses with icosahedral symmetry. The virus is approximately 28-35 nm in diameter and a genome size of approximately 7.4-7.7 kb *(247)*. There are currently seven genogroups that noroviruses are classified into. Genogroups I, II, and IV are associated with human infection whereas Genogroup III, GV, GVI and GVII have been shown to affect a variety of other animals including cattle and sheep, mice, canines and felines, respectively. Viruses are further classified into lineages within each genogroup, known as genotypes *(159, 334)*. Genogroup II genotype 4 (GII.4) is the most globally prevalent *(221)*.

The NoV genome is covalently linked to a viral protein genome. This consists of 3 open-reading frames (ORFs) that encode for viral proteins. ORF-1 encodes for the nonstructural proteins. The structural components, viral protein 1 (VP1) and viral protein 2 (VP2) are encoded in ORF-2 and ORF-3, respectively. VP1 is the major capsid protein and VP2 is the minor structural protein *(112)*. Virus-like particles (VLP's) are assembled through expression of VP1; VLP's have similar antigenicity and morphology to NoV and have been often used as a NoV surrogate *(88, 112, 127, 247)*.

Hepatitis A virus

Hepatitis A virus (HAV) is in the *Picornaviridae* family and the Hepatovirus genus. It is a non-enveloped, positive-sense, single-stranded RNA virus with icosahedral symmetry. The virus is approximately 27-32 nm in diameter with a genome size of approximately 7.5 kb *(34)*. There is a single species of HAV, categorized into 2 types: human HAV and simian HAV. Human HAV infects all species of primates, whereas simian HAV infects specifically cynomolgus and green monkeys *(128)*. There are 7 HAV

genotypes (genotypes I to VII). Genotypes I, II and III, which are further divided into subtypes A and B, infect humans *(60)*. Characterization of HAV into genotypes is essential in investigating outbreaks to trace foodborne outbreaks to the source *(60, 246, 251)*. HAV is stable outside of its host and can survive on contaminated surfaces, water and food. Transmission most often occurs between people, however food- and waterborne outbreaks have been recorded *(36, 227, 258)*.

Hepatitis E virus

Hepatitis E virus (HEV) is a non-enveloped, positive-sense single-stranded RNA virus with icosahedral symmetry. The virus is approximately 27-34 nm in dimeter and a genome size of approximately 7.2 kb *(136)*. HEV was classified in the *Caliciviridae* family due to its similar organization of the genome and structural morphology. The virus was later reclassified in the *Togaviridae* family because of similarities observed between replicative enzymes of togaviruses and HEV *(6, 276)*. The current classification of HEV is in the *Hepeviridae* family and Orthohepevirus genus with 4 species, A-D, that have varying host ranges *(75)*. Orthohepevirus A makes up the HEV variants that infect humans and eight genotypes have been identified within Orthohepevirus A. HEV1 and HEV2 only infect humans; HEV3 and HEV4 have been shown to infect humans and other domestic and wild animals; HEV5 and HEV6 are found to infect wild boars; and HEV7 and HEV8 have been identified in camels *(75, 99, 113, 283, 331)*.

Rotavirus

Rotaviruses (RV's) are non-enveloped, double-stranded RNA viruses in the *Reoviridae* family. They are often associated with causing gastroenteritis and diarrhea in children and infants *(62, 73, 144, 202)*. RV's have icosahedral symmetry and are

approximately 70 to 80 nm in diameter with a genome size of approximately 18 to 20 kb *(224)*. There are ten RV species that have been identified (A-J). The species most commonly linked to infection in children is RV species A, which is classified into different genotypes based on RNA sequence differences *(62)*. Although RV strains are distinctively different between humans and animals, cross-species infection has been observed due to strain similarity *(202)*. Outbreaks of water- and foodborne RV infections have occurred in several countries *(104, 157, 213)*.

## 2.3 Surrogate Viruses

Surrogates are microorganisms that are used in research instead of pathogens. Ideal surrogates should behave similarly to the target pathogen, non-pathogenic, have inactivation characteristics and kinetics that can be used to predict those of the target pathogen and be easily cultivated in the laboratory *(124, 274)*.

Many NoV surrogates have been explored due to the difficulty of growing NoV in the lab. Most NoV surrogates are closely related structurally or genetically to human norovirus *(65, 121)*. Before the discovery of animal caliciviruses *(12)*, poliovirus (PV), and bacteriophage MS2 (MS2) were used as NoV surrogates for inactivation studies; PV is often used as a surrogate for HAV as well *(14, 244)*. Research on human norovirus surrogates has established that non-enveloped viruses are more stable in the environment and foods than enveloped viruses *(95, 102)*.

### Bacteriophage MS2

Bacteriophage MS2 (MS2) is in the *Leviviridae* family, belonging to the Levivirus genus. It is a non-enveloped, positive-sense single-stranded RNA virus with icosahedral symmetry. MS2 is approximately 27 nm in diameter and has a genome size of

approximately 3.5 kb. The genome of MS2 is one of the smallest known, consisting of 3,569 nucleotides of single-stranded RNA. MS2 is a virus that mainly infects *E. coli*, and other members of Enterobacteriaceae. MS2 binds to the host cell, inject its genome and replicate once the genome is inside the host cell. MS2 has been used often as a surrogate organism for studies virus sensitivity to disinfectants and aerosolization properties *(191, 267, 309)*.

MS2 encodes four proteins: the coat protein (Coat), the maturation protein (A), the lysis protein (L), and the replicase protein (Rep). These proteins are arranged into an icosahedral shell to protect the genomic RNA. The structure of the coat protein is a five-stranded Beta-sheet with 2 alpha-helices and a hairpin *(156)*. When the capsid is assembled, helices and hairpin face the exterior of the particle and Beta-sheet face the interior. The coat protein is the primary structural component of the MS2 protein shell. The maturation protein is associated with attachment to the bacterial pilus, replication, RNA packing and infectivity. The maturation protein-RNA complex is the only viral component to enter host cells during infection. The replicase protein is involved in replication and the lysis protein is involved in the lysis of *E. coli*. The gene encoding the lysis protein overlaps the 3'-end of the upstream gene and the 5' end of the downstream gene, and was one of the first reported examples of overlapping genes *(304)*.

Murine norovirus

Murine norovirus (MNV) is in the *Caliciviridae* family, belonging to the NoV genus. MNV is a non-enveloped virus with a positive-sense single-stranded RNA genome with icosahedral symmetry. It is approximately 27-32 nm in diameter and has a genome size of approximately 7.5 kb *(295)*. It was identified in 2003 and is a species of NoV that

affects mice. MNV is the only NoV that replicates in a small animal and in cell culture *(56, 140, 324)*.

Feline calicivirus

Feline calicivirus (FCV) is in the *Caliciviridae* family, belonging to the Vesivirus genus. FCV is a non-enveloped virus, with a positive-sense single-stranded RNA genome with icosahedral symmetry *(27)*. It is approximately 27-35 nm in diameter and has a genome size of approximately 7.6 kb *(115)*. FCV causes disease in cat and is often used as a NoV surrogate due to its ability to be cultured in vitro and its phylogenetic similarity to NoV *(226, 310)*.

Tulane virus

Tulane virus (TV) is in the *Caliciviridae* family, belonging to the Vesivirus genus. TV is a non-enveloped virus, with a positive-sense single-stranded RNA genome and icosahedral symmetry. It is approximately 35-39 nm in diameter and has a genome of approximately 7.3 to 8.5 kb in size. TV was isolated from rhesus monkeys and can replicate in vitro in rhesus monkey kidney cells. TV is considered a suitable NoV surrogate due to its large genetic variability and its ability to recognize human histo-blood group antigens *(76, 300, 301)*.

Poliovirus

Poliovirus (PV) is in the *Picornaviridae* family, belonging to the Enterovirus genus. PV is a non-enveloped virus with a positive-sense single-stranded RNA genome and icosahedral symmetry. It is approximately 28-30 nm in diameter and has a genome of approximately 7.4 to 7.5 kb in size *(46)*. PV is a well characterized and has become useful for understanding the biology of RNA viruses. There are three serotypes of PV:

PV1, PV2 and PV3, each of which varies in its capsid protein. PV is made up of several capsid proteins, VP0 through VP4, each of which has a different purpose *(4)*. All forms of PV are infectious, though PV1 is most commonly found in nature *(193)*. Humans are the only known natural host of PV, however monkeys are able to be experimentally infected and have been used often to study PV *(123)*.

## 2.4 Emerging foodborne viruses

### Adenovirus

Adenoviruses (AdV) are in the *Adenoviridae* family and are divided into five genera. Human adenoviruses (HAdV) have been often linked to childhood gastroenteritis and belong to the genus Mastadenovirus. There have been seven identified HAdV species, HAdV A-G *(105, 134)*. These are double-stranded DNA viruses that are non-enveloped with icosahedral symmetry. HAdV are approximately 70 to 100 nm in diameter and a genome size of 28 to 45 kb *(105, 141)*. HAdV is most often transmitted via aerosolization or the fecal-oral route. Though infrequent, cases linked to foodborne outbreaks involving HAdV have been reported *(50, 303)*.

### Aichi Virus

Aichi virus (AiV) is in the family *Picornaviridae* in the genus Kobuvirus. AiV is made up of three species, AiV A, B and C, formerly known as Aichivirus, bovine kobuvirus and porcine kobuviruses, respectively. AiV A is comprised of three genetically unique members with varying host species – AiV in humans, known as AiV-1, canine kobuvirus and murine kobuvirus *(3, 139, 225)*. AiV-1 is a single-stranded, non-enveloped, positive-sense RNA virus with icosahedral symmetry. AiV genome is approximately 8.3 kb in length and 30 nm in diameter *(153)*. Though this virus has not

been linked to many outbreaks, it is often identified in sewage and is capable of being foodborne *(167, 179)*.

Astrovirus

Human astroviruses (HAstV) are small, non-enveloped, positive-sense single-stranded RNA viruses with an icosahedral symmetry, approximately 28-30 nm in diameter and a genome size of 6.8-7.0 kb *(35)*. HAstV is grouped within the *Astroviridae* family which is further divided into two genera: Mamastrovirus includes astroviruses that infect mammals and Avastrovirus includes astroviruses that infect avian species. HAstV are currently divided into eight serotypes (HAstV-1 to HAstV-8); however, HAstV-1 is the most prevalent worldwide *(93)*. Multiple large foodborne HAstV outbreaks have been reported *(254)*.

Highly Pathogenic Avian Influenza

Avian influenza is in the family *Orthomyxoviridae* in the genus Influenzavirus. There are 3 genera in the Orthomyxoviridae family; Influenzavirus A-C. These viruses are pleomorphic, negative-sense single-stranded RNA viruses and have a genome approximately 13.5 kb in size *(259)*. Avian influenza viruses are enveloped viruses and approximately 80 to 120 nm in diameter. Influenza A viruses infect humans as well as pigs and birds. Influenza B viruses infect seals as well humans (primarily children) and Influenza C infects humans. Type A influenza strains are classified by serological subtypes based on the primary viral surface proteins and undergo both antigenic drift and shift *(288)*. Highly pathogenic avian influenza (HPAI) is caused by the strain H5N1. Surveillance data has shown that H5N1 HPAI virus was detected on the surface and within eggs, as well as in imported frozen duck meat *(19, 111, 307)*. HPAI has also been

experimentally detected in breast and thigh meat, eggs, and blood and bones of HPAI virus-infected chickens *(290)*. This data suggests that HPAI can be transmitted to humans through consumption of contaminated poultry products *(52, 178)*.

Coronavirus

Coronaviruses (CoV) are large, enveloped, positive-sense single-stranded RNA viruses in the *Coronaviridae* family. These viruses are pleiomorphic with a genome size of 20 to 30 kb and a diameter of 80-220 nm. The most common human coronaviruses linked to infection are 229E (alpha coronavirus), NL63 (alpha coronavirus), HKU1 (beta coronavirus) and OC43 (beta coronavirus). Coronaviruses were initially divided into alpha, beta, gamma and delta based on their serology but are now divided by their phylogenetic clustering *(86)*. Two new coronaviruses have emerged over the last few decades: Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) *(7, 33)*. SARS-CoV may be spread to human from consumption of wild animals or food animals, believed to be derived from a different reservoir such as bats *(173, 325)*.

Nipah virus

Nipah virus (NiV) is an emerging virus found most commonly in Asia. It is in the *Paramyxoviridae* family and the Henipavirus genus *(182)*. It is a pleomorphic, enveloped, negative-sense single-stranded RNA virus. The virion size in the Henipavirus genome ranges from 40 to 600 nm with a genome size of approximately 18 kb *(1, 10)*. NiV is an enveloped virus, which are known to be less stable on food and in the environment *(71, 198)*.

Parvovirus

Parvovirus is in the family *Parvoviridae* and is a single-stranded DNA, non-enveloped virus with a virion size of 20 to 30 nm. Its genome size is approximately 5 to 6 kb and has an icosahedral capsid symmetry. *Parvoviridae* is divided into 2 subfamilies – Parvovirinae and Densovirinae that infect vertebrates and invertebrates, respectively. The first parvoviruses identified as pathogenic to humans include human parvovirus B19 (B19) and human bocavirus 1 (HBoV1) *(234)*.

Sapovirus

Sapovirus (SaV) is in the *Caliciviridae* family and like NoV, is known to be a major cause of viral gastroenteritis in humans and animals *(212, 217)*. It is a non-enveloped, positive-sense single-stranded RNA virus with icosahedral symmetry. The virus is approximately 30 to 38 nm in diameter with a genome size of approximately 7.3 to 7.5 kb *(54)*. SaV's have been classified into five groups, GI-GV, which is further divided into genetic clusters; GI-GIV have been known to infect humans. Outbreaks have been reported through foodborne transmission *(117, 155, 212)*.

## 2.5 Mechanism of Inactivation Treatments

### Ozone

*Introduction*

Ozone has been used as an inactivation treatment as an alternate to chlorine. It is advantageous over other chemical sanitizers since it has little effect on the environment – it rapidly dissociates into oxygen and does not form toxic byproducts *(122)*. In 1982, ozone was classified as general recognized as safe (GRAS) and has since then been used as a decontamination method in the food industry *(58)*. It is a strong oxidant that is capable of inactivating a range of organisms such as bacteria, fungi, protozoa and viruses,

but sensitivity to ozone varies based on type and strain. The ability of ozone to inactivate pathogens is dependent on ozone concentration, product matrix, contact time, temperature, pH and virus type *(49, 120, 146)*. Viral inactivation via ozone treatment appears to target the antigenic sites for host cell receptor attachment, viral capsid proteins and nucleic acids. The interaction of ozone with antigenic sites and viral capsid proteins could affect the adsorption of the virus onto host cells.

*Mechanism of action*

The mechanism of action of ozone on viruses is not fully understood. Most foodborne viruses are non-enveloped which is thought to allow the ozone to access the core nucleic acid and damage to the nucleic acid may prevent replication of virus following treatment *(142, 199, 313)*. Some researchers have hypothesized that ozone affects just the capsid *(222, 233, 333)*, but conflicting studies show that ozone can affect both the capsid and the viral genome. Damage to the genome is thought to occur because ozone can diffuse through the protein coat, damaging viral nucleic acid. Ozone has been shown to destroy the capsid via oxidation at higher concentrations *(143, 252, 267)*. It is believed that the interaction of ozone with antigenic sites and viral capsid proteins can affect the adsorption of the virus into the host cells *(120)*. It has also been suggested that in enveloped viruses the ozone destroys the double bond sites of the viral envelope, then goes on the interact with nucleic acids *(129, 289)*.

Research determined through electron microscopy that MNV was affected by ozone by damage to the outer protein capsid which then allowed the viral RNA to leak out, leaving empty viral particles remaining *(233)*. Several studies have observed the effect of ozone on bacteriophage f2 and MS2 and concluded that ozone affects the protein

capsid first to release RNA and which is subsequently inactivated by ozone. These studies also found that bacteriophage was more sensitive to ozone than enteric viruses *(84, 92, 110, 143, 282)*. They hypothesized that at higher ozone concentrations, degradation of the virus capsids occurs more quickly *(143)*. Reisser et al. (1977) found that when PV was treated with ozone, there was damage to the viral capsid *(242)*. Results from Herbold et al. (1989) indicate that PV showed increased resistance to ozone exposure than HAV, despite having similar structure *(116)*. In another study, it was observed that VP1 and VP2 virus capsid proteins of PV were damaged by the treatment of ozone, however the capsid protein most important for viral attachment to cells, VP4, was not affected. Results from this study suggest that the main mode of inactivation by ozone is nucleic acid damage as opposed to damage to the viral capsid *(252)*. Hudson et al. (2007) looked at the ability of ozone gas to inactivate NoV and FCV on surfaces and found similar decreases in viral RNA after ozone exposure, though no mechanism of action was suggested *(125)*.

Gamma Irradiation

*Introduction*

The use of gamma (ionizing) irradiation and electron beam generators on food products has been used in the food industry as a decontamination treatment. The product is exposed to gamma rays, which consists of high energy electromagnetic waves. The gamma rays are emitted from radioactive forms of cobalt 60 or cesium 137 *(98)*. Cobalt-60 is most often used for food irradiation because it is water soluble and presents little risk to the environment. This form of radiation has also been used on hygienic equipment used in the medical and dental industry. Gamma radiation doses of 2 to 4 kilogray (kGy)

have often been used in food products to inhibit the growth of bacteria and parasites, however research on inactivation of foodborne viruses continues to be of interest to researchers *(15)*. Gamma radiation is considered an ideal candidate vs. heat and chlorine for decontaminating products like fresh produce *(29)*, since it maintains the quality of the initial product and penetrates deeply, while delivering a uniform dose *(69)*. Electron beam generators create high energy electrons in an accelerator that is able to generate electrons to 99% of the speed of light *(87)*. These have also been used on food products, but are unable to penetrate food products as deeply as gamma radiation.

*Mechanism of action*

The inactivation properties of gamma rays have been explored for both enveloped and non-enveloped viruses and treatments are typically categorized as direct or indirect. Several studies have hypothesized that the effect of gamma irradiation on viruses is due to the alteration of nucleic acids, destroying the infectivity of the virus *(194, 312)*. However, researchers have also noted that the protein content of the viruses may have a stabilizing effect on radiation inactivation *(69)*. Direct inactivation involves damage to the nucleic acid and protein, as well as crosslinking of the genetic material or radiolytic cleavage *(88)*. Indirect inactivation of gamma ray treatment involves the action of free oxygen radicals, most often hydrogen peroxide and hydroxyl (OH) radicals *(205, 277, 279)*. Gamma radiation has been shown to be less effective when solutes, known as scavengers (e.g. proteins), are present since they are able to react with the OH radicals before they react with the virus *(279)*. De Roda Husman et al. (2004) found that when MS2 was dispersed in a high-protein versus a low-protein stock, the virus was inactivated

more when there was less protein. These results confirmed the role of OH free radicals in virus inactivation *(175)*.

Ultraviolet Light

*Introduction*

Ultraviolet light (UV) is a form of non-ionizing, electromagnetic radiation that has shorter wavelengths than visible light. This combination allows for damage in a variety of organisms such as bacteria, viruses and fungi *(158)*. UV light is divided into different groups by its wavelength and range, including UVA, UVB and UVC. UVA has a range of 400 to 315 nm, UVB has a range of 315 to 280 nm, and UVC ranges from 280 to 100 nm *(83, 240)*. This technology is appealing to the food industry due to its ease of use, low cost and lack of toxic byproducts. UV is often considered as an alternative to thermal pasteurization of beverages such as milk and juices *(55)*. UV has also been suggested as a surface disinfectant on fresh produce and ready-to-eat (RTE) meals *(30)*. Factors that have been shown to affect the success of UV light as a surface disinfectant for viruses include the virus strain, viral protein differences, viral nucleic acid type and the ability of the virus to form aggregates *(20, 94)*.

*Mechanism of action*

Many studies have been published on the effect of UV light on inactivating DNA and RNA viruses and varying UV effects. The generally accepted mechanism of inactivation via UV is through damage to the genome of viruses through breaking bonds and the formation of photodimeric lesions in DNA, RNA and nucleic acids *(21, 26)*. These lesions lead to inactivation through the prevention of transcription and replication of the virus *(94, 160, 207, 320)*. Most foodborne viruses contain genomic RNA and have

been observed to undergo morphological changes in the viral capsid after UV light exposure *(70)*.

A study by Wang et al. (2014) looked at the effect of UV on TV, a NoV surrogate, and determined that although UV treatment affected the capsid protein, inactivation occurred primarily through genomic damage, leading to the inability of the virus to replicate *(317)*. Bacteriophages are also considered to be more resistant to UV than other viruses and were found to be primarily inactivated by UV through damage to the RNA genome *(270, 305)*. Researchers observed how UV affected the single-stranded RNA virus PV, and found that it primarily attacks the viral nucleic acid *(20, 208, 209)*. UVs ability to inactivate viruses seems to be dose dependent. At doses >1000 $mWs/cm^2$, UV has been shown to affect the capsid protein and create RNA-protein linkages, leading to degradation *(70, 275, 321)*. UV light is thought to be capable of causing significant damage at the genomic level of RNA viruses *(70, 218, 275)*.

Researchers who studied the inactivation of several viruses by UV concluded that double-stranded DNA adenovirus (AdV) was more resistant than RNA enteric viruses, including PV and MNV *(103)*. Researchers also studied the effect of UV on the inactivation of AdV and found that the mechanism of inactivation involved both the capsid and the genome; noting that AdV has been known to continue to infect and replicate within a host even when there is damage to the DNA, as long as the structural proteins remain intact *(38)*. Double-stranded DNA viruses are also known to be capable of using host cell enzymes to repair the damage to DNA that is caused by UV light *(273)*. Another study observed that double-stranded RNA rotavirus (RV), was also more resistant than HAV and PV *(18)*. Several researchers have suggested that the improved

resistance of double-stranded viruses to UV treatment is linked to having an additional strand that remains undamaged and can act as a template for the host cell enzymatic repair *(103, 135, 154, 189, 299)*. Generally, double-stranded viruses have been found to be at least 10 times more resistant to UV than single stranded viruses for this reason *(119, 135, 241)*.

Fino et al. (2008) hypothesized that a large factor in UV ability to inactivate virus is the surface topography of the product. These authors studied 3 single-stranded RNA viruses, HAV, AiV and FCV, and found the larger differences in reduction between food products than between viruses. Lettuce had the greatest reduction vs. strawberries and green onions, attributed to product shadowing that disrupts the UV light path *(94)*. Kaoud et al. (2016) studied the inactivation of highly pathogenic Avian influenza (HPAI) by UV light in fecal matter and found no reduction after 48 hours, attributed to poor UV penetration *(138)*. This limitation of UV light is generally well known and restrict its adoption in the food industry *(5, 177)*.

## High Pressure Processing

### *Introduction*

High-pressure processing (HPP) has been increasingly used in the food industry and is considered a strong alternative to thermal processing *(97)*. Research has shown HPP can inactivate pathogenic microorganisms, maintain organoleptic and nutritional properties and extend the shelf-life of food products. HPP applies pressure uniformly, making it an effective method to inactivate pathogens on the surface or within a product *(239)*. Though most studies have looked at the ability for HPP to inactivate pathogenic and spoilage bacteria, some studies have investigated foodborne viruses. The mechanism

of action is believed to involve the alteration of the virus capsid that surrounds the RNA genome. A damaged viral capsid means that the virus is unable to attach to the host cell, and therefore is never able to release its nucleic acid into the cell and cause infection.

Viruses studied thus far have had varying resistances and sensitivities to HPP treatment, suggesting that a mechanism for inactivation via HPP may differ across viruses *(237)*. Kingsley et al. (2002) found that a 7-log reduction required an HPP treatment of 450 MPa for 5 min for HAV, whereas FCV treated at 275 MPa for 5 min at 22°C saw the same reduction in viral titer *(152)*.

*Mechanism of action*

As noted above, HPP is thought to alter capsid protein conformation and prevent virus attachment, host cell penetration or uncoating once the virus enters the cell *(109, 152, 181, 231, 237, 245)*. Studies have suggested that HPP may denature proteins of the viral coat of non-enveloped viruses and cause damage to the viral envelope of enveloped viruses *(119, 148, 152, 200, 269)*. Researchers found the capsid of HAV remained intact after 500 MPa HPP treatment and there was no release of RNA genome *(152)*. Other authors found damage to the attachment protein which can lead to a loss of ability to cause infection *(231)*.

Lou et al. (2011) also observed that HPP disrupted but did not degrade the viral capsid protein of MNV, and that the capsid protein remained antigenic. These authors also reported that primary and secondary structures of the major capsid structural protein VP1 was not disrupted *(181)*. In another study from the same lab further confirmed that HPP could disrupt the capsids from MNV and FCV, while virus-like particle (VLP) capsids of NoV were found the be highly resistant to HPP compared to its surrogates

*(180)*. Tang et al. (2010) found that HPP treated MNV was unable to bind to its target receptor and could not initiate infection in mouse RAW cells, and MNV capsid integrity was not affected by HPP treatment *(292)*. Other research has linked the inactivation of RV to the viral spike protein being altered when exposed to high pressure *(231)*.

Factors that influence HPP's effect on eliminating microorganisms include the product matrix, temperature, pressure applied, treatment length and morphology and size of the virus *(59, 97, 197)*. An increase in inactivation of NoV, TV and MNV was observed when HPP was applied at a lower temperature *(74, 150, 268)*. This effect of temperature and pressure when used together has been associated with maximizing protein denaturation from an increased water molecule density in the protein matrix *(17, 151, 161)*. The matrix of the suspension or food product has also been shown to influence the effectiveness of HPP.

Several studies have shown that properties such as fats, salt, protein and carbohydrates decrease the impact of HPP on viruses *(15, 17, 108, 147)*. It has been hypothesized that HPP leads to free radical formation, so antioxidants, like fruit polyphenols can decrease damage to the capsid that is caused by free radicals which can promote virus resistance to HPP *(24, 185)*. A study by Wilkinson et al. (2001) looked at the ability of HPP to inactivate PV and AdV and found that at 400 MPa, AdV was inactivated, however, at 600 MPa, PV saw no significant inactivation. The authors suggested that since PV is a small, spherical virus it is resistant when uniform pressure is applied while AdV is larger and icosahedral shapes so that the flat planes of this viral particle are thought to collapse or become damaged when a certain pressure is applied *(148, 323)*.

pH

*Introduction*

While acidification has often been used as a preservation technique in food microbiology *(15)*, viruses are generally stable at low pH, allowing them to tolerate the acidity of products and environments, such as berries, and the intestinal tract in humans *(174)*. Enveloped viruses are less resistant to low pH and experience denaturation more quickly than non-enveloped viruses *(95)*. Non-enveloped NoV can for example, survive acidic stomach conditions to be able to reach the small intestine, where the virus replicates within epithelial cells *(66)*.

*Mechanism of action*

Different viral capsids have shown different sensitivities to different pH environments. Studies have found that NoV GI.I and NoV GII.4 capsids were stable at acidic and neutral pH, while disassembly of the capsid occurred at pH greater than 8 *(13, 67, 229)*. NoV VLPs have been used as a NoV surrogate to understand the effect of pH on capsid stability. VLPs are composed of one or more capsid proteins but lack viral nucleic acids. Cuellar et al. (2010) found that the mechanical stability of NoV capsids were pH-dependent. The size of the NoV VLP and overall diameter increased when exposed to pH 10, eventually losing structural stability and disassembling *(66)*. Conversely NoV VLPs are stable at low pH *(13, 66, 126, 132, 256)*. It has been hypothesized that high pH changes to the capsid shell are linked to weakening of the capsomer-capsomer interaction. Jiang et al. (1992) found NoV VLPs dissociate under an alkaline pH into soluble capsid proteins and reassemble when returned to a neutral pH *(132)*. Ausar et al. (2006) found VLPs were stable from pH 3 to 7, but significant capsid

disruption at pH 8 was observed *(13)*. Lin et al. (2014) studied VLPs made with different structural proteins and found that VPLs made with VP1 and VP2 structural proteins were more stable at pH of 8 than those made with just VP1 *(176)*.

Researchers have also looked at other single-stranded RNA viruses similar to NoV. When HEV was studied in a low pH solution, it was found that the major capsid protein undergoes conformational changes which is thought to increase stability and allow the virus to retain infectivity *(332)*. Costafreda et al. (2014) concluded that the physical stability of HAV in the environment is related to capsid folding. HAV strains whose capsids vary in folding showed significant differences in its resistance to an acidic pH; where capsid folding is linked to mutations in the capsid coding region of viral nucleic acid *(61)*.

Changes in pH are also known to cause viral aggregation (multiple virus particles coming together) thought to occur because of viral capsid conformational changes. These conformation changes also effect viral entry into the host cell and release of RNA, affecting overall infectivity *(66, 164)*. Langlet et al. (2007) studied the effect of pH on the aggregation of MS2. MS2 phages showed significant aggregation when the pH was less than or equal to the phage isoelectric point (pI = 3.9); at a pH of 2.5, there was approximately a 2-log reduction measured of viral PFU *(164)*. Another study also observed the effect of pH on MS2 and showed that inactivation rates were lowest at a pH range of 6-8 *(89)*. Tian et al. (2013) observed the stability of TV when exposed to a range of pH and found that the virus remained stable from pH 3-8, however there was a clear reduction at pH 2.5 and 9, and no detected viruses at pH 10 *(300)*. The NoV surrogate MNV was found to be resistant at high and low pH; with a minimal reduction in

infectivity at a pH of 2. Compared to other enteric viruses, FCV has been found to be less stable at low pH *(48, 77)*.

Though studies have not been frequently conducted on NiV, one study looked at the effect of pH on virus survival in phosphate buffered saline (PBS) and showed that NiV was tolerant to a pH range from 3 to 11 *(96)*. It has been observed in AstV that a drop in pH leads to viral uncoating and studies on porcine astrovirus (PAstV) have showed that the virus retained its infectivity after 3 hours at pH 4, resulting in a 2-log reduction *(266, 318)*. Several studies have observed the stability of CoVs across a range of pH and concluded that CoVs are more stable at slightly acidic pH of 6-5.6 compared to an alkaline pH *(100, 102, 162, 228, 232, 286)*.

Pesavento et al. (2005) evaluated the effect of pH on conformational changes in rotavirus (RV) spike protein, VP4. This study observed that at increased pH, the VP4 protein has an irreversibly conformational change, affecting its cell binding ability *(223)*. An earlier study found that RV remained stable at a pH range of 3 to 10 but at a pH less than 3, the outer layer of the capsid collapsed *(190, 216)*. When exposed to a range of pH values the double-stranded DNA virus AdV has been shown to have damaged capsid proteins but not damage to its viral DNA *(214, 215, 243)*. Another DNA virus, Parvovirus B19 (B19), has been found to have an altered capsid at pH of 5 or lower, however the changes appear reversible and exposure to low pH is not associated with capsid degradation *(47, 184, 220, 272)*.

## Disinfectants

### *Introduction*

Sanitizers and disinfectants approved by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) are not always effective at inactivating or removing foodborne viruses. Viral inactivation by disinfectants depends on exposure time, disinfectant concentration, exposure temperature, food contact surface characteristics and/or surrounding food matrix. Capsid proteins, presence of a lipid envelope and the nucleic acid (DNA or RNA) are all susceptible to attack by chemical disinfectants *(263)*. Alcohols, chlorine, quaternary ammonium compounds, sodium hypochlorite, hydrogen peroxide and peroxyacetic acid are all relatively ineffective against many foodborne viruses; depending in large measure on whether a virus is enveloped or not *(68, 119, 195, 306)*. The EPA allows virucidal claims against NoV to be made from data that show effectiveness against FCV. There are also EPA-registered products that mention the effectiveness against MNV as the basis for anti-noroviral claims *(57, 80)*.

*Mechanism of action*

Alcohols

Alcohols (primarily methanol and ethanol) are often used disinfectants to reduce microbial load on a surface. Enveloped viruses have been found to be readily inactivated at 70% ethanol concentration, however, non-enveloped viruses are significantly more resistant *(137)*. The general mechanism of inactivation by alcohol is through the denaturation of the protein structure, leading to changes in virus conformation, and these inactivation mechanisms are largely based on the concentration of ethanol *(163)*. NoV surrogates, such as MNV, FCV, MS2 and TV have all been studied to see the extent of

inactivation using an alcohol-based product. Several studies have found MNV to be more susceptible to alcohols than other non-enveloped viruses *(22, 219, 262, 308)*.

NoV VLPs have also been used as a NoV surrogate to study the effects of alcohol. Sato et al. (2016) found that when NoV VLPs were treated with ethanol, the major capsid protein VP1, remained intact *(261)*. SaV, in the *Caliciviridae* family has been shown to be inactivated by ethanol through degradation of the viral capsid *(212, 319)*. Other studies have concluded that when alcohol-based disinfectants are used against enveloped viruses, they cause damage to the lipid envelope and rapid denaturation of the proteins, affecting the infectivity of the virus to the host cell *(82, 131, 145, 311)*.

Oxidizing agents

Oxidizing agents such as chlorine and chlorine dioxide have often been used as a disinfectant for their low cost, low toxicity to humans and wide range of application. The general mechanism of action of chlorine compounds against microbes is thought to involve the denaturation of proteins, as well as the ability to attack nucleic acids *(31, 163, 236)*. $ClO_2$ reacts with amino acids such as histidine, tyrosine and tryptophan, leading to the disruption of primary and secondary structures, and disruption of these structures results in degradation of the viral capsid protein *(201, 211, 285)*.

Several studies have looked at the effect of oxidizing agents on virus inactivation in suspension and on foods, however the mechanism of action is not entirely understood *(12, 196, 298, 300)*. MNV has been used often as a NoV surrogate to better understand the effect of $ClO_2$ on viruses and results showed that $CLO_2$ damaged the viral capsid structure which leads to the leaking and degradation of viral RNA *(114, 329)*. The inactivation mechanism of disinfectants on HAV is likewise not well understood but has

been suggested that inactivation occurs through capsid protein fragmentation and then damage to the nucleic acid *(210)*. In contrast, Li et al. (2002) suggested the initial target of chlorine inactivation was likely the HAV nucleic acid and not the capsid proteins, and that the 5' and 3' non-translating regions of HAV RNA are more sensitive to chlorine and the coding areas more resistant *(171, 172)*. Other studies have found that when viruses are aggregated, they exhibit a protective effect against inactivation when exposed to chlorine *(278, 296, 297)*.

Kingsley et al. (2017) studied the effect of chlorine treatment levels on the inactivation on NoV and MS2. The authors concluded a loss of binding to host cells was apparent and this might suggest a mechanism related to capsid binding *(149)*. Other research noted that binding of NoV viral capsid to histo-blood group antigen (HBGA) was significantly reduced by chlorine and suggests that the mechanism of inactivation was due to denaturation of the capsid proteins *(316)*. Wigginton et al. (2012) looked at the effect of disinfectants on function and structural integrity of MS2 and found that chlorine treatment affected the capsid protein, inhibiting the viral genome from being injected into the host cell but not in a way related to binding to host cells *(322)*. Inactivation of PV from exposure to sodium hypochlorite determined that there was no conformational change in the capsid, but that RNA was degraded *(9, 210)*. Additional studies have found that $ClO_2$ inactivated PV by disrupting the PV genome *(133, 271)*. It has been demonstrated that loss of AdV infectivity after chemical treatment was due to DNA degradation rather than to capsid denaturation *(264)*. AiV has been shown to be affected by sodium hypochlorite through disruption and damage to both the capsid and genomic RNA *(187)*. Several studies have looked at the effect of sodium hypochlorite on

the enveloped avian influenza virus and the mode of action seems to be damage to the genomic RNA *(287, 338)*.

Heat

*Introduction*

Heat is often used in the food industry as a food processing method to reduce pathogenic bacteria, spoilage microorganisms and viruses in food products *(166)*. D- and z-values are used to characterize the heat inactivation kinetics of microbes during thermal processing treatments *(43)*. Factors that have been seen to contribute the most to viral inactivation when exposed to heat include the temperature and the matrix *(25)*. In a study looking at heat inactivation in milk, the fats and proteins caused increased heat stability of HAV *(28)*.

*Mechanism of action*

As noted above, thermal inactivation of viruses is thought to involve conformational changes in the capsid structure *(119)*. Heat may induce structural changes to the capsid proteins which may lead to inactivation through changing the binding capacity to host cells *(25, 206, 235)*. Generally, heat disrupts the hydrogen bonds, destroying the structural integrity of the viral proteins *(41, 284, 315)*. Denaturation of the capsid proteins then leads to the release or denaturation of nucleic acids *(280)*. Studies on the mechanism of inactivation of AiV involved the degradation of the capsid and release of genetic material *(65, 255)*.

Pollard (1960) hypothesized that structural changes in the viral proteins occur as parts of the virus expand when heat is applied and that the enthalpy and entropy of the virus components (e.g. capsid and nucleic acid) differ, such that degradation rates can

vary over a wide temperature range *(230)*. Croci et al. (2012) found that exposure to 50°C led to damage of the FCV receptor binding site, and at increased temperatures, higher inactivation rates were associated with denaturation of capsid proteins, leading to the release and degradation of nucleic acids *(64)*.

Changes in the secondary, tertiary and quaternary structure has also been observed at increased temperatures *(13, 42, 63, 64, 281, 314, 322)*. Another study found that TV RNA levels were not significantly decreased when subjected to high heat (80°C) for 10 seconds. The authors concluded that these results show heat treatment did not physically degrade the genomic RNA, though a loss of capsid integrity and change in the virion symmetry was seen *(11)*. Studies on RNA viruses including FCV, MNV, HAV and PV concluded that heat changed the conformation of the capsid, leading to loss of infectivity, without a loss in RNA infectivity *(23, 44, 63, 118, 208, 209, 248)*.

A study by Brie et al. (2016) found that when MS2 was exposed to heat (72°C), particles were disrupted and the genome was susceptible to degradation. Another study observed that the capsid of MS2 was unable to recognize its receptor following heat treatment *(322)*. When RV was exposed to heat, the mechanism responsible for inactivation was associated with a disruption of viral RNA synthesis *(81, 249)*.

Enveloped viruses have been associated with increased susceptibility to harsh treatments. CoVs which are enveloped, single-stranded RNA viruses, have been studied with respect to heat treatment. Studies investigating both severe acute respiratory syndrome and Middle East respiratory syndrome (both caused by CoVs), have determined that thermal inactivation occurs through destabilization of the viral envelope,

affecting the viral capsid structural integrity and denaturation of the nucleic acid *(37, 51, 107, 165, 168, 170, 186)*.

Researchers looked at the effect of thermal resistance on viruses on the single-stranded DNA virus, PaV. Studies have shown that the mechanism of inactivation involves DNA release from an intact capsid *(184, 188, 265)*. When a double-stranded DNA virus (AdV) was heated, its capsid was degraded but with no observed effect on the infectivity of the DNA *(253)*.

Cold Plasma

*Introduction*

Plasma (the fourth state of matter) has become a promising food industry non-thermal technology *(39, 91, 169)*. It has been shown to be effective against a variety of yeasts, spores, viruses and fungi *(16, 45, 72, 79, 90, 183, 192, 238, 302, 337)*. Plasma is made up of photons, electrons, positive and negative ions, and reactive species such as radicals, atoms and molecules. The reactive species often include reactive oxygen species (ROS) and reactive nitrogen species (RNS) *(106)*. Factors that greatly influence the efficacy of plasma as an inactivation method against viruses include the gas composition, pH of the solution and treatment time, virus suspension medium and virus type *(2, 78)*.

*Mechanism of action*

The general mechanism of microbial inactivation via cold plasma is a synergistic effect of ROS and the chemistry of the air produced by cold plasma, leading to oxidation of cellular components. It can be hypothesized that following oxidation, the capsid protein (for non-enveloped viruses) or lipid membrane (for enveloped viruses) are attacked, leading to subsequent nucleic acid degradation and virus inactivation *(40, 260)*.

The inactivation of viruses by plasma on has not been studied as much as other microbes *(72, 90, 101, 336)*. Aboubakr et al. (2015) hypothesized that plasmas mode of action against FCV in suspension was that the ROS and RNS react with the capsid protein leading to peroxidation and capsid destruction. Researchers have found that virucidal activity of plasma against NoV surrogates were significantly enhanced by adding oxygen such that the inactivation rate of cold plasma is primarily a function of the oxygen concentration in the mixture of carrier gas *(2, 8, 328, 336)*. Other studies looking at AdV and several bacteriophages have also shown that plasma affects the capsid protein of a virus, leading to subsequent damage to the nucleic acids *(291, 326, 327)*. Niemira (2012a, 2012b) suggested that components of plasma like ROS, photo-inactivating light and gaseous antimicrobials may act on both the viral capsid and genome, depending on the structural integrity of the virus *(203, 204)*. An earlier mechanism proposed by Roy et al. (1981) and Kim et al. (1980) suggested that ROS may inactivate viruses through specific damage to the capsid polypeptide chains after which the ROS diffuse through the damaged capsid and react with the virus nucleic acid *(143, 252)*.

Table 2.1: Characteristics of foodborne viruses

| Virus Genus or Species | Family | Nucleic acid type | Envelope | Morphology Symmetry | Size of virion (nm) | Culturable? | Genome Size (kb) | Disease caused | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Influenzavirus | Orthomyxoviridae | (-) ssRNA | Y | Helical | 80-120 | Y | 13.5 | Respiratory, eye, and gastroenteritis infection | Sangsiriwut 2018 |
| Henipavirus | Paramyxoviridae | (-) ssRNA | Y | Helical | 40-600 | Y | 18 | Respiratory infection, encephalitis | Ang 2018 |
| Astrovirus | Astroviridae | (+) ssRNA | N | Icosahedral | 28-39 | Y | 6.8-7 | Gastroenteritis | Bosch 2014 |
| Norovirus | Caliciviridae | (+) ssRNA | N | Icosahedral | 28-35 | N | 7.4-7.7 | Gastroenteritis | Robilotti 2015 |
| Vesivirus | Caliciviridae | (+) ssRNA | N | Icosahedral | 27-35 | Y | 7.6 | Gastroenteritis | He 2016 |
| Sapovirus | Caliciviridae | (+) ssRNA | N | Icosahedral | 30-38 | N | 7.3-7.5 | Gastroenteritis | Choi 2015 |
| Hepatovirus | Picornaviridae | (+) ssRNA | N | Icosahedral | 27-32 | Y | 7.5 | hepatitis, inflammation of liver | Bondarenko 2013 |
| Orthohepevirus | Hepeviridae | (+) ssRNA | N | Icosahedral | 27-34 | N | 7.2 | hepatitis, inflammation of liver | Kamar 2014 |
| Enterovirus | Picornaviridae | (+) ssRNA | N | Icosahedral | 28-30 | Y | 7.2-7.5 | Poliomyelitis, meningitis, encephalitis | Brown 2003 |
| Levivirus | Leviviridae | (+) ssRNA | N | Icosahedral | 27-28 | Y | 3.5 | | Bollback 2001 |
| Kobuvirus | Picornaviridae | (+) ssRNA | N | Icosahedral | 30 | | 8.3 | Gastroenteritis | Kitajima 2015 |
| Recovirus | Caliciviridae | (+) ssRNA | N | Icosahedral | 35-37 | Y | 6.7 | | Yu 2013 |
| Coronavirus | Coronaviridae | (+) ssRNA | Y | Helical | 80-220 | Y | 20-30 | Gastroenteritis, resipiratory infections | Fehr 2015 |
| Adenovirus | Adenoviridae | dsDNA | N | Icosahedral | 70-100 | Y | 28-45 | Respiratory, eye, and gastroenteritis infection | Ghebremedhin 2014 |
| Rotavirus | Reoviridae | dsRNA | N | Icosahedral | 70-80 | Y | 18-20 | Gastroenteritis | Phan 2016 |
| Parvovirus | Parvoviridae | ssDNA | N | Icosahedral | 20-30 | N | 5-6 | Gastroenteritis | Qiu 2017 |

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# Chapter 3: Virus risk in the food supply chain

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## 3.1 Abstract

Enteric viruses are an important food safety concern and have been associated with many foodborne disease outbreaks. Norovirus and Hepatitis A virus have been implicated in majority of outbreaks; however, other foodborne viruses such as Hepatitis E virus, Sapovirus and Rotavirus can also present a risk to humans. Viral foodborne disease outbreaks have typically been associated with foods served raw including shellfish, fruits and vegetables. The contamination of food by viruses can occur anywhere in the supply chain. Unlike bacteria, viruses cannot replicate in food, so the ability of a contaminated food to serve as a vehicle for infection depends on virus stability and host susceptibility. The burden of foodborne enteric viral disease is often difficult to estimate as many illnesses are mild and go unreported. Molecular assays have been developed for foodborne viruses, and the sensitivity of theses assays has significantly improved throughout the last decade. Surrogate viruses are often used in laboratory research to further understand virus behavior as many foodborne viruses are difficult or impossible to culture outside a human host. This review provides an overview of the epidemiology and detection of foodborne viruses, and most summarizes the state of the science in quantitative microbial risk assessment as applied to foodborne viruses, including the use of viral surrogates.

## 3.2 Introduction

Viruses are increasingly recognized as important causes of foodborne disease worldwide in recent years. Viruses have properties that make them quite different than

more commonly studied foodborne bacterial pathogens. Viruses consist of nucleic acids, enclosed within a protein coat called a capsid. Viruses are not free-living and are only capable of replicating within the living cells of humans, other animals, plants or bacteria. As such, foodborne viruses (unlike foodborne bacteria) are unable to replicate in food. Most foodborne viruses lack a viral envelope, and thus are extremely stable in the environment.

Some foodborne viruses are able to withstand some food processing techniques which control bacterial pathogens in foods. Foodborne viruses may able to survive in foods, on hands, in feces, and on food contact surfaces and floors for long periods of time. Some foodborne viruses, such as norovirus (NoV) may be shed in very high numbers ($10^5$–$10^{11}$ virus particles/g of stool or vomitus) from infected individuals, and have a relatively low median infective dose, which can lead to large outbreaks in short periods of time *(54)*. The burden of illness from foodborne viruses is highest in young children, the elderly and immunocompromised. Viruses cause a wide range of diseases in plants, animals and humans, and each group of viruses has its own typical host range and cell preference *(44)*. Since viruses are increasingly recognized as a common cause of foodborne illness, significant progress has been made in recent years regarding the methodology available for detection and identification of viruses in food and clinical samples *(23)*.

## 3.3 Foodborne viruses involved

There has been an increase in data published on the epidemiology of viruses in the last decade *(41)*. Foodborne viral infections are typically caused by enteric viruses, and these infections are spread via virus particles shed in the stool or vomit of infected

individuals. Norovirus (NoV) and hepatitis A virus (HAV) are the two most common causes of viral foodborne illness in the United States *(16)*. Hepatitis E virus (HEV), sapoviruses, rotaviruses, astroviruses, and Aichi viruses are less commonly associated with outbreaks of foodborne gastroenteritis *(17, 37, 42, 50, 56)*. NoV is generally considered to be the most prominent cause of viral-associated foodborne illness worldwide. NoV is linked to an estimated 19–21 million cases of acute gastroenteritis annually in the United States *(56)*. This review is focused on the viruses causing most foodborne viral disease, NoV and HAV, as well the emerging foodborne viral hazard, HEV (Table 1).

## 3.4 Common foods and notable outbreaks

Foods served raw or lightly cooked are considered at greatest risk of causing enteric viral illnesses. Shellfish, fresh fruits and fresh vegetables are among the foods most commonly linked to foodborne virus outbreaks *(3, 15, 32)*. Shellfish, fruits and vegetables are at increased risk of transmitting enteric viruses since they can undergo extensive human handling, may not undergo further processing and are subject to environmental contamination pre-harvest and post-harvest. Fresh fruits and vegetables are at a high risk for contamination by foodborne viruses because these commodities normally undergo a little or no processing and can be contaminated at any step from pre-harvest to post-harvest. Major routes of contamination include contaminated water for irrigation or washing, or by infected workers handling the food during harvesting, processing or distribution *(14)*. Fresh produce usually undergoes a brief sanitization step after harvest from the field, though commonly used sanitizers are ineffective in removing viral contaminants from these foods *(4, 24, 30)*. Shellfish may be harvested from waters

subject to human fecal contamination or sewage discharge and may be handled by infected shellfish harvesters. Shellfish are usually eaten with their digestive tracts in place and are often eaten raw, and both practices increase risk *(12, 53)*. Contamination of foods associated with food and waterborne viruses can occur at any point in cultivation, harvesting, processing, distribution, or preparation.

The proportion of foodborne disease caused by viruses has historically been difficult to estimate due to difficulties in culturing or detecting viruses (versus bacteria). Under reporting due to the mild and self-limiting nature of some viral infections (NoV), or lack of an identified vehicle due to long onset times and the delayed appearance of symptoms for other infections (HAV) can also occur. Factors contributing to the increase and spread of viral foodborne disease include lack of clean water, globalization of the supply chain and changes in eating habits, including increased consumption of food commonly eaten raw. The 2018 Winter Olympics in PyeongChang, South Korea were struck by a NoV outbreak with 194 confirmed cases. The likely source of the outbreak was contaminated cooking water *(29)*. Frozen raspberries imported from China were the cause of more than 700 confirmed cases of NoV in Quebec between March and July in 2017 *(22)*. Royal Caribbean cruise line made headlines in December 2017 after more than 500 people fell ill with NoV on two of the company's cruise ships *(49)*. A multistate outbreak of HAV linked to frozen strawberries imported from Egypt resulted in 143 cases in 9 states in 2016 *(18)*.

## 3.5 Means of disease spread

Viruses can be transmitted directly and indirectly and may contaminate a wide variety of foods pre-harvest or postharvest. The most common route for transmission of

foodborne viruses is termed fecal-oral, where viruses in microscopic particles of feces are orally ingested by another person. Some viruses can also be transmitted by aerosolization of vomitus particles from an infected person and subsequently ingested by the next victim. Patients with viral gastroenteritis are capable of shedding high numbers of viruses in their feces, sometimes reaching over $10^{10}$ NoV genome copies/g (gc/g) feces, and it is estimated that as many as 3 x $10^7$ virus particles are released in a single vomiting episode *(2, 34, 54, 64)*. Feces from those shedding of HAV may contain >$10^6$ infectious virus particles per gram and virus excretion in feces can occur 2-3 weeks before symptoms appear and last for at least 6 weeks after onset *(48, 58, 62)*. Peak shedding of HEV (about $10^8$ gc/g) occurs during the incubation period and early, acute phase of the disease *(61)*. Indirect transmission of these viruses can occur via water and food or from contact with contaminated fomites including serving utensils, plates, tables, and so on.

## 3.6 Challenges of detecting foodborne viruses in foods

Significant efforts have been made to develop tools for virus detection in foods in the last decade. Detection of viruses in foods can be a greater challenge than culturable bacteria because viruses do not replicate outside the host and, therefore, cannot be enriched for or otherwise cultured *(19)*. Viral loads present in food samples are typically much lower than those found in clinical samples and thus detection methods must be quite sensitive. The original methods for detection of human enteric viruses in food are based on mammalian cell culture assays. This method is expensive, time-consuming and some enteric viruses grow poorly or not at all in cultured cells *(55)*. Current virus detection methods for foods are based on molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) or next generation sequencing (NGS). Other

laboratory assays for the detection of viruses include electron microscopy (EM) and immunological enzyme immunoassay. Electron microscopy has the ability to detect multiple viral pathogens, but it is insensitive, requires expensive equipment and involves training. Immunological enzyme immunoassay results in high specificity, high throughput but has low sensitivity *(36)*.

RT-PCR detection of viral nucleic acid is currently one of the best approaches for the detection of foodborne viruses in food matrices because it has high specificity, sensitivity and throughput. RT-PCR also has several important limitations. Viruses must first be extracted and concentrated from the food matrix before application of RT-PCR *(38, 68)*. Extraction and concentration steps are often inefficient and can result in loss of virus and low recovery. NGS offers new potential methods for sensitive detection of viruses in food and clinical samples. As the sensitivity of NGS methods are improved, they may be the ideal laboratory method to investigate foodborne outbreaks *(19)*.

## 3.7 Microbiological risk assessment

### General factors to consider

Risk assessment is a process that can be used to under- stand and manage biological or chemical hazards associated specific foods. It is commonly presented as consisting of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization. Risk assessments can be categorized as qualitative, semi-quantitative, and quantitative, each requiring more data than the last. In quantitative microbial risk assessment, mathematical models are used to describe pathogen prevalence and concentration, as well as their growth and inactivation kinetics in foods as a result of transport, handling, and processing *(43, 69)*. Parameters important in viral risk

assessment include the stability of foodborne viruses in food and in response to processing stresses (e.g. heat, freezing, sanitizer exposure) as well as transfer and spread via cross-contamination.

Most microbial risk assessments have focused on bacterial pathogens, but there is increased interest in risk assessments for enteric viruses, in part because measures used to control or bacterial contamination in food or water are not always effective for controlling viruses *(20)*. Viral risk assessments should consideration such as virus inactivation over time, susceptibility to disinfectants, differences in host immunity, differences in clinical symptoms and health outcomes (including the potential for asymptomatic and secondary infections), genetic diversity and emergence of novel viral strains *(10)*. Over the last decade, almost two dozen foodborne or waterborne viral risk assessments have been published, often focusing on commonly contaminated foods and environmental sources associated with outbreaks (Table 2). Early risk assessments focused on irrigation water quality and a variety of viruses, while more recently published risk assessments have focused on Norovirus in a variety of food products.

## 3.8 Viral surrogates

Some enteric viruses infecting humans lack an easy or suitable cell culture-based assay system or pose a high risk to laboratory staff, so surrogate viruses are often used *(27)*. Surrogates microorganisms are typically selected on the basis of their morphological similarities and/or similar physiological characteristics to the pathogens of interest *(13)*. The ideal surrogate should be equivalent or slightly more resistant to treatments than the target organism, and should be nonpathogenic, with similar survival and persistence characteristics *(9, 13, 35)*. Surrogates for pathogenic foodborne enteric

viruses include feline calicivirus, murine norovirus, bacteriophage MS2, Tulane virus, porcine sapovirus, and poliovirus *(11, 57)*. There is no one universal one-to-one relationship between specific foodborne viruses and their surrogates. This is because a surrogate suitable for one stress (i.e. heat) might not be ideal for another stress (i.e. sanitizers), and there are multiple other stresses or situations that need to be studied (frozen survival, pH stress, cross-contamination, etc.).

## 3.9 Risk management strategies

The decision-making process for risk managers should be transparent and based on sound science and risk assessment *(10)*. Viruses associated with foodborne disease tend to be those where humans are the natural hosts; therefore, effective control measures need to include preventing exposure of foods to human feces or vomitus. Effective control of water treatment, food processing, cleaning and disinfection of surfaces, personal hygiene and hand washing, and/or sanitation are all required in order to control the spread of viruses along the food chain, although the efficiency of current control measures is poorly characterized *(10)*. Vaccination can also be an important risk management strategy, and vaccines against HAV and rotavirus have already been implemented and there are now several candidate vaccines for NoV, although none have currently made it to market *(21)*.

## 3.10 Conclusion

Foodborne viruses cause considerable morbidity and mortality. Controlling these viruses means relying on good personal and food hygiene, good agricultural practices, appropriate post-harvest controls and effective management of human sewage to prevent further transmission. More data on foodborne viruses and their surrogates, and well as

risk assessments incorporating such data are needed to assist risk managers in controlling foodborne viral disease.

Table 3.1: Characteristics of foodborne viral infections

| Frequency of foodborne transmission | Virus name | Virus family | Genome | Transmission routes | | |
|---|---|---|---|---|---|---|
| | | | | Fecal-oral | Person to person | Water and environment |
| High | Hepatitis A virus | Picornaviridae | ssRNA | X | X | X |
| | Norovirus | Caliciviridae | ssRNA | X | X | X |
| Low/Unknown | Hepatits E virus | Hepeviridae | ssRNA | X | | X |
| Rare | Aichi virus | Picornaviridae | ssRNA | X | | X |
| | Astrovirus | Astroviridae | ssRNA | X | X | X |
| | Rotavirus | Reoviridae | dsRNA | X | X | |
| | Sapovirus | Caliciviridae | ssRNA | X | X | |

Table 3.2: Summary of recent viral risk assessment related publications

| Year published | Virus(es) studied | Food, viral source or study focus | Citation |
|---|---|---|---|
| 2000 | Enteric viruses | Irrigation water | *(67)* |
| 2001 | Enterovirus | Irrigation water | *(51)* |
| 2005 | Hepatitis A virus | Irrigation water | *(60)* |
| 2006 | Rotavirus | Irrigation water | *(31)* |
| 2007 | Rotavirus, Norovirus | Irrigation water and soil | *(40)* |
| 2008 | Norwalk virus | Dose response study | *(63)* |
| 2008 | Hepatitis A, Norovirus | Fresh fruit | *(7)* |
| 2009 | Norovirus | Foodservice | *(47)* |
| 2009 | Hepatitis A virus | Clams | *(52)* |
| 2010 | Rotavirus, Norovirus | Irrigation water and soil | *(39)* |
| 2012 | Poliovirus, Rotavirus | Leafy greens post-irradiation | *(26)* |
| 2012 | Hepatitis A virus | Oysters | *(65)* |
| 2013 | Norovirus | Irrigation water and soil | *(5)* |
| 2013 | Norovirus | Oysters | *(66)* |
| 2013 | Norovirus | Greywater irrigated lettuce | *(28)* |
| 2014 | Norovirus | Wastewater irrigated vegetables | *(46)* |
| 2014 | Norovirus | Wastewater irrigated vegetables | *(6)* |
| 2015 | Norovirus | Deli sandwiches | *(59)* |
| 2015 | Norovirus, Hepatitis A virus, Adenovirus | Fresh produce, farm to fork | *(8)* |
| 2015 | Norovirus, Adenovirus | Wastewater and soil | *(1)* |
| 2017 | Norovirus | Foodservice | *(25)* |
| 2017 | Norovirus | IQF raspberries and raspberry puree | *(33)* |
| 2018 | Norovirus | Frozen berries | *(45)* |

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# Chapter 4: Survival of bacteriophage MS2 on frozen strawberries and raspberries

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## 4.1 Abstract

Norovirus is a foodborne enteric virus and has been linked to outbreaks following consumption of fresh and frozen fruits. Experiments were performed to determine the efficacy of freezing and frozen storage (-20°C) on the survival and persistence of norovirus surrogate, bacteriophage MS2, on frozen strawberries and raspberries at three different starting titers. Frozen storage at -20°C was used to represent a storage scenario in consumers home freezer. There was a decline rate of approximately 0.22 log PFU/month for strawberries and 0.21 log PFU/month raspberries, respectively, at a high starting titer. The decline in log PFU/month was increased as the starting titer increased. The pH did not change significantly during frozen storage for either berry. The color of the berries and pH did not significantly change throughout the freezing process and frozen storage.

## 4.2 Introduction

Frozen foods are one of the largest sectors of the food industry and large quantities of frozen foods are consumed year-round. The United States (U.S.) frozen food industry is valued at over $6 billion in annual sales *(36)*. Freezing is typically considered superior to dehydration and canning because foods retain more of their fresh sensory attributes and nutritional properties *(42)*, but ice crystals can form in fruits, leading to a breakdown of the fruit structure, affecting overall appearance *(12, 16, 29)*. Batches of frozen berries are often packed in small bags in large cases and have a typical "best-quality" shelf-life of around 18 to 24 months, as observed on packages purchased at local New Jersey supermarkets.

Norovirus (NoV) is the leading cause of acute gastroenteritis in the world causing an estimated 21 million illnesses in the U.S. annually *(5)*. Many foodborne outbreaks involving fresh and frozen berries have been linked to viruses *(2, 19, 22, 24, 26, 33, 34, 37)*. The largest outbreak on record arising from NoV-contaminated berries occurred in Germany in 2012, where over 11,000 people fell ill with NoV after consuming contaminated frozen strawberries sourced from China *(28)*. Viruses can survive frozen storage, which can contribute to geographically and temporally widespread outbreaks. NoV is usually spread through feces or vomit of infected individuals and can be passed through contaminated water, food or direct person-to-person contact. Contamination can occur pre- or post-harvest or during handling by workers or consumers *(30)*. Many berries may require more handling by hand than most fruits or vegetables due to their delicate nature. A survey of NoV contamination in commercial frozen berry fruits from

2016 to 2017 in the leading berry-producing area in China found the prevalence of NoV was 9% when sampling 900 frozen retail berry samples *(13)*.

NoV was unable to be propagated in cell culture until recently *(11)* so NoV is often studied using surrogate organisms such as Murine norovirus (MNV), Feline calicivirus (FCV), Tulane virus (TV) and bacteriophage MS2 (MS2) *(6, 32)*. Freezing is a common practice to preserve berries commercially and at home, but survival characteristics of foodborne viruses on frozen fruits is not well documented. The objective of this study was to determine the effect of freezing and frozen storage on frozen berries contaminated with bacteriophage MS2 over a two-year time period.

## 4.3 Materials and methods

### Bacteriophage MS2 Propagation

MS2 (ATTC 15597-B1) and *E. coli* C3000 (ATCC 15597) were obtained from American Type Culture Collection (Manassas, VA). MS2 was propagated using *E. coli* C3000 as a host on tryptic soy agar (TSA) plates using the double agar overlay method (Difco, Becton Dickinson, Sparks, MD) *(20)*. MS2 stocks were prepared on the cell lawns of *E. coli* C3000 by U.S. Environmental Protection Agency method 1601*(9)*. One tenth (0.1) ml of overnight *E. coli* C3000 culture was added to 5 ml of molten TSB soft agar, in addition to 0.1 ml of MS2 (~11 log PFU/ml). The inoculated culture was spread on the surface of a TSA plate and incubated overnight at 37°C. MS2 was recovered by scraping the top of the plate (soft agar layer) and placing it in TSB. The MS2 phage in TSB was separated from the host cells by centrifugation (5,000 x g, 4°C for 25 min), followed by filtration of the supernatant through a 0.45-μm pore-size filter (Fisher), stored at 4°C prior to use.

Frozen Storage Experiments

Fresh strawberries and raspberries were purchased from a local Stop & Shop and stored at 4°C prior to use (Stop & Shop, Somerset, NJ). The strawberries and raspberries had pH (Accumet Basic AB15 pH Meter, Fisher Scientific) values of 3.40 ± 0.02 and 3.27 ± 0.05, water activities ($a_w$) of 0.986 and 0.984 (Rotronic Instrument Corp., Hauppauge, NY), and 14 and 12 °Brix, respectively. Fresh strawberries and raspberries had initial L*, a* and b* values of 29, 33, 10 and 34, 36, 10, respectively (Konica Minolta Sensing, Inc., Chroma Meter CR-410). °Brix content was obtained by taking three measurements of sliced and whole berries, varying berry surface angles and averaging the L*, a* and b* values. Virus dilutions in phosphate buffered saline (PBS) were inoculated onto the surfaces of each 15 g portion of fresh berries (one large strawberry, three raspberries) by dispensing 50 µl over approximately 10 spots with approximately 11 log PFU/ml of MS2. Inoculated samples were air-dried in a biosafety cabinet for 1 hour. Individual berries were placed in sterile sample bags and placed in the freezer to avoid cross-contamination between berries and reduce freezer burn.

Berry samples were analyzed on the day of inoculation, in triplicate, and the remaining samples were stored at -20°C. Batches of three samples were thawed and processed as described above, over a two-year period. Additional uninoculated berries were stored for each individual sampling time to determine the pH and $L$ (lightness), $\Delta a$ (redness), $\Delta b$ (yellowness) values over time. The total color difference, $\Delta E$, was calculated as well to determine if there were noticeable differences in color measurement from the initial fresh berries (Equation 1).

Equation 1 $\Delta E = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)}$

The freezing time from a whole fresh strawberry and raspberry to when the center was frozen was estimated to be approximately 1.15 hrs and 0.37 hrs, respectively, using Pham's method *(31)*. This method is more accurate than Plank's method *(23)* because it considers the diameter of the product. The inputs used for the calculation were -20°C for air temperature, 0°C for final center temperature, product diameter of ¾" (0.01905 m) and ¼" (0.00635 m) for strawberry and raspberry respectively, as well as a convective heat transfer of 12.2 $W/m^2°C$ as estimated using numerical simulation from the published literature *(7)*. Strawberries and raspberries were tested to determine when the product reached freezing by inserting a thermometer (Thermistor Thermometer, Grainger) into the berry; the temperatures of the berries reached freezing (0°C) at 1.02 ± 0.18 and 0.23 ± 0.07 hrs, respectively.

Data Analysis

Regression analysis was performed to estimate the rate of decline in virus concentration over time (Microsoft Office Excel; Microsoft, Redmond, WA, USA). The D-value, or decimal reduction time, is determined by the best fit on the slope of the viral survival curve obtained by plotting the log of the viral titer after frozen storage against the time the frozen berries are stored. The reciprocal of the slope of the regression line is the D-value. The decline rate (log PFU/month) was calculated.

## 4.4 Results

Figures 1 and 2 show survival of MS2 on frozen strawberries and raspberries stored at -20°C with high, medium and low starting titers. The D-value for frozen strawberries at high starting titer was approximately 139 days, whereas the D-values for medium and low titer were about 182 and 250 days, respectively. The decline rate per

month for high, medium and low titer on frozen strawberries were 0.22, 0.17 and 0.12 log PFU/month, respectively. The D-value for frozen raspberries at high starting titer was approximately 147 days, whereas the D-values for medium and low titer were about 217 and 270 days, respectively. The decline rate per month for high, medium and low titer on frozen raspberries were 0.21, 0.14 and 0.12 log PFU/month, respectively. There was a significant decrease in viral titer during the freezing process that ranged from 0.84 to 1.42 log PFU/berry by day 1.

No significant change in pH of the frozen strawberries and raspberries was observed over the two-year time period (Fig. 3). Colorimeter values for the frozen strawberries and raspberries are shown in Figure 4 and Figure 5. L* and a* values fluctuated during the freezing process and throughout frozen storage. The b* value fluctuated less throughout freezing and frozen storage for both berry types. The total color difference (Delta E*) is calculated from the other three parameters and thus shows fluctuations similar to L*, a* parameters.

## 4.5 Discussion

The freezing process is believed to have little effect on foodborne pathogens, including enteric viruses. Similarly, frozen storage at typical commercial conditions (e.g. -20°C) produces only a gradual decline in viability over time *(3, 18)*. The occurrence of foodborne viral disease outbreaks from consumption of fruit frozen for prolonged periods of time indicates that if the food is contaminated prior to freezing, viruses survive and can remain infectious long enough through frozen shelf life to cause disease.

Mattison et al. (2007) researched the survival of FCV inoculated on lettuce and strawberry disks stored at 4°C. Greater than a 2.5 log reduction on strawberries was

observed after 6 days at 4°C and an average of a 2 log reduction was observed on lettuce following 7 days of storage at 4°C *(25)*. Though FCV is commonly used as a NoV surrogate, it has been shown to be an unreliable surrogate when exposed to acidic conditions, like those found in fruits. Studies have reported that the inactivation of FCV increased with decreasing produce pH *(4, 17, 35)*.

Dawson et al. (2005) used the surrogate bacteriophage MS2 to observe virus survival on a variety of fresh produce. At 4°C, < 1 log reduction was observed after 7 days of storage on strawberry, lettuce, pepper, carrots, cabbage, and tomato and < 2 log reduction was seen prior to produce deterioration *(8)*. In a study using human norovirus GII.4 and GI.4, MNV and human adenovirus (hAdV), virus survival on fresh strawberries and fresh raspberries depended on the storage temperature and fruit type – viruses typically showed similar persistence patterns *(39)*. The largest difference among persistence was observed at 21°C; there was a rapid decline of infectious MNV-1 and hAdV particles on strawberries after 1 day (approximately 1.5 log reduction), whereas raspberries saw no decline over this period. This study demonstrated that NoV was less persistent on strawberries than on raspberries. Our research, as well as Verhaelen et al. (2012), demonstrate the importance of considering differences between different fruits when assessing risk *(39)*.

The freezing process has shown a reduction of viral load on food. FCV and canine calicivirus (CaCV) showed a decline in infectivity of $0.34 \pm 0.18$ *D* and $0.44 \pm 0.12$ *D*, respectively, after 5 cycles of freeze-thawing *(10)*. While we did not study repeated freeze-thaw cycles, we saw little decline from the single freeze thaw cycle we used. A study by Kurdziel et al. (2001) observed a reduction of < 2 log of Poliovirus (PV) in

frozen strawberries after 15 days of storage at -20°C, with a D-value of 8.4 days; while no decline was observed for fresh raspberries under the same conditions *(21)*. PV is known to be more resistant to environmental conditions than other enteric viruses, therefore the authors expected little affect during freezing *(40)*. No significant reduction was observed of MNV-1 infectivity in deep-frozen spinach and deep-frozen onions over 6 months of storage at -21°C *(1)*.

Richards et al. (2012) reported that long-term frozen storage at -80°C did not decrease NoV RNA titers over the 17-week study period *(32)*. Butot et al. (2008) studied the effect of frozen storage at -20°C on HAV, NoV, FCV and rotavirus (RV) when inoculated on strawberries, raspberries and blueberries. No significant reduction in the viability of any of the viruses was observed during freezing, except for FCV in strawberries, raspberries and blueberries. The $TCID_{50}$ (50% tissue culture infective dose) values on these samples were reduced by more than 1 $\log_{10}$ unit. Over a 3-month period, frozen storage had a limited effect on the survival of HAV and rotavirus RV in all products tested, whereas FCV infectivity declined by more than 2 $\log_{10}$ units on strawberries, raspberries and blueberries, likely due to their lower pH *(3)*.

Over a two-year a time period, the pH for both frozen raspberries and strawberries did not significantly change. A study by Grzeszczuk et al. (2007) looked at the effect of blanching, freezing and frozen storage on spinach plants found that the pH significantly changed after 9 months of frozen storage *(15)*. Another study *(38)* recorded the effect of ascorbic acid treatment on frozen strawberries and raspberries and observed a 10-13% decrease in pH following 3 months of storage, regardless of the ascorbic acid treatment received. Experiments by Mgaya-Kilima et al. (2014) reported on the effect of storage

temperature on roselle-fruit juice blends at 4°C and 28°C. At both temperatures, the pH was observed to increase due to the decrease in acidity of the juices *(27)*. Several studies found that after freezing and frozen storage of strawberries and raspberries found that the L*, a* and b* values did not change significantly *(14, 41)*, consistent with our observations.

Our findings suggest that if MS2 is used as a surrogate for foodborne pathogenic viruses for fruits contaminated before freezing, predicted risk of disease decreases very slowly and consistently over a 2 year shelf. Unfortunately, the published literature on viral survival in frozen fruits shows wide variability in virus survival rates. These may be true differences between viruses, which means selecting a surrogate is challenging. Although risk from viruses on frozen berries may go down during storage, prevention of contamination and/or processing to reduce risk still have the best potential to decrease the risk of future outbreaks.

Table 4.1: Survival kinetics of MS2 on Frozen Strawberries and Frozen Raspberries

| Food | pH | Water Activity | °Brix | Starting titer (log PFU/g) | $R^2$ of regression line | Decline rate (log PFU/month) | D-value (days) |
|---|---|---|---|---|---|---|---|
| Strawberries | 3.40 ± 0.02 | 0.986 | 14 | High (9.89) | 0.9618 | 0.216 | 139 |
| | | | | Medium (8.21) | 0.9183 | 0.165 | 182 |
| | | | | Low (6.51) | 0.9720 | 0.120 | 250 |
| Raspberries | 3.27 ± 0.05 | 0.984 | 12 | High (9.47) | 0.9364 | 0.204 | 147 |
| | | | | Medium (8.65) | 0.9392 | 0.138 | 217 |
| | | | | Low (6.56) | 0.8781 | 0.120 | 250 |

Figure 4.1: Survival of MS2 on frozen strawberries

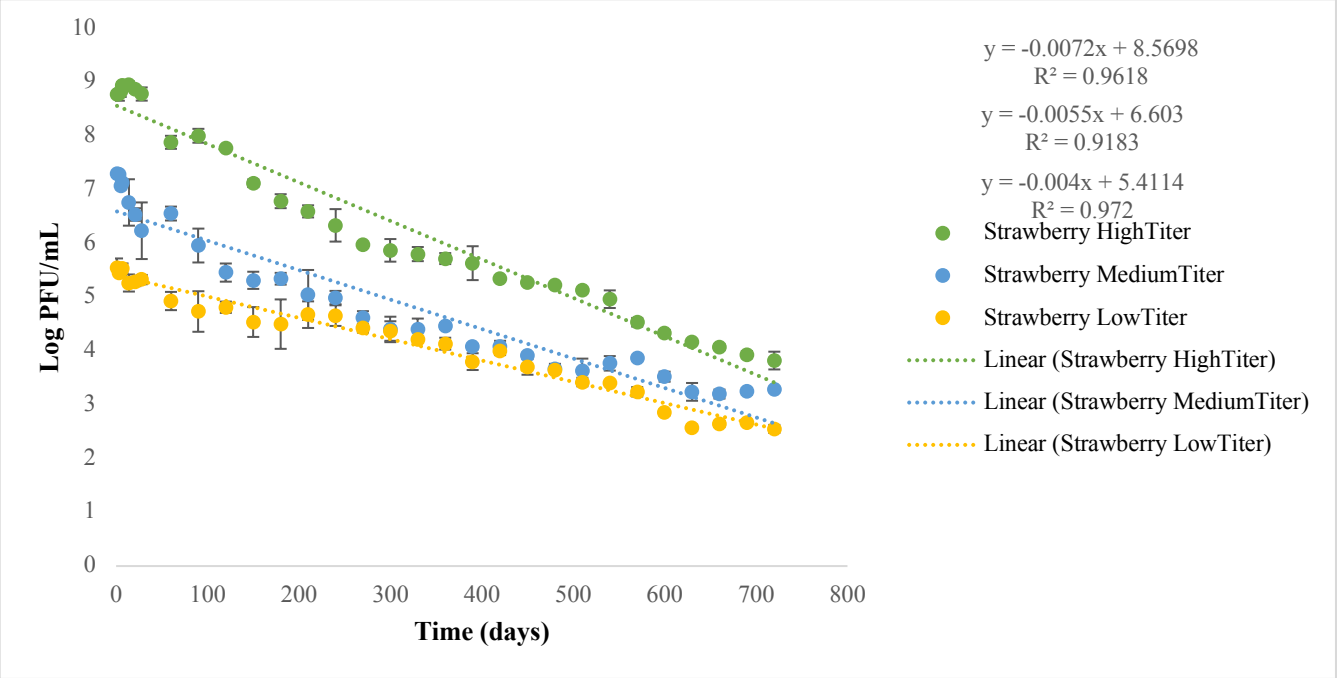


Figure 4.2: Survival of MS2 on frozen raspberries

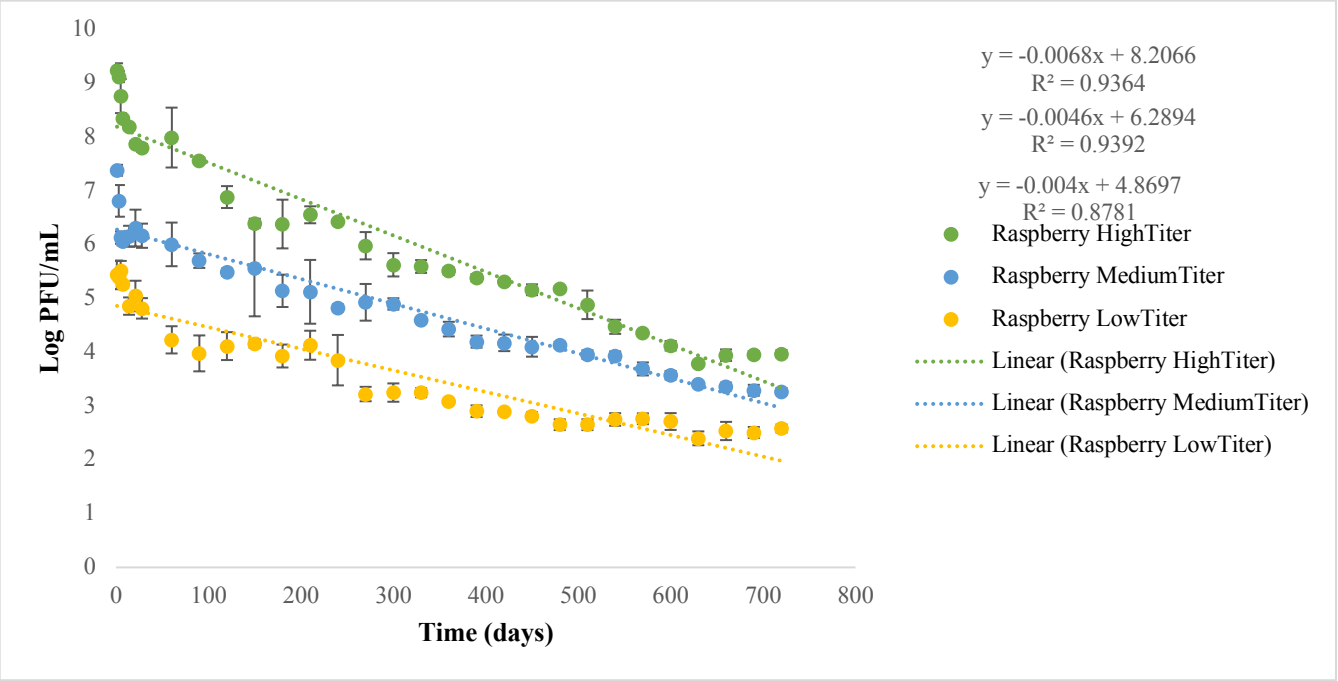


Figure 4.3: pH change in frozen strawberries and frozen raspberries over time

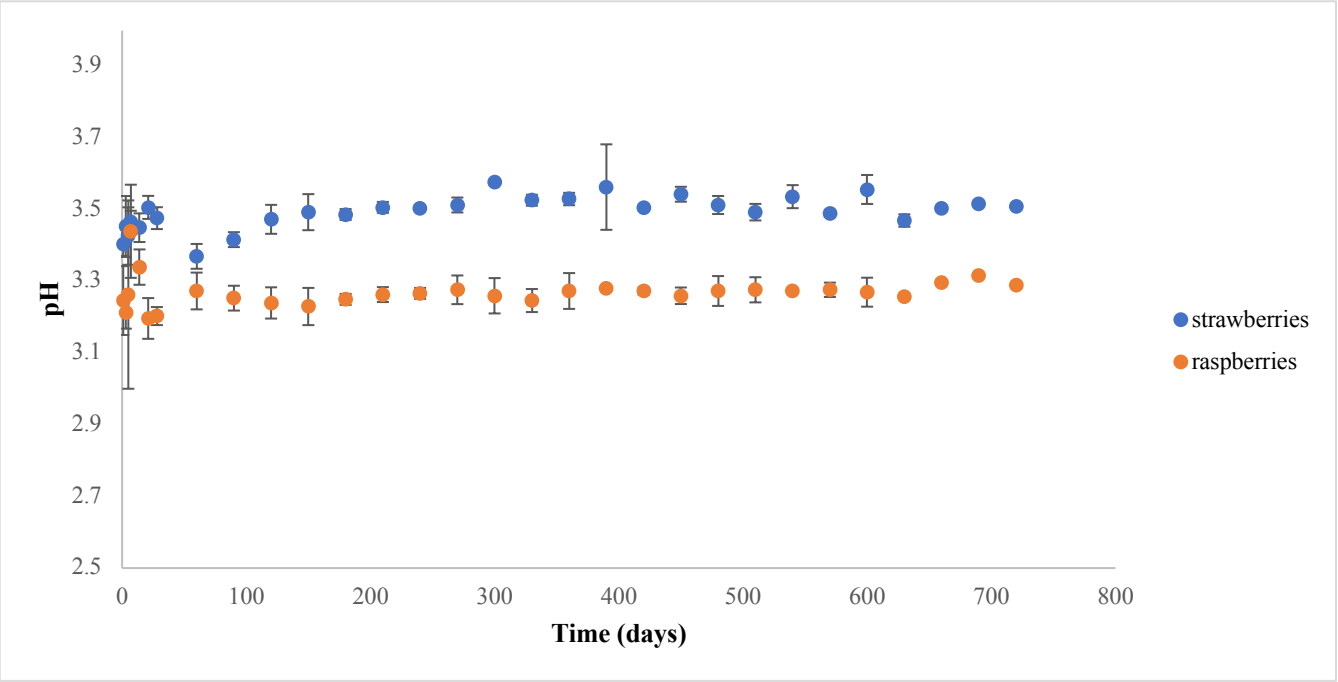


Figure 4.4: Colorimeter values for frozen strawberries over time

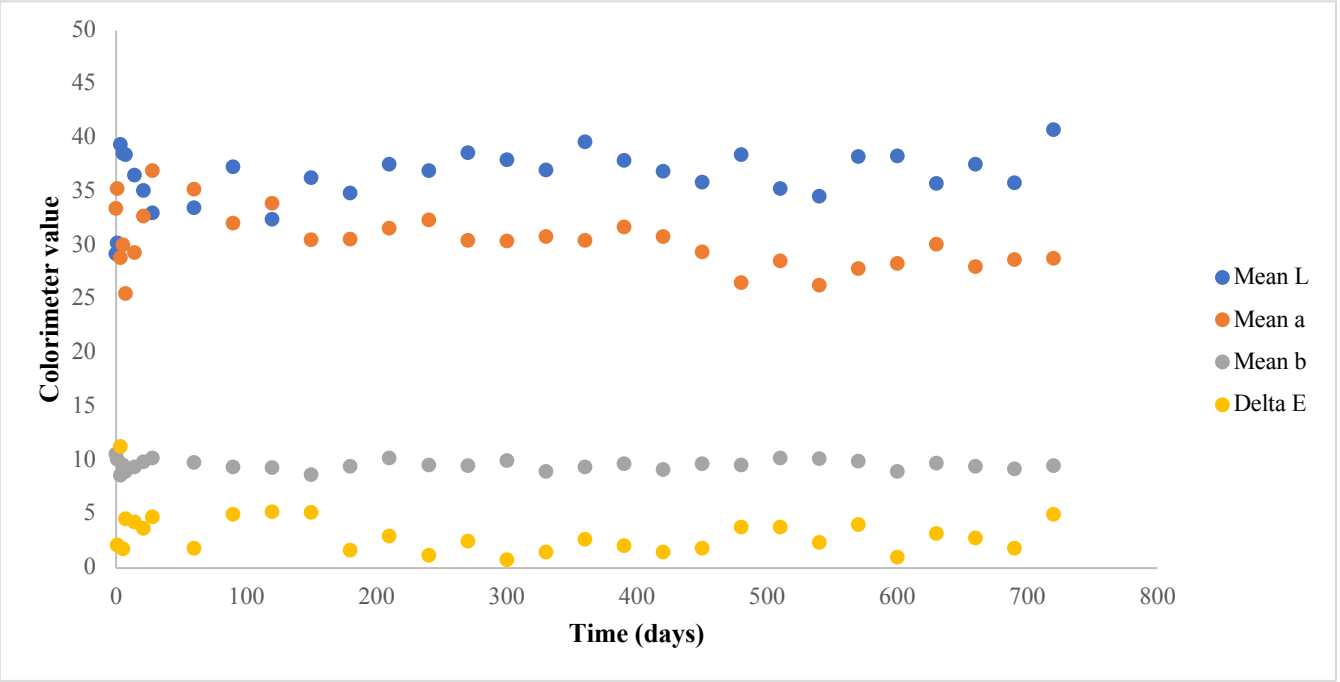
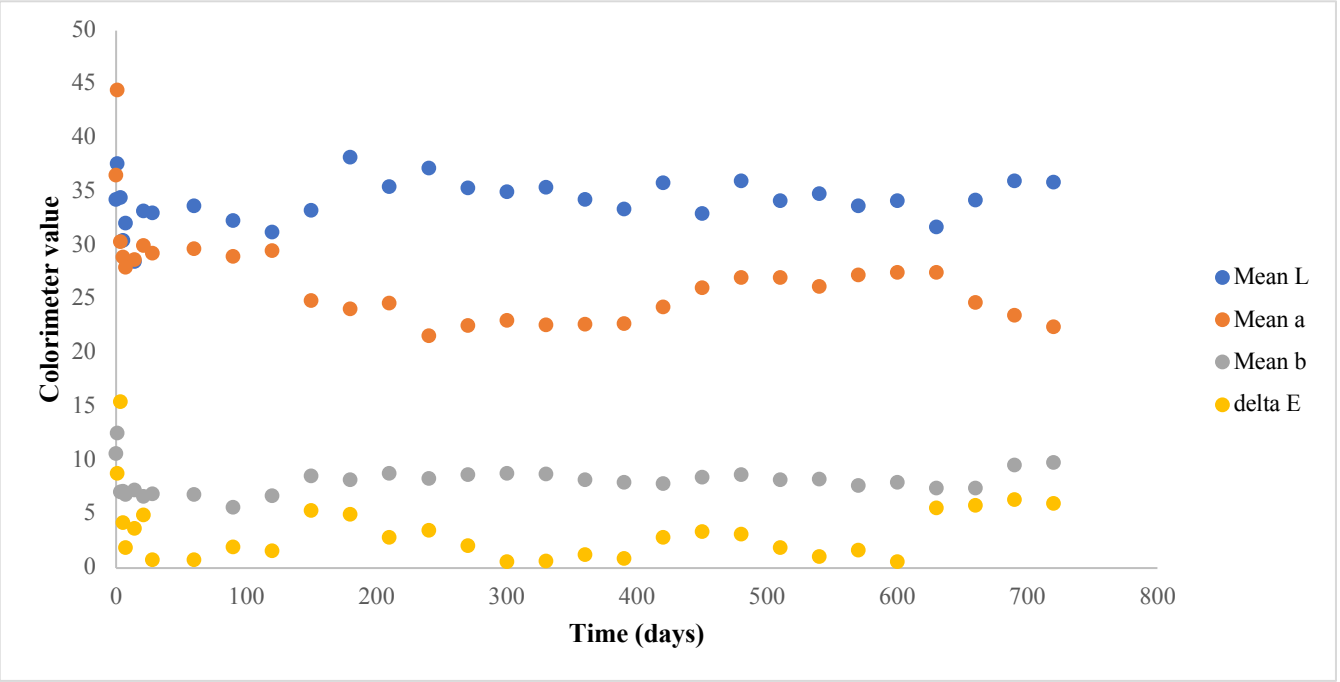


Figure 4.5: Colorimeter values for frozen raspberries over time



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# Chapter 5: Thermal Inactivation of bacteriophage MS2 on Frozen Berry Puree

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## 5.1 Abstract

Norovirus is the leading cause of foodborne illness worldwide. There is little information regarding thermal inactivation characteristics of norovirus due to the complexity of cell culture systems necessary for propagation. Thermal inactivation of microorganisms remains a key measure in the food industry, therefore understanding the thermal inactivation kinetics of human norovirus surrogates could be valuable. The objective of this study was to evaluate the thermal inactivation kinetics of norovirus surrogate, bacteriophage MS2, in frozen berry purées. Strawberry and raspberry purées were inoculated with MS2 (~10 PFU/ml purée) and exposed to three temperatures (55, 60 and 65°C) over a range of suitable times in a water bath. The *z*-values for raspberry purée at 10, 20 and 30°Brix were 10.9, 16.3 and 21.9°C, respectively. The *z*-values for strawberry purée at 10, 20 and 30°Brix were 10.1, 16.3 and 22.2°C, respectively. The results of this study will assist in developing suitable thermal processing procedures of frozen berries to ensure delivery of a safe product to consumers.

## 5.2 Introduction

Norovirus (NoV) is the leading cause of acute gastroenteritis worldwide *(14)*. NoV infections are primarily transmitted through fecal-oral contamination, by aerosolized vomit from an infected person or by contaminated food, water or surfaces. Fruits and vegetables, such as minimally processed berries, have been recognized as vehicles of viral gastroenteritis. Frozen raspberries contaminated with NoV were reported to be the probable cause of several outbreaks in Denmark, France and Sweden *(16, 24, 28, 31)*. Frozen strawberries sourced from China were linked to over 11,000 illnesses to children in Germany in 2012 *(25, 33)*.

Fresh produce may become contaminated with foodborne viruses' pre-harvest, following contact with contaminated irrigation water or contaminated water used to dilute fertilizer applied to the produce. Contamination can also occur throughout several stages of the food chain including harvesting, processing, storage, distribution or preparation by coming in contact with contaminated water or surfaces or infected workers *(26, 27, 36, 42)*. Berries can be individually quick frozen (IQF) or processed into purées for use in other products. Berry purées can be further processed into products such as smoothies, jams, jellies or fruit fillings.

Heat treatment is applied to manufacture fruit purées which helps to increase the shelf-life and to inactivate pathogenic and spoilage microorganisms that may be present. Raspberry purée is generally pasteurized by heating for approximately 2 min at 88°C *(38)*. Studies have shown that this pasteurization process would be sufficient to inactivate high levels of NoV *(3, 12, 40)*. Consumer demand for minimally processed foods with nutritional and flavor profiles closer to fresh have resulted in a modification of the berry

pasteurization processes to use more mild heating conditions, like 30 s at 65°C or 15 s at 75°C *(3)*. Several studies have been performed on heat inactivation of viruses in various foods *(1, 2, 37)* using small volumes of homogenized food samples under controlled heating conditions *(41)*. Only a few studies have specifically observed inactivation of viruses using heat for berries *(3, 5, 13, 20, 30)*.

Viruses are affected by thermal inactivation when the capsid of the virus is altered. The capsid encloses the viral genome and additional components that are needed for virus structure or function needed to bind to the host *(21)*. Song et al. (2011) determined that the mechanisms of thermal inactivation involve the denaturation of viral proteins and disassembly of virus particles into noninfectious viral subunits *(39)*. Experimental studies relevant to human NoV are often performed using surrogate organisms because NoV could not be cultured until recently, and even now requires a complex cell system *(23)*. Commonly used surrogates for foodborne viruses include Feline calicivirus (FCV), Murine norovirus (MNV), Tulane virus (TV), Poliovirus (PV) and bacteriophage MS2 (MS2) *(19, 29, 43)*. MS2 is similar in shape and size to NoV and is also a single-stranded RNA virus. MS2 is adapted to the intestinal tract and has been shown to be a successful indicator for NoV in water and aerosolization studies (Tung-Thompson 2015; Dawson 2005). MS2 is typically used as an internal control for validation of recovery and detection methods for NoV *(34)*. Bacteriophages, like MS2, are useful surrogates because they are relatively easy to propagate and require a shorter incubation period for detection than other virus plaque assays. The reduction of bacteriophage MS2 by mild heat treatments on frozen raspberry and strawberry purées was investigated in this study. This data may have a variety of uses, but we will

specifically use it to predict the effectiveness of different time/temperature treatments for microwaving frozen berries.

## 5.3 Materials & Methods

### Bacteriophage MS2 preparation

MS2 (ATTC 15597-B1) and *E. coli* C3000 (ATCC 15597) were obtained from American Type Culture Collection (Manassas, VA). MS2 was propagated using *E. coli* C3000 as a host on tryptic soy agar (TSA) plates using the double agar overlay method (Difco, Becton Dickinson, Sparks, MD) *(32)*. MS2 stocks were prepared on the cell lawns of *E. coli* C3000 by the U.S. Environmental Protection Agency method 1601 *(22)*. One tenth (0.1 ml) of overnight *E. coli* C3000 culture was added to 5 ml of molten TSB soft agar along with 0.1 ml of MS2 (~11 log PFU/ml). The inoculated culture was spread on the surface of a TSA plate and incubated overnight at 37°C. The MS2 phage was recovered by scraping the top of the plate (soft agar layer) and placing it in TSB. The MS2 phage in TSB was separated from the host cells by centrifugation (5,000 x g, 4°C for 25 min), followed by filtration of the supernatant through a 0.45-µm pore-size filter (Fisher) and stored at 4°C, prior to use.

### Berry preparation

Frozen raspberry purée (100% raspberries) and strawberry purée (100% strawberries) were purchased from a local supermarket (Stop and Shop, Somerset, NJ). The pH and °Brix of samples were measured using a surface pH probe (Accumet Basic AB15 pH Meter, Fisher Scientific) and a °Brix refractometer (Fisherbrand Handheld Analog Brix/Sucrose Refractometer, Fisher Scientific), respectively. The initial °Brix measurements were 10 and 8 °Brix for raspberry and strawberry purée, respectively. The

purées were modified as needed by directly adding sucrose (Sucrose, Fisher Scientific) and homogenizing the samples until a final concentration of 10, 20 and 30 °Brix for raspberry and strawberry purée was obtained. The pH of the initial frozen raspberry and strawberry purée samples were 3.01 ± 0.09 and 3.41 ± 0.03, respectively.

Thermal treatment

The frozen purées were thawed for 30 min and 10 g samples were placed into sterile filter bags for immediate use following appropriate addition of sucrose to reach desired °Brix measurements. Each berry purée sample was inoculated with MS2 to obtain a concentration of approximately 10 log PFU/ml. One ml of the virus stock solution was used to inoculate individual samples (10 g of berry purée) and these samples were then placed in a water bath at respective temperatures (55°C, 60°C and 65°C), with negligible come-up time. The virus extraction procedure was performed on all heat-treated samples after a maximum storage of 20 min on ice to cease further thermal inactivation. Raspberry and strawberry purée inoculated with 1 ml virus stock solution at room temperature (RT) served as a positive control. Uninoculated un-heat-treated raspberry and strawberry purée served as a negative control.

Data analysis

Thermal resistance of MS2 at 55°C, 60°C and 65°C was evaluated by calculating the D-value, the time required at a specific temperature to obtain a one log reduction of the viral titer. Using linear regression, the D-value, or decimal reduction time, is given by the best fit on the slope of the viral inactivation curve. The viral inactivation curve is created by plotting the log of the viral titer after heat treatment against the time of exposure. The relative heat resistance at different temperatures (*z*-value) was also

calculated. A categorical regression was performed in R Studio to model the effect of temperature as a continuous variable, with categorical variables for berry type and °Brix to determine significance of interactions on the D-value ($p < 0.05$) (RStudio, Inc., Boston, MA).

## 5.4 Results & Discussion

Frozen raspberry purée had a measured pH of 3.01 and 10 °Brix, while the strawberry purée had a measured pH of 3.41 and 8 °Brix. The calculated D-values and *z*-values for the variables are shown in Table 1. The pH of the samples remained constant when sucrose was added. As the sucrose concentration increased, the D-value decreased in both berry purées. The D-values for raspberry purée at 55°C were 1.27, 1.11 and 0.99 min for sucrose concentrations of 10, 20 and 30 °Brix, respectively. The D-values were for strawberry purée at 55°C were 1.52, 1.12 and 0.97 min for 10, 20 and 30 °Brix, respectively. The calculated z-values for raspberry purée were 10.9, 16.3 and 22.0°C for 10, 20 and 30 °Brix, respectively. The z-values were for strawberry purée were 10.1, 16.4 and 22.2°C for 10, 20 and 30 °Brix, respectively.

The results of the categorical regression showed which variables and variable interactions had a significant effect on the D-value. Temperature was the only variable that had a significant effect on the D-value ($p = 0.0023$). Interactions between berry type, °Brix and temperature did not significantly affect the D-value ($p > 0.05$). The effect of 30 °Brix, was not statistically significant ($p = 0.1873$) and neither was the interaction of temperature with 30 °Brix ($p = 0.1802$) as shown in Table 2.

Several thermal inactivation studies have been conducted using NoV surrogates and foodborne enteric viruses and are summarized in Table 3. Table 3a summarizes

thermal inactivation data available in the published literature involving fruits and vegetables and Table 3b in cell culture media. The most recent study by Bartsch et al. (2019) researched the thermal inactivation of human NoV and surrogates in strawberry purée. Both MNV and TV were inactivated to below detection (> 7 $\log_{10}$ reduction) after treatment for 8 s at 80°C *(5)*. MNV inactivation has been studied in spinach and raspberry purée *(3, 4, 9)*. Baert et al. (2008) researched the effectiveness of blanching spinach at 80°C and the survival of MNV during this process. The come-up time of the blanching process was not considered and the researchers did not specify the final temperature of the spinach following treatment and no thermal inactivation kinetics were given *(4)*.

Bozkurt et al. (2015) reported thermal inactivation kinetics of MNV and FCV in spinach of temperatures at 50, 56, 60, 65, and 72°C. D-values for MNV were between 0.16 and 14.57 min with a z-value of 10.98°C *(9)*. These findings were similar other research from the same group *(10, 11)* who reported similar z-values (9.31, 11.62, 10.37°C) of FCV and MNV with the same temperature profiles. Another study also from this group *(8)* investigated thermal inactivation kinetics of HAV (50 to 72°C) in spinach and reported D-values ranged from 34.3 to 0.91 min at 50 to 72°C with a z-value of 13.92°C. HAV is another commonly researched foodborne virus; calculated D-values for this organism in strawberry mashes with varying brix values, 28 and 52° brix at 80°C, were 1.22 and 8.94 min, respectively *(20)*. These results indicate that an increase in °Brix increases the thermal resistance of HAV. This effect was also seen in strawberry mash with 28 and 52°brix heated at 85°C with reported D-values of 0.96 and 4.98 min, respectively *(20)*.

As Table 3 shows, thermal resistance of foodborne enteric viruses can be influenced by the food matrix. Significant differences can be seen across food types and between food and cell culture media suspensions. Inactivation results vary by the compositional different in the food matrix since the environment that viruses are found in impacts their thermal inactivation sensitivity. To gain a better understanding of how viruses can be inactivated in foods, the interaction between temperature and matrix must be considered *(6)*. Certain food components such as the protein, fat and sugar content have been shown to play a protective role against thermal inactivation of viruses. *(7, 17, 18, 20, 35)*.

The container geometry has also been shown to influence the thermal inactivation behavior of viruses; using larger container sizes can affect the time to reach the ideal temperature, known as the come-up time *(15)*. The influence of sample size on thermal resistance of human norovirus surrogates, like FCV and MNV has been reported *(10, 11)* and at high temperatures, the increased come-up time might contribute to variances in the measured D-value.

Foodborne viruses are generally more heat resistant than most foodborne non-spore-forming bacterial pathogens. Therefore, processing recommendations based on data for pathogenic bacteria may not eliminate foodborne enteric viruses. A detailed understanding of the thermal inactivation behavior of NoV and its surrogates has significant importance for understanding the role that thermal processing can play in managing risk. This study will contribute to developing appropriate thermal processing procedures to manage NoV risk from frozen berries.

Table 5.1: Inactivation kinetics of bacteriophage MS2 in Raspberry and Strawberry Purees with modified sugar content

| Berry type | °Brix | Temperature (°C) | D-value (min) | z-value (°C) | $R^2$ |
|---|---|---|---|---|---|
| Raspberry | 10 | 55 | 1.276 | 10.9 | 0.999 |
| | | 60 | 0.462 | | |
| | | 65 | 0.155 | | |
| | 20 | 55 | 1.113 | 16.3 | 0.971 |
| | | 60 | 0.677 | | |
| | | 65 | 0.272 | | |
| | 30 | 55 | 0.997 | 22.0 | 0.973 |
| | | 60 | 0.686 | | |
| | | 65 | 0.350 | | |
| Strawberry | 10 | 55 | 1.524 | 10.1 | 0.998 |
| | | 60 | 0.447 | | |
| | | 65 | 0.155 | | |
| | 20 | 55 | 1.115 | 16.4 | 0.975 |
| | | 60 | 0.670 | | |
| | | 65 | 0.273 | | |
| | 30 | 55 | 0.970 | 22.2 | 0.975 |
| | | 60 | 0.667 | | |
| | | 65 | 0.345 | | |

Table 5.2: Categorical regression analysis

| Variable and Interaction type | p-value |
|---|---|
| Berry temperature | 0.0023 |
| Brix 20 | 0.4373 |
| Brix 30 | 0.4209 |
| Temperature – Berry Type, strawberry | 0.1873 |
| Temperature – Brix 20 | 0.4583 |
| Temperature – Brix 30 | 0.4049 |
| Berry Type, strawberry – Brix 20 | 0.1802 |
| Berry Type, strawberry – Brix 30 | 0.5792 |
| Temperature – Berry Type, strawberry- Brix 20 | 0.5972 |
| Temperature – Berry Type, strawberry – Brix 30 | 0.5648 |

Table 5.3A. Summary of literature data on thermal inactivation of foodborne enteric viruses in foods

| Virus | Citation | Sample | Temp (°C) | D-value (min) | SD | z-value (°C) |
|---|---|---|---|---|---|---|
| Feline calicivirus (FCV) | Bozkurt et al 2015 | Turkey deli meat | 50 | 9.94 | | |
| | | | 56 | 3.03 | | |
| | | | 60 | 0.82 | | |
| | | | 65 | 0.43 | | |
| | | | 72 | 0.14 | | |
| | Bozkurt et al 2014b | Spinach | 50 | 17.39 | | 9.89 |
| | | | 56 | 5.83 | | |
| | | | 60 | 0.78 | | |
| | | | 65 | 0.27 | | |
| | | | 72 | 0.15 | | |
| | Bozkurt et al 2014 | Mussels | 50 | 5.20 | | 11.39 |
| | | | 56 | 3.33 | | |
| | | | 60 | 0.77 | | |
| | | | 65 | 0.33 | | |
| | | | 72 | 0.07 | | |
| | Butot et al 2009 | Basil | 75 | 0.63 | | |
| | | Chives | | <0.63 | | |
| | | Mint | | <0.63 | | |
| | | Parsley | | 0.68 | | |
| | Allowood et al 2004 | Cabbage | 4 | 1.50 | | 176 |
| | | | 25 | 1.00 | | |
| | | | 37 | 1.00 | | |
| | | Lettuce | 4 | 1.50 | | 176 |
| | | | 25 | 1.00 | | |
| | | | 37 | 1.00 | | |
| Murine Norovirus (MNV) | Bartsch et al 2019 | Strawberry puree | 50 | 31.40 | 4.8 | 8.6 ± 0.6 |
| | | | 56 | 5.90 | 0.7 | |
| | | | 63 | 2.20 | 0.3 | |
| | | | 72 | 0.10 | 0 | |
| | | | 80 | 0.00 | 0 | |
| | Shao et al 2018 | Oyster homogenate | 49 | 28.17 | | |
| | | | 54 | 14.41 | | |
| | | | 58 | 4.60 | | |
| | | | 63 | 1.82 | | |
| | | | 67 | 0.86 | | |
| | Bozkurt et al 2015 | Turkey deli meat | 50 | 21.00 | 0.8 | |
| | | | 56 | 7.30 | 0.8 | |
| | | | 60 | 2.70 | 0.6 | |
| | | | 65 | 0.90 | 0.1 | |
| | | | 72 | 0.20 | 0 | |
| | Bozkurt et al 2014 | Mussels | 50 | 20.19 | | |
| | | | 56 | 6.12 | | |
| | | | 60 | 2.64 | | |
| | | | 65 | 0.41 | | |
| | | | 72 | 0.18 | | |
| | Bozkurt et al 2014b | Spinach | 50 | 14.57 | | |
| | | | 56 | 3.29 | | |
| | | | 60 | 0.98 | | |
| | | | 65 | 0.40 | | |
| | | | 72 | 0.16 | | |
| | Hewitt et al 2009 | Milk | 63 | 0.70 | | |
| | | | 72 | 0.50 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Baert et al 2008 | Spinach | 80 | 0.74 | | |
| | | Raspberry puree (9.2 Brix) | 65 | 0.44 | | |
| Poliovirus | Strazynski et al 2002 | Milk | 72 | 0.44 | | |
| Tulane virus | Shao et al 2018 | Oyster homogenate | 49 | 18.18 | | |
| | | | 54 | 8.64 | | |
| | | | 58 | 3.14 | | |
| | | | 63 | 1.56 | | |
| | Bartsch et al 2019 (PA) | Strawberry puree | 50 | 23.20 | 2.6 | |
| | | | 56 | 5.40 | 0.3 | |
| | | | 63 | 0.70 | 0 | |
| | | | 72 | 0.10 | 0 | |
| | | | 80 | 0.00 | 0 | |
| | Bartsch et al 2019 (PCR) | Strawberry puree | 50 | 126.30 | 18.8 | |
| | | | 56 | 19.50 | 2.6 | |
| | | | 63 | 4.60 | 0.6 | |
| | | | 72 | 0.20 | 0 | |
| | | | 80 | 0.00 | 0 | |
| | Ailavadi et al 2019 | Spinach | 50 | 7.94 | 0.21 | 10.74 |
| | | | 54 | 4.09 | 0.04 | |
| | | | 58 | 1.43 | 0.02 | |
| Hepatitis A virus (HAV) | Bozkurt et al 2015 | Spinach | 50 | 34.40 | | 13.92 |
| | | | 56 | 8.43 | | |
| | | | 60 | 4.55 | | |
| | | | 65 | 2.30 | | |
| | | | 72 | 0.91 | | |
| | Bozkurt et al 2015 | Turkey deli meat | 50 | 42.08 | | |
| | | | 56 | 20.62 | | |
| | | | 60 | 5.91 | | |
| | | | 65 | 2.27 | | |
| | | | 72 | 1.01 | | |
| | Bozkurt et al 2014 | Mussels | 50 | 54.17 | | 12.97 |
| | | | 56 | 9.32 | | |
| | | | 60 | 3.25 | | |
| | | | 65 | 2.16 | | |
| | | | 72 | 1.07 | | |
| | Butot et al 2009 | Basil | 75 | 1.34 | | |
| | | Chives | | <0.83 | | |
| | | Mint | | 1.46 | | |
| | | Parsley | | 1.21 | | |
| | | Basil | 95 | <0.83 | | |
| | | Chives | | <0.83 | | |
| | | Mint | | <0.83 | | |
| | | Parsley | | 1.03 | | |
| | Hewitt et al 2009 | Milk | 63 | 1.10 | | |
| | | | 72 | <0.3 | | |
| | Deboosere et al 2004 | Strawberry mash (28 Brix) | 85 | 0.96 | | |
| | | Strawberry mash (52 Brix) | 80 | 4.98 | | |
| | | Strawberry mash (52 Brix) | 85 | 8.94 | | |
| | Bidawid et al 2000 | Milk | 85 | 0.01 | | |
| | | 1% fat milk | 71 | 1.64 | | |
| | | 3.5% fat milk | 71 | 2.08 | | |
| | | 18% fat milk/cream | 71 | 3.16 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Human Norovirus GI | Butot et al 2009 | Basil | 75 | 5.20 | | |
| | | Chives | | <0.83 | | |
| | | Mint | | 2.57 | | |
| | | Parsley | | 1.56 | | |
| | | Basil | 95 | 4.90 | | |
| | | Chives | | <0.83 | | |
| | | Mint | | <0.83 | | |
| | | Parsley | | 1.58 | | |
| Human Norovirus GII | Bartsch et al 2019 | Strawberry puree | 50 | 584.10 | 189.5 | 6.1 ± 0.1 |
| | | | 56 | 59.80 | 8.1 | |
| | | | 63 | 8.00 | 1 | |
| | | | 72 | 0.60 | 0.2 | |
| | | | 80 | 0.00 | 0 | |
| | Croci et al 2012 | Mussels | 60 | 25.00 | | |
| | | | 80 | 4.84 | | |
| | Butot et al 2009 | Basil | 75 | 1.71 | | |
| | | Chives | | 1.85 | | |
| | | Mint | | 1.58 | | |
| | | Parsley | | 1.64 | | |
| | | Basil | 95 | 1.55 | | |
| | | Chives | | 1.08 | | |
| | | Mint | | <0.83 | | |
| | | Parsley | | 0.89 | | |
| | Hewitt et al 2006 | Mussels | 100 | 1.30 | | |

Table 5.3B. Summary of literature data on thermal inactivation of foodborne enteric viruses in cell culture media

| Virus | Citation | Temp (°C) | D-value (min) | SD | z-value (°C) |
|---|---|---|---|---|---|
| Feline calicivirus (FCV-F9) | Bozkurt et al 2014 | 50 | 19.95 | | 10.97 |
| | | 56 | 6.37 | | |
| | | 60 | 0.94 | | |
| | | 65 | 0.72 | | |
| | | 72 | 0.21 | | |
| | Bozkurt et al 2013 | 50 | 20.23 | | 9.29 |
| | | 56 | 6.36 | | |
| | | 60 | 0.56 | | |
| | | 65 | 0.32 | | |
| | | 72 | 0.11 | | |
| | Gibson et al 2011 | 37 | 599 | | 14.01 |
| | | 50 | 50.6 | | |
| | | 60 | 14.1 | | |
| | Cannon et al 2006 | 56 | 6.72 | | 9.46 |
| | | 63 | 0.41 | | |
| | | 72 | 0.12 | | |
| | Duizer et al 2004 | 37 | 480 | | 9.87 |
| | | 56 | 2.7 | | |
| | | 71.3 | 0.17 | | |
| | Doultree et al 1999 | 56 | 8 | | |
| | | 70 | 0.49 | | |
| | | 100 | 0.13 | | |
| Murine Norovirus (MNV) | Bozkurt et al 2014 | 50 | 36.28 | | 10.37 |
| | | 56 | 3.74 | | |
| | | 60 | 1.09 | | |
| | | 65 | 0.77 | | |
| | | 72 | 0.25 | | |
| | Bozkurt et al 2013 | 50 | 34.49 | | 9.31 |
| | | 56 | 3.65 | | |
| | | 60 | 0.57 | | |
| | | 65 | 0.3 | | |
| | | 72 | 0.15 | | |
| | Hirneisen et al 2013 | 50 | 2.47 | | 22.83 |
| | | 55 | 1.18 | | |
| | | 60 | 0.64 | | |
| | | 65 | 0.56 | | |
| | Hewitt et al 2009 | 63 | 0.9 | | |
| | | 72 | <0.30 | | |
| | Cannon et al 2006 | 56 | 3.47 | | 12.23 |

| | | | | | |
|---|---|---|---|---|---|
| | | 63 | 0.44 | | |
| | | 72 | 0.17 | | |
| Sapovirus (SaV) | Wang et al 2012 | 56 | 12.6 | | |
| Hepatitis A virus (HAV) | Bozkurt et al 2014 | 50 | 56.22 | | 12.51 |
| | | 56 | 8.4 | | |
| | | 60 | 2.67 | | |
| | | 65 | 1.73 | | |
| | | 72 | 0.88 | | |
| | Cappelozza et al 2012 | 50 | 56.22 | | |
| | | 60 | 2.19 | | |
| | | 70 | 1.09 | | |
| | Gibson et al 2011 | 50 | 385 | | 9.99 |
| | | 60 | 74.6 | | |
| | | 70 | 3.84 | | |
| | Hewitt et al 2009 | 63 | 0.6 | | |
| | | 72 | <0.30 | | |
| Tulane Virus (TV) | Ailavadi et al 2019 | 52 | 4.59 | 0.05 | 9.09 |
| | | 54 | 2.91 | 0.05 | |
| | | 56 | 1.74 | 0.07 | |

## 5.5 Bibliography

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# Chapter 6: The Inactivation of bacteriophage MS2 on Frozen Berries during microwave heating

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Keywords: thermal inactivation, bacteriophage MS2, frozen strawberry, microwave, human norovirus

## 6.1 Abstract

Human norovirus is the most common causative agent of foodborne illness in the United States. Frozen berries have been repeatedly linked to acute gastroenteritis caused by norovirus. Many guidelines recommend that frozen berries be microwaved for at least 2 min, but it is unclear if this thermal treatment is effective at inactivating human norovirus. Epidemiological investigations have recognized improper handling and heating prior to consumption as a common consumer practice that has led to illness. The objective of this study was to assess the effect of microwave heating at varying power levels in the survival of bacteriophage MS2, when inoculated in frozen strawberries. D- and *z*-values collected previously were used to predict the effect of microwave heating in a variety of scenarios. Bacteriophage MS2, a norovirus surrogate, was inoculated into frozen strawberries with a starting concentration of approximately 10 log PFU/g. Samples were heated in a 1,300-Watt research microwave oven at power levels ranging from 30-100% (full power) to determine the inactivation of the surrogate organism. Temperatures at the surface of berries were monitored during heating, using fiberoptic thermometry. Our findings indicate that microwave power levels and heating times significantly affect final berry temperatures reached and estimated reduction in the concentration of MS2. Following heat treatment of 60 s for 3 berries, log reductions of 1.1 ± 0.4, 1.5 ± 0.5, 3.1 ± 0.1 and 3.8 ± 0.2 log PFU/g were observed for 30, 50, 70 and 100% microwave power levels, respectively. These finding will also be useful in future quantitative microbial risk assessments when predicting the effect of microwave heating of berries in the home or foodservice.

## 6.2 Introduction

Norovirus (NoV) is the leading cause of acute gastroenteritis worldwide, causing approximately 685 million cases annually *(5)*. NoV outbreaks throughout the United States (U.S.) and Europe have implicated frozen berries as transmission vehicles over the last decade. Frozen berries may be consumed in a frozen or thawed state without subsequent heating which would reduce NoV risk. The largest NoV outbreak on record occurred in 2012 in Germany, where over 11,000 people fell ill after consuming contaminated frozen strawberries *(2)*. Data from that same outbreak also indicated that those consuming heated berries were less likely to become ill. Following large outbreaks of NoV and hepatitis A virus (HAV) elsewhere around Europe, a regulation has been implemented requiring caterers to heat-treat frozen berries prior to serving and recommending that consumers also heat frozen berries prior to consumption *(2, 9, 24)*. Microwave heating can be fast and efficient, but standardizing microwave oven cooking instructions has proven difficult due to the uneven heating profiles inherent in the technology *(23)*.

Microwave energy inactivates microorganisms as a function of the heat that is produced. Microwave heating is faster heating compared to conventional oven heating, but a major drawback can be the non-uniform product temperature distribution and resulting hot and cold spots that affect pathogen survival *(13, 28)*. Stirring or turning the foods during microwave heating can help to reduce the effects of unbalanced heating and allow for more consistent microbial reduction. Food geometry, food location in the oven, oven wattage, oven age, and differing dielectric and thermal properties can significantly affect the temperatures reached in different sections of the product *(20)*.

The U.S. Federal Communications Commission has designated two microwave frequencies for food processing and industrial microwave heating: 915 and 2,450 megahertz (MHz). U.S. domestic microwave ovens operate at a frequency of 2,450 MHz and several different common wattages (600, 700, 800, 1,000 and 1,200) *(7, 12)*. Microwave power levels are adjustable and determine the amount of microwave energy directed at the food product. An oven set at 50% power means the oven is producing microwaves 50% of the time and not producing microwaves the other 50% of the time *(27)*.

Cooking instructions should be validated for different oven wattages due to variations in heating profiles. The objective of this study was to assess the effect of microwave heating on the inactivation of the NoV surrogate, bacteriophage MS2 (MS2), and develop validated heating instructions for microwave heating of frozen strawberries.

## 6.3 Materials and Methods

### Frozen Strawberry Product Characteristics

Whole frozen strawberries were purchased from a local supermarket and stored in the freezer (-20°C) prior to use (Stop & Shop, Somerset, NJ). Each strawberry weighed approximately 15 g and was approximately ¾" in diameter.

The pH and °Brix of samples were measured using a surface pH probe (Accumet Basic AB15 pH Meter, Fisher Scientific) and a °Brix refractometer (Fisherbrand Handheld Analog Brix/Sucrose Refractometer, Fisher Scientific), respectively. Thawed frozen strawberries were ~8 °Brix. The pH of thawed frozen strawberries was 3.65 ± 0.03. The water activity of thawed frozen strawberries measured 0.972, using a water activity meter (Rotronic Instrument Corp., Hauppauge, NY).

Microbiology

MS2 (ATTC 15597-B1) and *E. coli* C3000 (ATCC 15597) were obtained from American Type Culture Collection (Manassas, VA). MS2 was propagated using *E. coli* C3000 as a host on tryptic soy agar (TSA) plates using the double agar overlay method (Difco, Becton Dickinson, Sparks, MD) *(18)*. MS2 stocks were prepared on the cell lawns of *E. coli* C3000 by a method described by the U.S. Environmental Protection Agency method 1601 *(8)*. Briefly, 0.1 ml of overnight *E. coli* C3000 culture was added to 5 ml of molten TSB soft agar along with 0.1 ml of MS2 (~11 log PFU/ml). The inoculated culture was spread on the surface of a TSA plate and incubated overnight at 37°C. The MS2 phage was recovered by scraping the top of the plate (soft agar layer) and placing it in TSB. The MS2 phage in TSB was separated from the host cells by centrifugation (5,000 x g, 4°C for 25 min), followed by filtration of the supernatant through a 0.45-μm pore-size filter (Fisher) and stored at 4°C prior to use.

Inoculation procedures

Whole frozen strawberries (3 or 5) were removed from the freezer and placed in a sterile plastic dish and one ml of the virus stock solution was spot inoculated on the 3 or 5 strawberries, to obtain a concentration of approximately 10 log PFU/g of strawberry. The inoculated frozen berries were placed at the center of the microwave carousel and heated for the selected times (15s - 300s) and power levels (30 - 100%) as shown in Table 1. Product temperatures were initially determined by placing two fiber optic probes at the berry surface and berry center.

Microwave oven

A Panasonic Model NNS760WA with 1300W of nominal power (Panasonic North America, Secaucus, New Jersey) was used in this study. The FISOCommander OSR System ® (FISO Technologies, Quebec, Canada) including fiber optic sensors, and the FISOCommander Microwave Workstation Edition ® (Fiso Technologies, Quebec, Canada) software were used to monitor the temperature of the frozen strawberry throughout microwave heating. Probes were placed to record surface temperatures since it was the surface of the frozen whole strawberries that was inoculated with MS2. Surface temperatures were recorded until frozen berry thawed such that the fiber optic probe was dislodged due to melting of the berries. Probes were also used to record internal berry temperature.

Heating instructions specified on the packaged, whole frozen strawberries stated: "Place desired amount of frozen fruit into a 1.5 quart microwave-safe dish and cover. Defrost on defrost setting 1 to 2 minutes, or until thawed. Do not thaw completely." These heating instructions, as well as those collected from a variety of local supermarkets (Table 2), were used as a baseline for time and temperature conditions to validate.

Survival of bacteriophage MS2 after microwave heating

Samples were transferred to a sterile filter bag with 100 ml phosphate buffered saline (PBS) immediately after microwaving, and then homogenized, serially diluted and plated on an *E. coli* lawn on TSA to enumerate PFU. Data were recorded until the detection limit (2 log PFU/g) was reached. Frozen strawberries inoculated with 1 ml virus stock solution and held at room temperature were a positive control and used to calculate the concentration before microwaving. Uninoculated, unheated frozen strawberries were a negative control to ensure MS2 was not present on uninoculated berries.

## 6.4 Integrated Lethality Analysis

The *z* (10.1°C) and D-values ($D_{55}$ = 1.52 min) from the water bath study using fruit purées and the microwave berry surface temperatures were used to estimate integrated lethalities from microwaving.

## 6.5 Results

Microwave heating profiles

The heating profiles seen in Figure 1 are representative of the surface temperatures recorded. The temperature increased quicker when 3 berries were heated vs. 5 berries, regardless of microwave power level. The initial surface temperatures of whole frozen strawberries prior to heat treatment was -2.3 ± 1.9°C. Inoculated berry surface temperature was higher than freezer temperature (-20°C), since the MS2 stock was at 4°C before inoculation.

Survival of bacteriophage MS2 after microwave heating

Uninoculated, unheated frozen strawberries showed no detectable MS2 as expected. Frozen strawberries inoculated with 1 ml virus stock solution and held at room temperature was used to calculate virus concentration prior to microwaving, and was 8.9 ± 0.5 PFU/g berry. The results across all power levels (30-100%) showed greater log reduction at increased power levels, and when 3 berries were heated versus 5 berries (Fig 2). When 3 and 5 berries were heated at 30% power level for 240 s (4 min), log reductions of 5.2 ± 0.3 and 4.1 ± 0.1 PFU/g were observed, respectively. When 3 and 5 berries were heated at 50% power level for 180 s (3 min), log reductions of 5.2 ± 0.1 and 5.1 ± 0.4 PFU/g were observed, respectively. At 70% power level for 105 s (1.75 min), log reductions of 5.4 ± 0.3 and 5.1 ± 0.1 PFU/g were observed for 3 and 5 strawberries,

respectively. Lastly, when 3 and 5 strawberries were heated at the highest microwave power level, 100%, for 90 s (1.5 min), log reductions of 5.7 ± 0.5 and 4.7 ± 0.4 PFU/g were observed, respectively.

## 6.6 Discussion

The heating instructions on frozen strawberry packages we sampled suggest a serving size of ~1 cup (Table 2). It is important to take into consideration the number of berries consumers may be microwaving at one time. Vilayannur et al. (1998) reported that the volume of the product affects microwave heating, with higher volumes resulting in longer heating times compared to smaller volume products to reach target temperatures *(29)*, and these finding are consistent with ours which show slower heating of five berries compared to three berries.

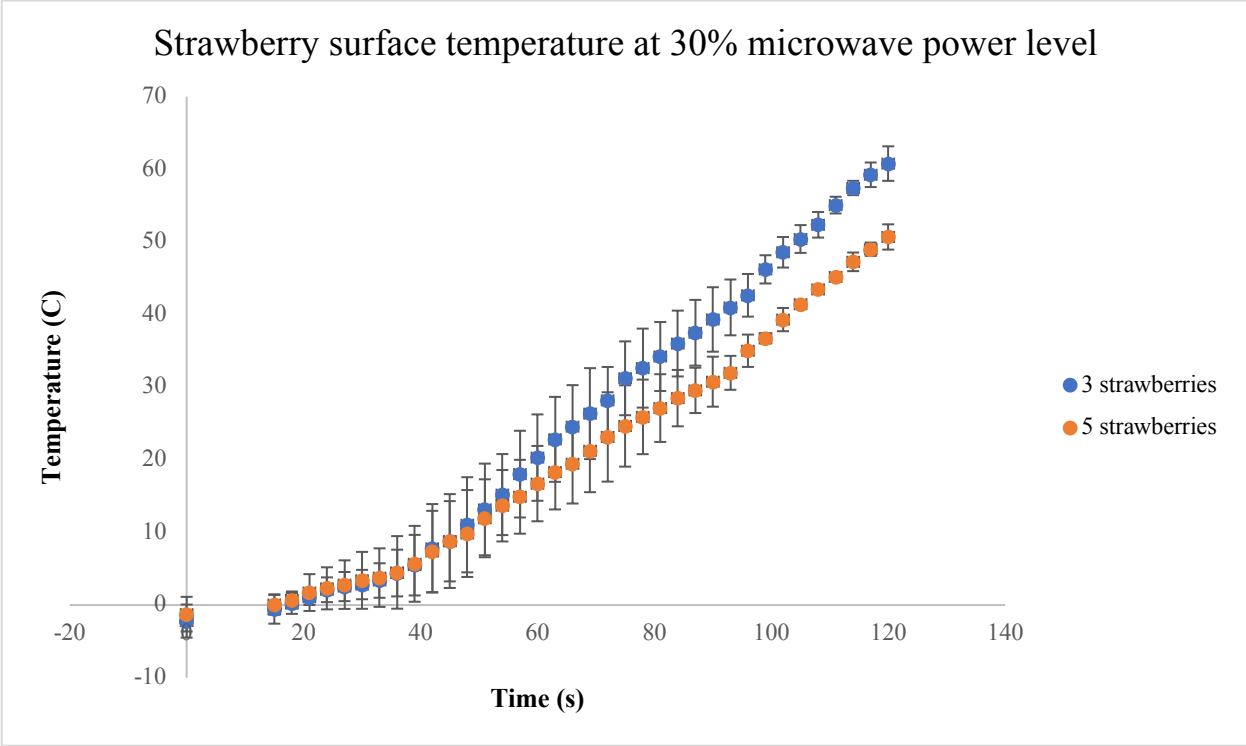
Majority of microwave heating instructions for frozen strawberries advise using low power or the defrost setting and range from 60 s (1 min) to 300 s (5 min) depending on the brand and amount of strawberries being heated (Table 2). These heating instructions are typically intended to produce thawed fruit of acceptable quality. The increase in outbreaks and recalls linked to NoV and HAV on frozen fruit show it may also be necessary to validate these heating instructions for food safety. Published studies on the effect of microwave heating to inactivate pathogens on foods has typically been conducted using a variety of pathogenic bacteria including *Salmonella* spp., *Listeria* spp. and *E. coli* O157:H7 *(1, 14-17, 21, 22, 25, 26, 30-32)*. Published results have shown both log-linear and non-log-linear inactivation patterns. Pucciarelli et al. (2005) investigated the inactivation of *Salmonella* Enteritidis on microwaved raw poultry starting at a temperature of 25°C and concluded that bacteria are inactivated log-linearly with

microwave heating time *(22)*. Another study using *E. coli* (ATCC 11775) in peptone water starting at 50°C found that microwave heating under a vacuum resulted in log-linear inactivation with microwave heating time *(31)*. A study looking the inactivation of *Salmonella* Typhimurium in salsa observed a non-log linear reduction with microwave heating time, where reduction rate increased dramatically with heating time *(26)*.

The information on microwave inactivation of viruses on foods is very limited. A study by Misbu et al. (1990) suggested that HAV can be inactivated by microwaving, following an outbreak of HAV in sandwiches where those ate sandwiches were not microwaved had nine times the chance of acquiring HAV as those that ate microwaved sandwiches *(19)*. Another study found that microwaving reduced PV to below detection levels in infant milk *(17)*. Many thermal inactivation studies have been conducted on NoV surrogates, including MNV, FCV and HAV in food products including blue mussels, turkey deli meat and spinach *(3, 4, 10, 11)*, but none of these studies investigated inactivation kinetics in microwave ovens.

Recent research has shown the complexities associated with modeling the thermal profile of even very simple matrices which approximate food during microwave heating *(6)*. Our research reveals some of these complexities, and it is not surprising that the theoretical integrated lethalities we calculate using water bath derive D- and *z*-values for berry purée and limited thermal data from the surface of microwave berries do not match the experimentally measured MS2 survival during microwave heating. Until these complexities are understood and overcome, validation of microwave cooking directions will have to be done empirically on an experiment by experiment basis as we have done here.

Figure 6.1: Microwave heating profiles



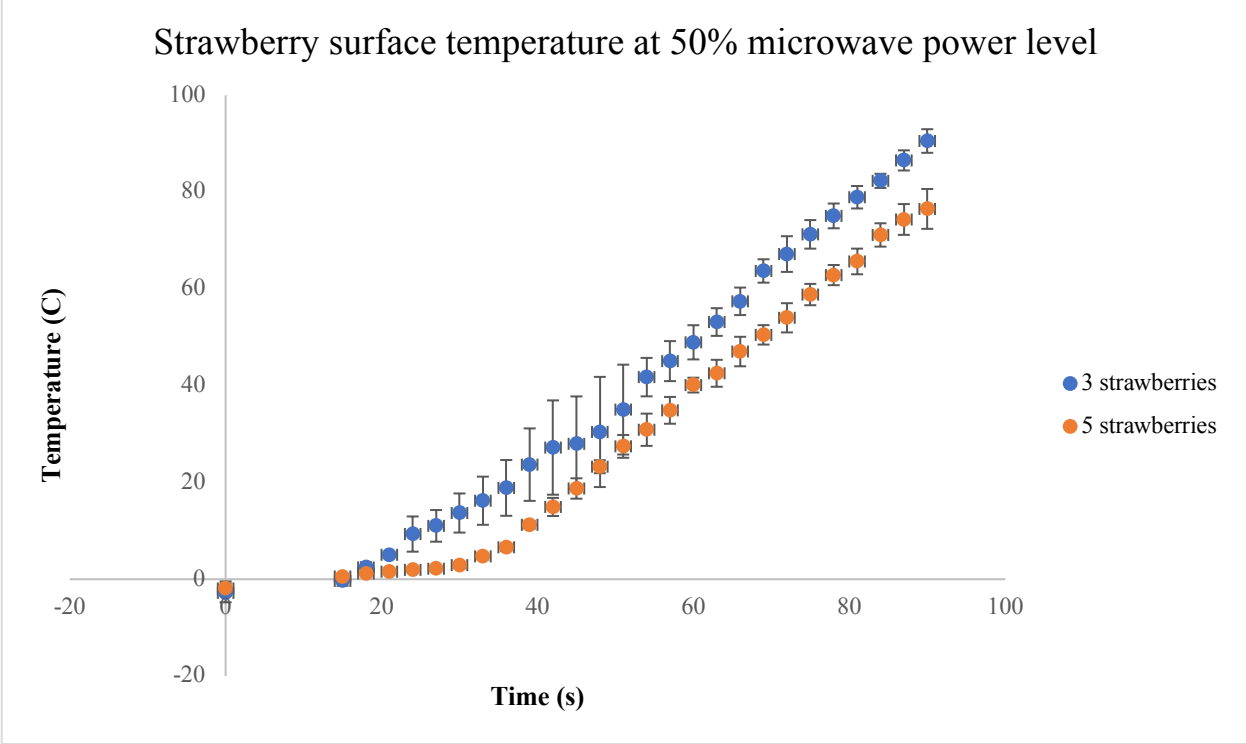
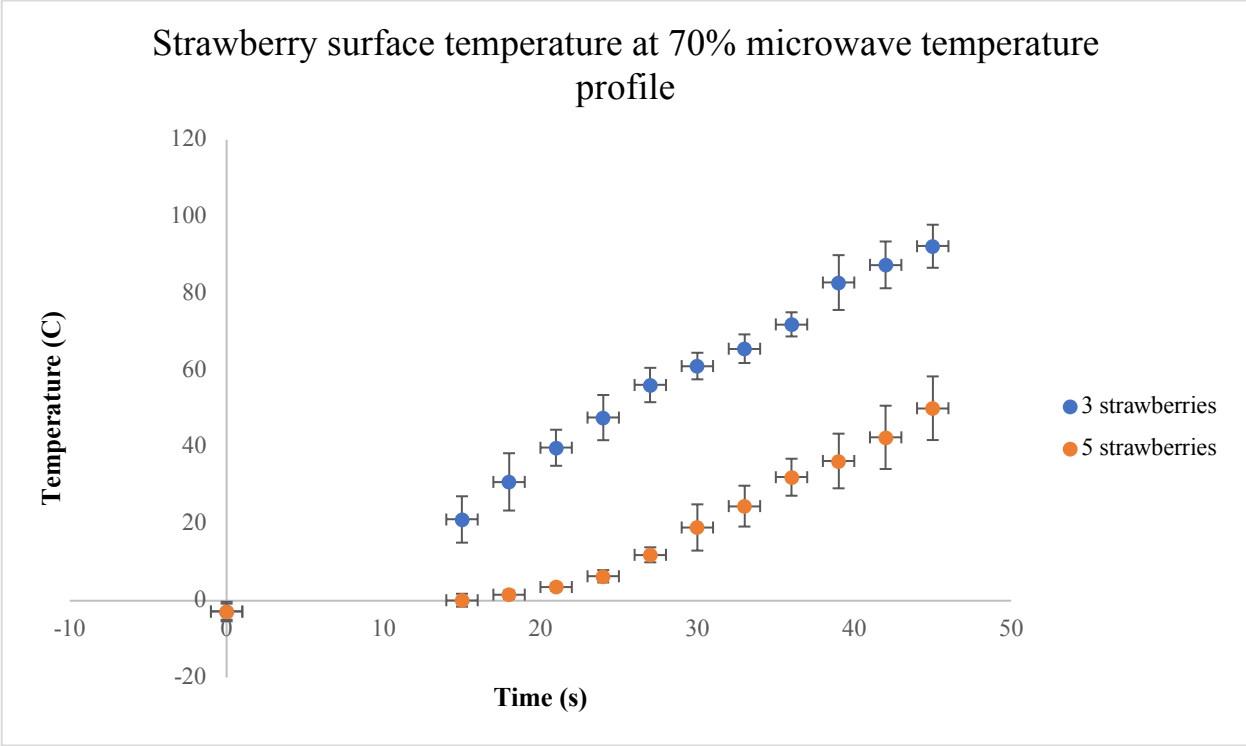



Figure 6.1: Microwave heating profiles



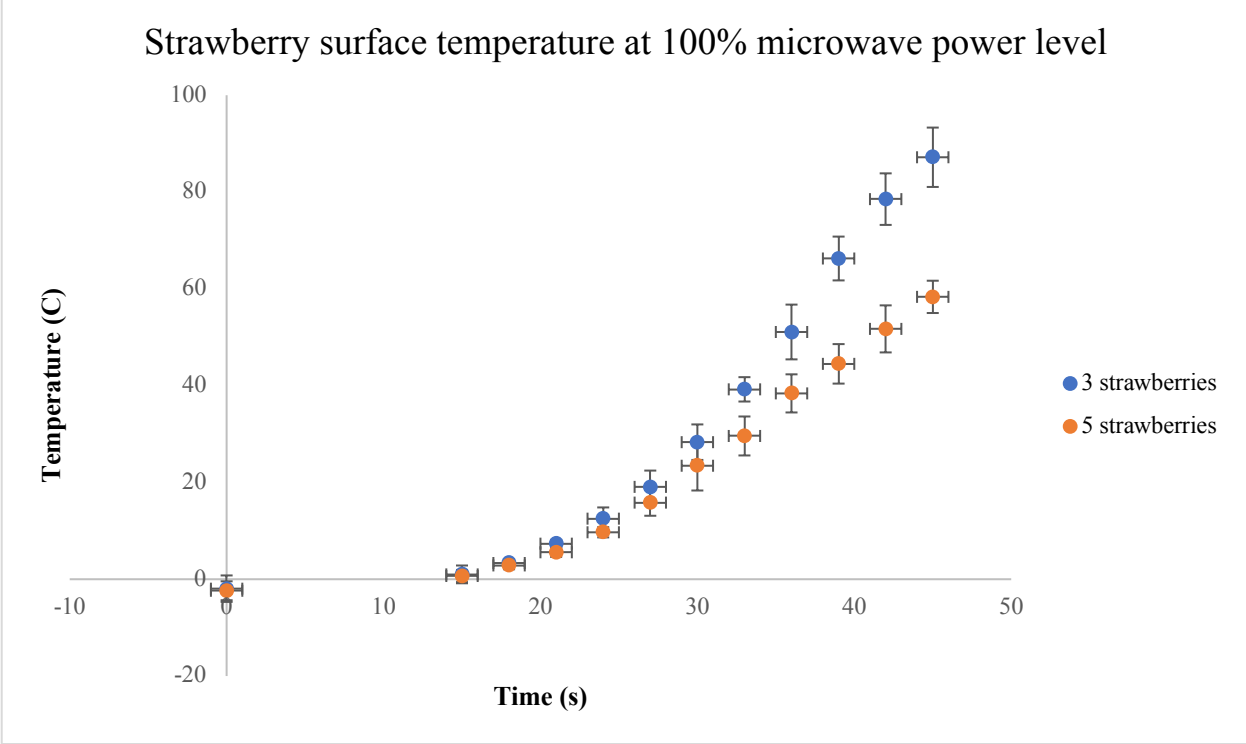
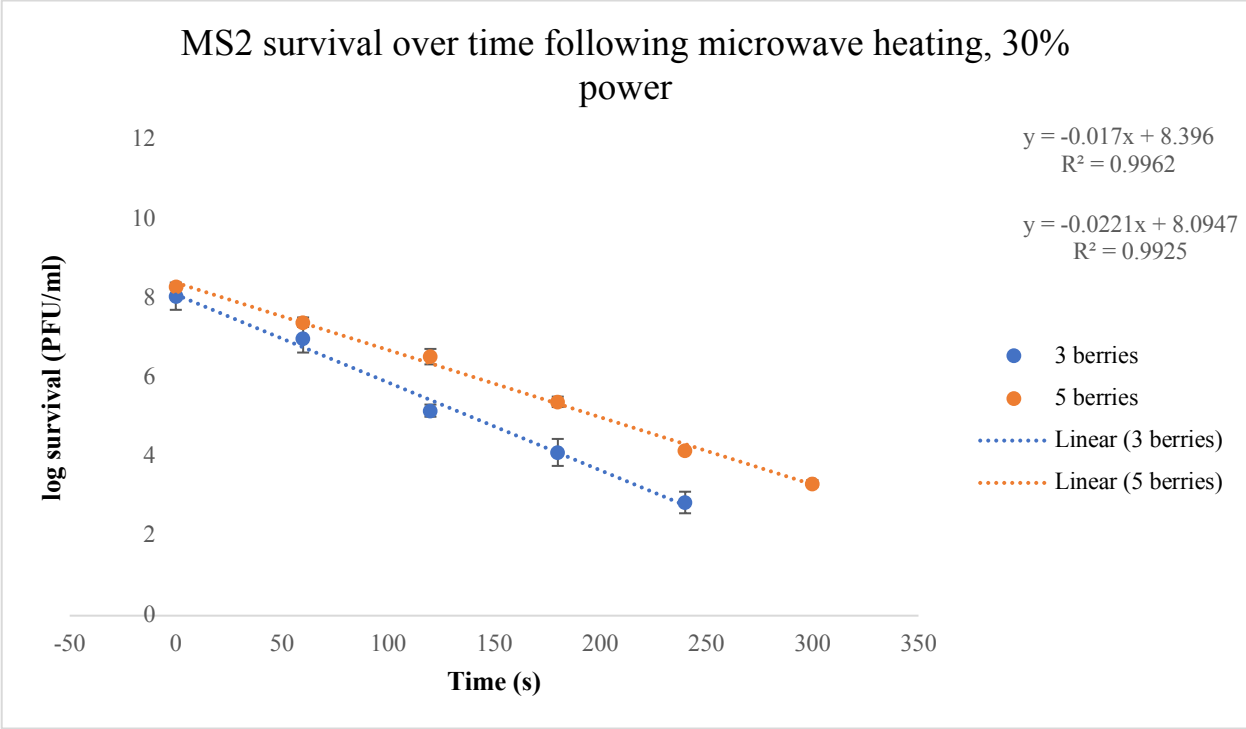



Figure 6.2: Microwave inactivation of bacteriophage MS2 on frozen, whole strawberries



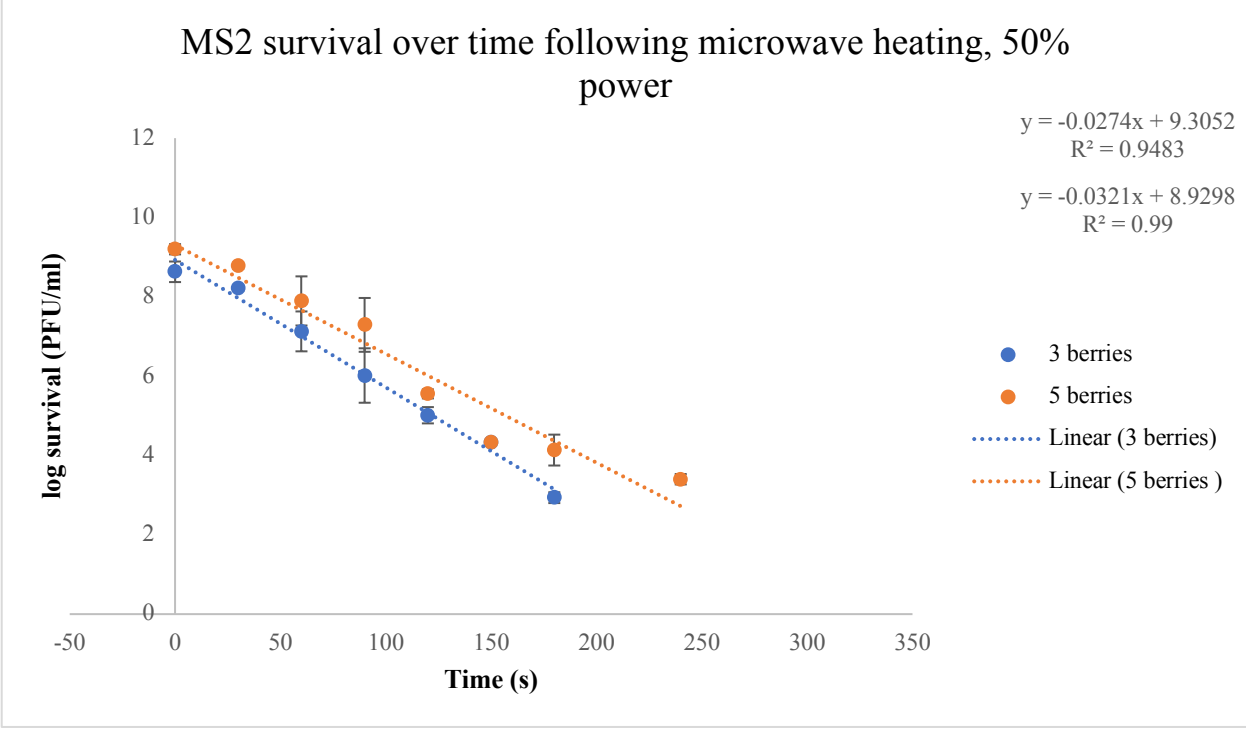
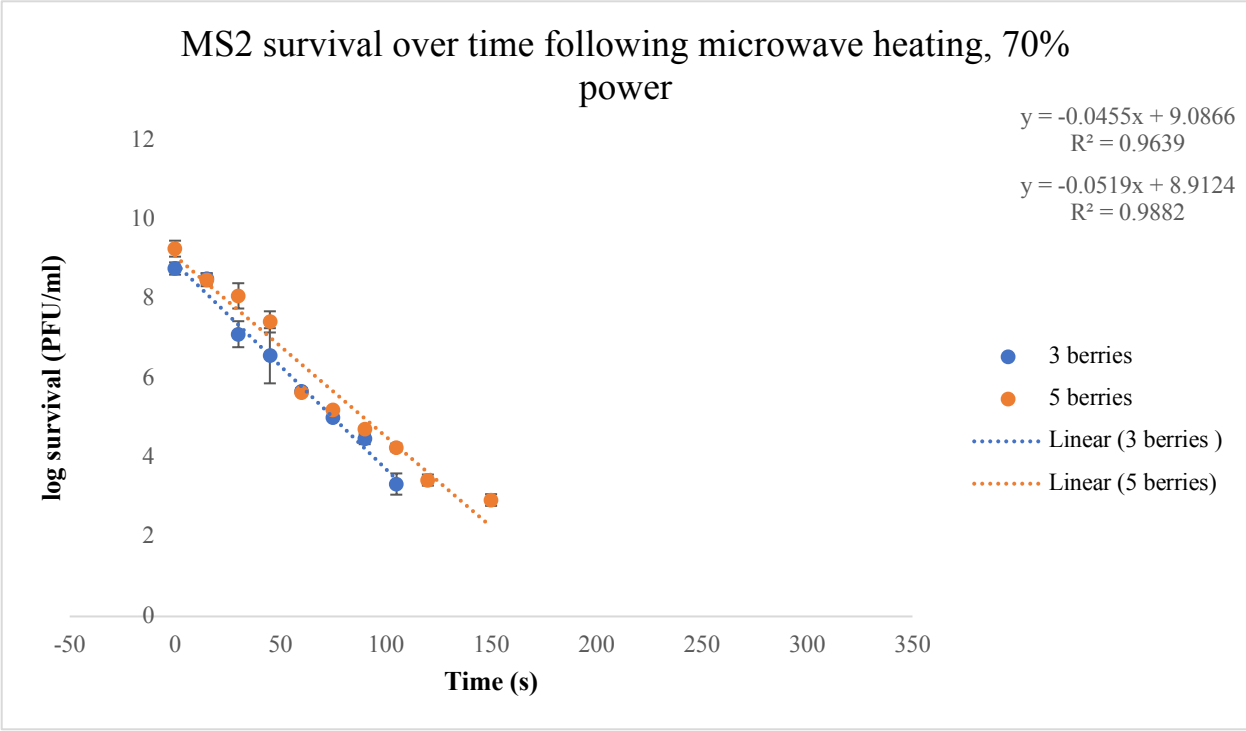



Figure 6.2: Microwave inactivation of bacteriophage MS2 on frozen, whole strawberries



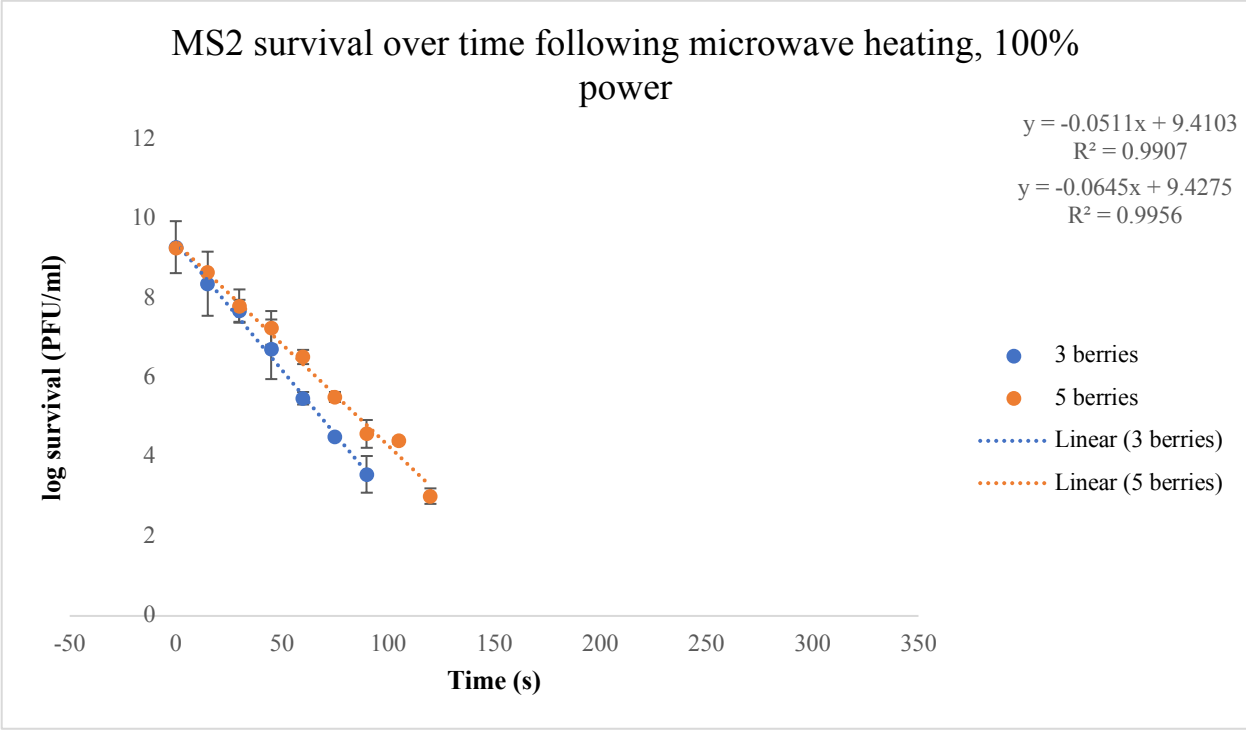



Table 6.1: Experimental microwave heating parameters

| | **Microwave Power Level (%)** | | | |
|---|---|---|---|---|
| | **30** | **50** | **70** | **100** |
| Heating Time (s) | 60 | 30 | 15 | 15 |
| | 120 | 60 | 30 | 30 |
| | 180 | 90 | 45 | 45 |
| | 240 | 120 | 60 | 60 |
| | 300 | 150 | 75 | 75 |
| | | 180 | 90 | 90 |
| | | 240 | 105 | 105 |
| | | | 120 | 120 |
| | | | 150 | |

Table 6.2: Microwave package instructions for heating whole frozen strawberries

| Brand | Power level | Time | Serving Size (1 cup = 140g) | Heating Instructions |
|---|---|---|---|---|
| Great Value | 30% power | 1 min | 1 cup | "Thaw desired amount at room temperature for approximately 30 minutes, or thaw in microwave on defrost setting (30% power) for 1 minute. " |
| Nature's Promise | Defrost | 1-2 min | 1 cup | -* |
| Cascadian Farm | Defrost | 4 min | 1.25 cup | "Do not microwave in bag. For a 1200 watt microwave, heat quarter bag for 2 minutes on defrost or half bag for 6 minutes on defrost. For a 1000 watt microwave, heat quarter bag for 4 minutes on defrost or half bag for 8 minutes on defrost. For a 700 watt microwave, heat quarter bag for 6 minutes on defrost or half bag for 10 minutes on defrost. Microwave wattages vary; adjust defrost time for the wattage of your microwave. Do not leave microwave unattended." |
| Dole | Defrost | 1 min | 1 cup | "…place in microwave dish and thaw on Defrost setting for 1 minute. Serve slightly frozen. Do not refreeze." |
| Stop & Shop | Defrost | 1-2 min | 1 cup | "Place desired amount of frozen fruit into a 1-1/2 quart microwave-safe dish and cover. Defrost on defrost setting 1 to 2 minutes, or until thawed. Do not thaw completely." |
| Shoprite | Defrost or low | 2-3min | 1 cup | -* |
| Wegmans | Defrost (30% power) | 2-5min | 1 cup | "Microwave (1100 Watt): Place ½ package or less of fruit in microwave safe dish and cover. Set to defrost (30% power) 2-5 minutes, stirring halfway through or until desired softness." |
| Woodstock | Defrost | 1 min | 1 cup | "…thaw in a microwave on defrost setting for 1 minute. Serve while there are a few ice crystals on the fruit for a firmer texture." |

* no other directions provided

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# Chapter 7: Farm to Fork Quantitative Microbial Risk Assessment for Norovirus on Frozen Strawberries

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## 7.1 Abstract

Foodborne illness outbreaks have been increasingly linked to the consumption of fresh and frozen berries that were contaminated with pathogenic viruses, such as human norovirus (NoV). Contamination of berries is assumed to take place at harvest by the use of contaminated water for pesticide dilution, irrigation water source or by shedding berry pickers in the field. A quantitative microbial risk assessment simulation model was built to replicate the largest known NoV outbreak which sickened about 11,000 people over a 3-week period. The outbreak occurred in Germany in 2012 when contaminated frozen strawberries were served at nearly 400 schools and daycare centers. The risk model explicitly assumed that all contamination would arise from NoV contamination of surface water used for pesticide dilution. Input data was collected from the published literature, observational studies and assumptions. The model starts with contamination of the berries in the field, and proceeds through transportation to processing facility, washing, sanitizing, freezing, frozen transport to cargo ship, transport view of cargo ship, transport to distribution center, frozen storage at the distribution center, transport to the catering facility, food service preparation and consumption, dose response, and predicted illnesses. A total of 21 scenarios were chosen to evaluate the impact of model parameters on the number of illness associated with NoV contamination of berries. Scenarios evaluated include the initial level of NoV in surface water, the effect of seasonality on the prevalence of NoV in surface water, the strength of the pesticide used, the volume of water used to dilute the pesticide, temperature during transportation to processing facility, washing and sanitizing conditions at processing facility and preparation (heat-treatment) of berries prior to consumption. Scenarios were compared via the Factor Sensitivity

technique where the logarithm of the ratio of mean illnesses was used to compare different assumptions. The input that had the greatest effect on increasing in the number of illnesses was a high NoV concentration in the water (8 log Genome Copies/L) when compared to the baseline scenario with resulting mean illnesses of 7,964 illnesses and ~2 illnesses, respectively. This assumption about the concentration of virus in the pesticide makeup water was the only variable capable of producing an outbreak similar to that observed in Germany in 2012. Heat-treatment of the berries, use of a pesticide with strong antiviral effect, and assumption about the virus concentration in the pesticide make-up water had the largest impact on decreasing illnesses.

## 7.2 Introduction

Norovirus (NoV) is the leading cause of foodborne disease worldwide, causing an estimated 685 million cases of acute gastroenteritis annually *(31, 37, 54)*. Although most deaths occur in developing countries, NoV continues to be a significant burden to high-, middle- and low-income countries *(11)*. NoV are transmitted primarily from person-to-person via the fecal-oral route or from aerosolized vomit. The virus may also be transmitted indirectly through contaminated food, water, fomites and environmental surfaces.

The average incubation period for NoV-associated gastroenteritis is 12 to 48 hours and is typically followed by symptoms including nausea, vomiting and diarrhea with abdominal cramps. The average probability of infection for a single NoV particle was estimated to be near 50% (0.5), exceeding any other virus studied thus far *(61)*. Viral load from an infected person has been shown to range from $10^8$ to $10^{12}$ viral particles per gram of feces *(5, 6, 61)*. Shedding of NoV can start in the pre-symptomatic phase as early as 3 to 14 hours before onset and those who are infected with NoV can continue to shed it in their feces for several months after initial infection *(1, 6, 47)*. NoV stability in the environment is thought to be due to its lack of a viral envelope; it can survive freezing and heating, can survive for weeks on surfaces and is resistant to many common chemical disinfectants that are effective for bacteria *(25, 36)*.

The market for frozen berries has continued to succeed because of the availability to consume the product year-round *(4)*, even though fresh and frozen berries have been linked to NoV and Hepatitis A virus (HAV) foodborne disease outbreaks around the world *(12, 26, 34, 39, 43, 55, 57, 60)*.

The risk factors for contamination of berry fruits at primary production with NoV are not well documented in the published literature due to limited data. Suggested risk factors based on what is known for other pathogens associated with fresh produce include (1) environmental factors such as heavy rainfall that increase the transfer of NoV from sewage runoff to irrigation water sources or fields (2) use of sewage-contaminated agricultural water as irrigation water or for the application of agricultural chemicals such as pesticides and (3) poor food handlers health and hygiene or contaminated equipment at harvest or post-harvest *(42, 60)*.

Temperature is considered a major factor influencing virus persistence, although it is not considered an effective mitigation strategy for fresh berries because persistence of enteric viruses is higher at low temperatures and quality loss (e.g. decay) generally increases with an increase in temperature *(53)*. Some berries undergo washing (strawberries, blueberries) prior to freezing, while more fragile berries (e.g. raspberries and blackberries) may not get washed as it can lower product quality. The presence of NoV in frozen berries has been linked to many outbreaks of gastroenteritis throughout the world, which clearly shows these viruses survive and remain infectious after freezing *(12, 33, 39, 43, 55)*.

Quantitative microbial risk assessment (QMRA) is used to better understand and manage food safety risks. Models are developed to describe the transmission of pathogens over a specified food production chain. These models may cover the complete farm to fork pathway or only a portion of it. De Keuckelaere et al. (2015) analyzed published risk assessments that studied viruses, fresh produce, irrigation and wash water from food safety and water management perspectives *(19)*. Several studies have

presented quantitative risk assessments showing the impact of contaminated water on the spread of NoV on leafy greens and other crops consumed raw *(10, 40, 45)*. Other risk assessments and exposure assessments have focused on the spread of NoV by ill food handlers, highlighting the importance of hand hygiene measures in foodservice facilities *(21, 23, 28, 44, 46)*. A quantitative farm-to-fork exposure model was developed describing the spread of NoV and Hepatitis A during the harvesting and processing of leafy greens and berry fruits *(15)*.

Here we consider the source of the contamination, NoV inactivation and survival on berries, as well as processing at the facilities and preparation of the berries prior to consumption. Our QMRA is designed to simulate the largest known outbreak arising from NoV-contaminated berries, which occurred in 2012 in Germany and was linked to frozen strawberries sourced from China.

## 7.3 Materials and methods

Overview of the development of the risk model.

Data from the peer reviewed literature regarding NoV behavior in fresh and frozen fruit were used to develop the model. The model parameters and their corresponding probability distributions are described in Table 1. Inputs were assumed to be independent, although some inputs may have dependencies (e.g. strength of pesticide diluted in a specific volume of water). The risk model assumes that contamination of strawberries strictly arises from NoV contamination in the surface water. Other sources of contamination will be explored in subsequent research.

Contamination source.

Berries are susceptible to contamination with NoV through spraying with pesticides mixed with contaminated water. Sources of water used for agriculture applications can be ranked by risk of microbiological contamination and are in order of increasing risk: rain water, ground water from wells, surface water, and raw or inadequately treated wastewater *(35)*. The main sources of NoV in surface and groundwater are sewage discharge and human fecal waste. Pesticides are often diluted in different volumes of water depending on the crop. Although NoV does not replicate in water, it can remain infectious in water for prolonged periods of time. Seitz et al. (2011) found through human challenge studies that NoV remained infectious in water for at least 61 days *(56)*.

We assumed the use of drip irrigation in our model. Drip irrigation itself is an unlikely point of microbial contamination because water is applied to the soil or directly at the roots of the plant, far from the edible fruit. Drip irrigation is a preferred method for berries since berries are particularly susceptible to mold growth which is likely to occur if overhead irrigation is used *(28)*. Limited information on NoV adherence to and persistence on strawberries exists, and therefore were not considered in this model.

Our model assumes that all of the pesticide applied adheres to the edible fruit, which implied no run-off (or pesticide drift). Pesticide drift occurs in many crops *(22)*, including strawberries, and is complex and multi-faceted *(14)*. Given the complexity of modeling this aspect of agricultural production, we have chosen the simplifying assumption above.

Our model also assumes that contaminated the water is applied immediately before harvest. Many pesticides have a prescribed pre-harvest interval, which specifies

the length of time after application that is required prior to harvest. During this pre-harvest interval any virus particles present on the berries would be subject to environmental stresses, including exposure to sunlight and drying, and thus would lose viability. Unfortunately, we have no knowledge of the pre-harvest interval period between pesticide treatment and picking of strawberries in China. Information on pre-harvest interval for strawberries in the US *(38)* shows that pre-harvest intervals of 0 to 1 days are quite common for many of the pesticides used on strawberries. Given the lack of the published data on pre-harvest intervals in China, common short pre-harvest intervals in the US, and minimal declines observed over these short intervals, we have chosen to make the simplifying assumption that no reduction in virus population occurs pre-harvest.

Effects of washing and chlorine application.

Data from Butot et al. (2008) and Predmore et al. (2011) on the effect of washing and sanitizing berries prior to freezing were extracted from the scientific literature and analyzed for inclusion in the model *(17, 51)*. Wash water as a source of contamination was not considered in this model. Some berries (e.g. strawberries) are washed with water before freezing, but more fragile fruits (e.g. raspberries) are not *(59)*. Washing fruits or vegetables with water alone generally yields no more than a 2-log reduction in microbial concentration *(13)*. Excessive chlorine concentrations must be avoided as they can affect sensory quality *(49)*. It has been shown that prolonged treatment of berries with chlorinated water did not result in a significant increase in the effectiveness, although various surrogates have been shown to be affected differently *(20, 24, 48)*.

Time and temperature during transportation and storage.

Strawberries were assumed to be transported on a refrigerated truck after harvest to a processing facility. The baseline simulation used 4 °C as the temperature for transporting strawberries to the processing facility, as literature data showed that the fruit quality is not adversely affected at this temperature *(58)*. Data on NoV survival and inactivation at various storage temperatures was used to determine the concentration of NoV on strawberries over time *(18, 29, 32)*. The simulation assumed that once the strawberries arrived at the processing facility, they were exposed to washing and sanitizing steps, followed by individually quick freezing (IQF). The frozen strawberries were transported by cargo ship, assuming a transportation time of 25-30 days from the port in China to the port in Germany *(3)*.

Process of freezing berries.

Although all processing steps are important in maintaining the quality of berries, the freezing process in the most critical. The primary goal in freezing fruit is to maintain the original characteristic product quality. This is best achieved by freezing rapidly and careful handling before and after freezing. If freezing is slow, large ice crystals will form and can break down food structures. This results in high drip losses and a deterioration in product quality. Several factors that affect freezing rates include the type of freezing equipment used, initial berry temperature and product characteristics (e.g. size, shape and structure). Individually quick freezing (IQF) is one of the quickest ways of freezing small fruits. Advantages of the IQF process include short freezing times, efficient heat transfer and less product dehydration *(27)*. Freezing has no significant effect on the infectivity of NoV, and virus particles appear to retain their structural and genome integrity after

freezing and during multiple freeze-thaw cycles *(52)*. Data from Butot et al. (2008) were used to determine the log reduction of NoV during frozen storage *(17)*.

Preparation at catering facility and consumption.

The serving size was selected after an internet search of similar recipes and it was decided that ~4 strawberries per serving of strawberry compote was an appropriate serving size. While we could have used a more complex assumption regarding serving size, since our specific objective was to simulate the 2012 German outbreak (rather than for example all domestically consumed frozen strawberries in the United States), this simplified assumption suits our purpose. Should a future risk assessment need to address more complex scenarios, food consumption databases could be used to estimate variable serving sizes.

The baseline model assumed that strawberries were not heated prior to consumption. Different heat treatment scenarios were considered based on data available in the literature. The effect of mild heat treatment (30s at 65°C) was simulated with a normal distribution with mean log reduction and standard deviation of 1.86 ± 0.32 *(9)*. High heat treatment (15s at 75°C) resulted in a mean log reduction and standard deviation of 2.81 ± 0.39 *(8, 9)*. NoV inactivation data was based on NoV surrogates including feline calicivirus F9 (FCV) and the murine norovirus 1 (MNV-1).

Dose-response modeling.

Dose-response models mathematically link exposure to probability of infection and/or illness, where exposure represents the dose ingested *(50)*. Illness (i.e. symptoms of vomiting and/or diarrhea) is the endpoint of this risk assessment. An existing dose response model for the probability of illness among infected subjects was used with

parameters η and r as given by Teunis et al. (2008) *(61)*. The risk of illness is considering the dose as the sum of dose from GI and GII and the parameters, η and r, were assumed to be independent of the NoV genogroup (GI or GII), as well as secretor status *(50, 62)*. The values for η and r in this model are 2.55 x $10^{-3}$ and 0.086, respectively Equation 1:

$$P(\text{ill/dose}, \eta, r, \text{inf}) = 1 - (1 + \eta \times \text{dose})^{-r} \qquad (1)$$

Simulation modeling.

Extracted data and user inputs were entered into an Excel (Microsoft, Redmond, WA) spreadsheet as described in Table 1, discussed in detail in the results and discussion section below. The Excel add-in @Risk (Palisade Corporation) was used to perform Monte Carlo simulations of 100,000 iterations for each scenario evaluated. Scenarios were constructed to reflect the best estimates of the number of servings (~100,000) believed to be involved in the outbreak. The baseline simulation condition is shown in detail in Table 1, but briefly: The concentration of NoV in water was modeled as a uniform distribution from 1.27 to 4.84 log genome copies (GC)/L. The volume of liquid used to apply pesticides was 200 L/ha. The seasonality for prevalence of NoV in surface water was represented by a triangular distribution assuming a minimum of 12%, a most likely value of 12% and a maximum value of 95% per L. The effect of pesticide on reduction in NoV concentration was described by a lognormal distribution with mean 0.35 and standard deviation of 0.56 log GC/L where the resulting is shifted by -0.21. The truck temperature for transport from the field to the freezing location was assumed to by 4 °C, with no change in NoV concentration at that temperature. We assumed that the strawberries were washed in cool (18 °C) water resulting in a reduction of NoV concentration following a normal distribution (mean 0.67, standard deviation 0.33) log

GC NoV. We also assumed the strawberries were sanitized using 200 ppm chlorine, resulting in a reduction of NoV concentration following a normal distribution (mean 1.35, standard deviation 0.24) log GC NoV. The baseline assumed no heating step during foodservice preparation resulting in no change in NoV concentration prior to consumption.

Sensitivity analysis.

A total of 21 scenarios were chosen to evaluate the impact of model parameters on the number of illness associated with NoV contamination of strawberries, and these are shown in Table 2. Scenarios evaluated include the initial level of NoV in surface water, the effect of seasonality on the prevalence of NoV in surface water, the strength of the pesticide used, the volume of water used to dilute the pesticide, temperature during transportation to processing facility, washing and sanitizing conditions at processing facility and preparation (heat-treatment) of strawberries prior to consumption. Each scenario was selected to explore variations around each of the 8 parameter baselines to test the individual impact of a given parameter on the change in the number of illnesses. Equation 2, adapted from Zwietering et al. (2000), represents the scenario factor sensitivity (FS), which is the order of magnitude of the importance of each scenario relative to the baseline *(64)*. High factor sensitivity values equate to a high sensitivity to the variations and reveal what factors have greater effects on the number of illnesses (N).

$$FS_k = \log \frac{N_k(variation)}{N_k(baseline)} \qquad (2)$$

## 7.4 Results and Discussion

As noted above, Table 1 summarizes the Excel spreadsheet used for risk calculations and explains how the variables are linked in the risk assessment. The first

column represents the spreadsheet cell designation for the variable on that line of the table. The next column is a description of the variable in words, with bold headers describing each section of the risk assessment. The third column is either a number, formula or an @Risk formula representing the value of a given cell. The fourth column shows the units of the variable in the third column. The last column represents the source of the information used to determine the value of the variable. The source can be either user input, from the published literature or calculated from other cells in the spreadsheet.

The first section of Table 1 (In field) represents variables describing the environmental factors that influence NoV contamination on strawberries in the field. Information involving conditions strawberries were exposed to during the outbreak in Germany from strawberries harvested in China is limited *(39)*. Because much of this information is unknown, important variables were included from the published literature or as user input. Key in-field variables include starting concentration and prevalence of NoV in surface water, fraction of positive water liters used for pesticide delivery and the ability of pesticides to reduce NoV concentration in the pesticide water. The NoV concentration in water is expressed as a uniform distribution *(30)* and the prevalence of NoV in the water is expressed as a triangular distribution based on the published literature *(7)*. Surface water (river water, lake water, canal water, etc.) is typically used for diluting the pesticide that will be sprayed onto strawberry plants *(16, 28, 41)*. The effect of pesticides on the reduction in NoV from water used to dilute pesticides is expressed as a log normal distribution based on data extracted from Verhaelen et al. (2013) *(30)*. Various pesticide strengths were evaluated in the sensitivity analysis. A binomial distribution was used to determine how many liters were positive which was

used to calculate the effective concentration per liter, considering positive and negative liters. The level of NoV on the strawberries at harvest was determined by calculation of the effective concentration per liter, considering positive and negative liters multiplied by the volume of water sprayed on strawberries.

The next section (Transportation to processing facility) presents data extracted from Kurdziel et al. (2001), Verhaelen et al. (2012) and Dawson et al. (2005) to estimate the effect of temperature on the persistence and survival of NoV during transportation to the facility. Relevant data extracted include mean log reduction and the standard deviation of the log reduction at 3 different temperatures (4, 10 and 21°C). Refrigeration temperature (4°C) was used as the baseline with no log reduction of NoV observed *(18, 32, 63)*.

The simulation assumes strawberries were washed and sanitized after receipt at the processing plant. Data were extracted from the peer reviewed literature *(17)* to estimate the degree to which washing reduces NoV contamination on strawberries (Table 1, Washing log reduction). Although the primary purpose of the washing step is to remove dirt and debris rather than achieve a microbial reduction, reductions in NoV concentration has been shown when berries were washed with warm or cold water *(17)*. The simulation assumed that sanitizer was applied to the strawberries after washing. The baseline used for spray sanitizer data on berries was a 200 ppm chlorine solution (Table 1, Sanitizer log reduction). Chlorine concentrations for produce and wash water are generally ≤ 200 ppm *(13)*. The variables in this section, as well as the washing section, express the variability in log reduction by using the RiskNormal function using the mean log reduction and standard deviation from the published literature *(17)*. Scenarios with

varying sanitizers (5ppm $ClO_2$ and 10ppm $ClO_2$) using the RiskNormal function, as well as no application of sanitizers, were considered (Table 2). The washed and sanitized strawberries undergo the IQF method (Table 1, Freezing process). Although commercial IQF is generally thought to cause little change in the concentration of microorganisms, no peer reviewed data on survival of NoV during the IQF process was found. A single non-food related study that examined the effect of freeze-thaw cycles on NoV titers found that both capsid integrity and viral RNA titers remained stable through repeated freeze/thaw cycles *(52)*, so we assumed that freezing had no effect on NoV concentration.

The next three sections of Table 1 (Truck to cargo ship, Transport via cargo ship and Transport to distribution center) model the expected change in NoV level on strawberries during these three phases of frozen storage. Data for log reduction after 90 days frozen storage was extracted and calculated from Butot et al. (2008) using a normal distribution (mean 0.4, standard deviation 0.18), and this was adjusted to estimate the log reduction per day. A uniform distribution was used to model the variability in each leg of transport. Depending on the transportation step, the length of storage was either determined by data from the literature or user input. Ranges of 0.5 to 2 days during transport from China distribution center to cargo ship, 25 to 30 days on cargo ship from China to Germany *(3)* and 0.5 to 5 days on a truck from the cargo ship to distribution center in Germany, were selected from uniform distributions.

The Frozen storage at distribution center section estimates the time and corresponding log reduction during storage at the German frozen food distribution center. The time at the distribution center was expressed as a uniform distribution ranging from 0.5 to 90 days. The simulation then assumed the product was transported on a truck,

frozen, to the catering facility. The time for transport from the frozen food distribution center to the catering facility was expressed as a uniform distribution ranging from 0.5 to 5 days. All the frozen transport time variables, except for the transport by cargo ship are designated as user inputs, as no good source for these data were readily available. Since frozen strawberries can maintain their quality for 14-18 months *(2)*, the values selected here may underestimate the declines in NoV populations observed during frozen storage.

The next section of Table 1 (Foodservice preparation and consumption) represents the expected change in NoV level on frozen strawberries depending upon preparation method. Strawberry compote made with unheated or cold frozen strawberries was the food type associated with the large NoV outbreak in Germany *(12, 39)*. German kitchens not associated with the outbreak almost exclusively served the strawberries after "heating", but the temperatures reached during that heating processes were unknown. Our baseline model assumes no heat step was applied to the strawberries prior to serving, and thus no thermal inactivation of NoV. Two different preparation steps were used to represent alternative scenarios where frozen berries were heated prior to consumption: mild heat treatment for 30s at 65°C by using the @Risk function RiskNormal (1.54, 0.32) and high heat treatment for 15s at 75°C with a log reduction of RiskNormal (2.81, 0.39).

The next section of Table 1 (Serving and dose response) includes the serving size of the number of strawberries consumed per dessert, calculations that convert the log genome copies (GC) per strawberry to the dose per serving, and the parameters of the dose-response model from Teunis et al. (2008). Based on the dessert implicated in the outbreak (strawberry compote) and extensive search of strawberry compote recipes for one serving, we assumed that 4 strawberries constituted a serving *(39)*. The model output

was the probability of illness, which was used to calculate the number of illnesses. The probability of illness given the dose from the previous section was combined with the number of servings used per iteration in a binomial distribution to predict the number of illnesses arising from those servings.

Figure 1 shows a tornado plot representing a sensitivity analysis of the risk assessment. Since risk assessment models can be complex and may have intricate interactions between various inputs, it may be difficult to determine which model parameters contribute the most to variation in the output. Although there are many different approaches to sensitivity analysis, we used the method of Zwietering et al. (2000) because the resulting Factor Sensitivity values distinctly show the sensitivity to individual variants *(64)*. Figure 1 shows the log relative change in mean number of illnesses from alternative scenarios compared to the baseline scenario from 100,000 iterations. The scenario that had the greatest impact on the number of illnesses relative to the baseline was the assumption of a high level of NoV present in the water (8 log GC/L). This resulted in a mean of 7,694 illnesses (Table 2), whereas the baseline risk model resulted in a mean of only 1.89 illnesses. The top bar for Figure 1 shows a factor sensitivity of 3.6 calculated from these two values using equation 2: log (7,694/1.89). Mild- and high-heat treatment to strawberries had a significant reduction on the illnesses relative to the baseline, with 0.02 mean illnesses and 0 mean illnesses, respectively. Since it was not possible to calculate log (0/1.89), it was assumed that the minimum number of possible illnesses occurred in the high-heat treatment scenario (i.e. 1 illness in 100,000 iterations, log(0.00001/1.89) or a factor sensitivity of -5.3). Use of a pesticide with a strong antiviral effect also impacted the probability of illnesses (0.02 mean illnesses, for a

factor sensitivity of 1.9). Relative to the other scenarios, seasonality of NoV prevalence in water and the truck temperature had the least effect on the outcome of illnesses, with factor sensitivities ranging from -0.5 to 0.2. Other key scenarios evaluated the volume of water used to dilute pesticides. Pesticides sprayed using large volumes of water may lead to a greater risk of viral contamination of the crop because the probability of contaminated water coming in contact with crops is higher and the concentration of pesticides is lower due to dilution, resulting in potentially greater viral persistence *(30)*. The baseline model assumed a volume of 200 L/ha was applied to the strawberries, and the sensitivity analysis showed that illnesses were 10-fold lower and higher (factor sensitivities of -1 and 1) vs. the baseline when 20 L/ha and 2,000 L/ha, respectively were used.

Figure 2 compares the distribution of predicted illnesses over the 100,000 iterations for the baseline and worst case (8 log GC/L in the surface water) scenarios, using both illnesses and log(illnesses). Figure 2a shows that for the baseline scenario most iterations (~70%) result in no illnesses, the average number of illnesses is ~2, and the distribution is highly skewed with one iteration resulting in over 400 illnesses. Figure 2b shows a much different picture for the 8 log GC/L in the surface water scenario. In this case the most frequent result is still no illnesses, but many more scenarios result in illness, with much less skewed distribution, mean illnesses over 7,000 and one scenario resulting in almost 40,000 illnesses. Figure 2c shows the baseline scenario on a log(illness) scale. Most iterations (~70%) result in no illnesses, but because log(0) is undefined, those iterations are indicated as such. Figure 2c makes it clear that when illnesses occur, the most common number of illnesses is 1, shown as 0 on the log illness

scale, with frequency declining steadily. Figure 2d shows a similar log(illness) plot for the high illness scenario (8 log GC/L in the surface water). As with Figure 2b, the most common result is no illnesses. Figure 2d makes it clear that (as also seen in Figure 2c) that when illnesses occur, the most common number of illnesses is also 1 (shown as 0 on the log illness scale). The frequency of various illness rate declines and remains fairly constant from about 1.5 log (31 illnesses) to 3 log (1,000 illnesses), when the frequency increases to around the mean of 3.5 log, followed by a steady decline to the maximum number of illnesses (~4.5 log).

Figure 3 shows a comparison of the distribution of simulated virus particles per serving from 100,000 iterations of quantitative microbial risk assessment for Norovirus in frozen strawberries. The y-axis represents the logarithm of the relative frequency of observation of specific virus particle concentrations. A logarithmic transformation is used on this axis to better visualize frequency of low probability events, where zero represents 100% (i.e. all iterations of the simulation), -1 represents 10%, -2 represents 1%, etc. Panel (A) baseline scenario shows the baseline distribution of virus particles. As with all of the other scenarios, the most frequent prediction was for a serving to contain zero virus particles. The next most common prediction was for a serving to contain a single virus particle in about 4% of the iterations. as the predicted number of virus particles increases, the predicted frequency decreases. The highest predicted concentration of virus particles per serving in the baseline scenario was 17. Since that figure represents 100,000 iterations, those predictions showing a frequency of -5 represent a single iteration of the simulation. Panel (B) represents the baseline conditions plus high heat (15s at 75 °C) use during food service preparation. The highest number of virus particles

predicted per serving in this scenario was only two, which occurred in less than 10 iterations of the simulation. Panel (C) shows the results from the scenario where highly contaminated (8 Log GC/L) water was used for pesticide application, and dramatically higher virus particle concentrations for serving were predicted, with the highest concentrations in excess of 80,000 virus particles. The pattern of contamination is however consistent with those shown in panel A and B, where the most frequent simulation result is still a serving containing zero virus particles. Panel (D) shows a scenario where the interaction between the use of highly contaminated (8 Log GC/L) water plus high heat (15 s at 75 °C) use during food service preparation is presented. The most contaminated serving contains almost 800 virus particles, but this was only observed during one iteration of the simulation, and more than 97% of servings contained zero virus particles.

This risk assessment was undertaken to simulate the German 2012 NoV outbreak linked to frozen strawberries sources from China *(12)*, but we also believe it can be adapted to other berry types. It was possible to develop a working QMRA model, which has identified available data and data gaps, and which is able to provide simulation results which approximate the German outbreak. The data gaps identified include information on persistence and survival of human NoV strains (instead of surrogates) in fresh and frozen strawberries and in response to heating. Our model shows that the German outbreak in 2012 could have resulted from the use of a highly contaminated water source applied to a large number of strawberries prior to harvest. Our model also predicts that thorough heating of frozen strawberries prior to serving would have a dramatic effect on risk. Following the outbreak that sickened ~11,000

people in Germany, the European Union (EU) regulation now requires 5% of consignments of frozen strawberries imported from China into the EU to be tested for norovirus, as well as recommending to the catering sector to heat-treat berries prior to consumption *(12)*. These two interventions appear to have prevented the recurrence of an outbreak the size of the German 2012 event. The use of a model-based risk assessment supports these risk management measures and would likely assist in comparison of the utility of additional intervention measures.

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**Competing interests**

The authors have no competing interests to declare.

Figure 7.1: Comparison of Norovirus in frozen strawberry scenario assumptions on factor sensitivity. Factor sensitivity is defined as the logarithm of the ratio of the mean number of illnesses for the relevant factor versus the baseline scenario.

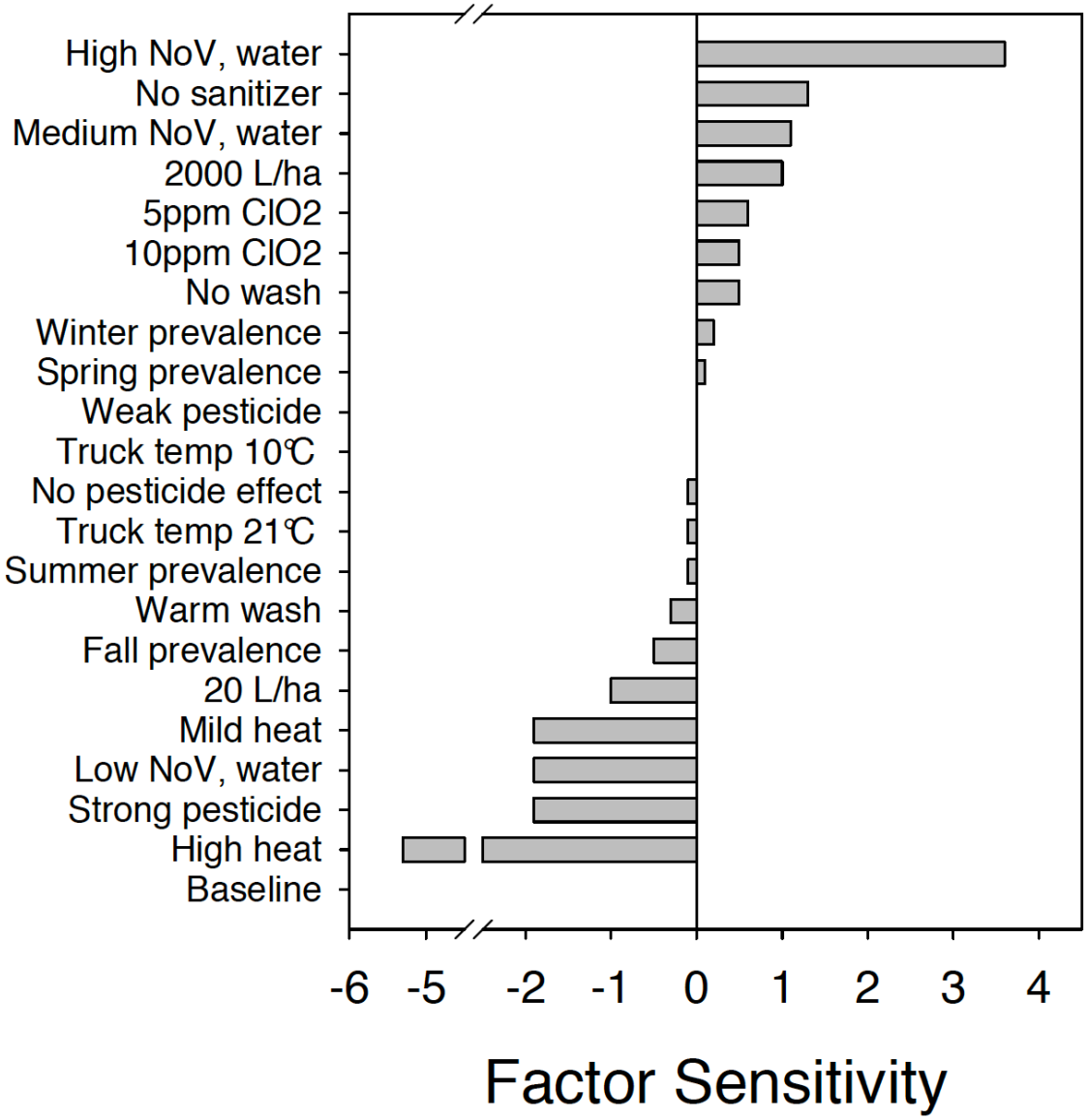

Figure 7.2: Comparison of distribution of predicted illnesses from 100,000 iterations of quantitative microbial risk assessment for Norovirus in frozen strawberries. Leftmost panels represent baseline scenario (A and C) versus highly contaminated (8 log GC/L) pesticide makeup water (B and D). Topmost panels (A and B) show data as illnesses, while bottommost panels (C and D) show the same data expressed as logarithm of the number of illnesses.

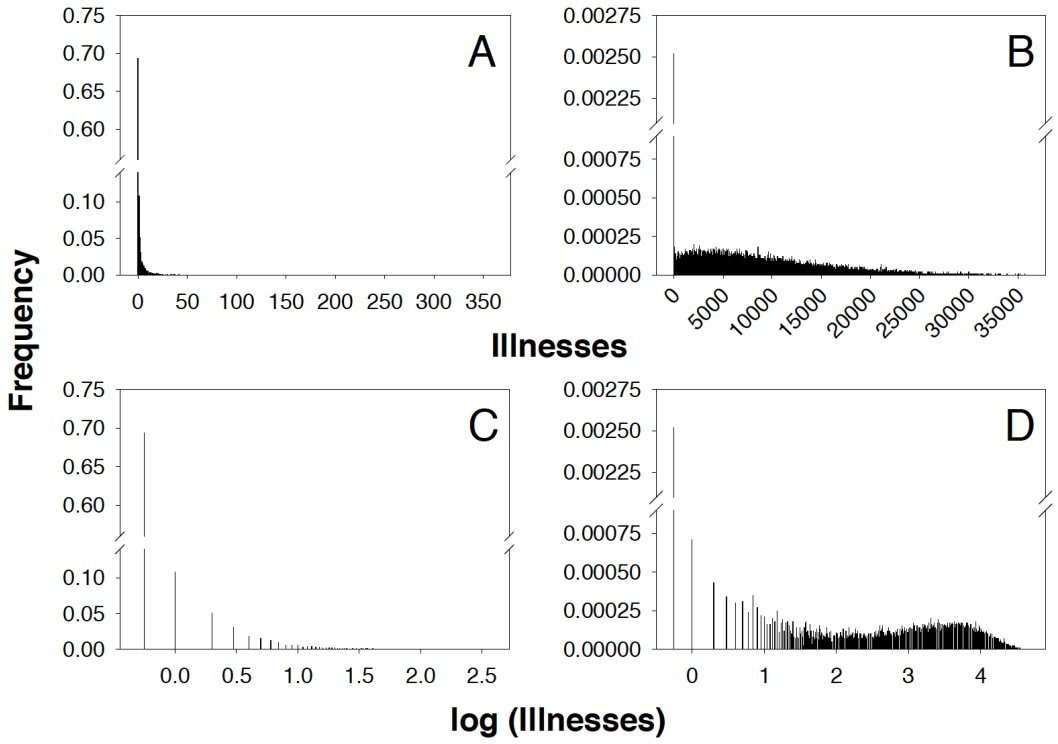

Figure 7.3: Comparison of distribution of simulated virus particles per serving from 100,000 iterations of quantitative microbial risk assessment for Norovirus in frozen strawberries. (A) baseline scenario, (B) baseline plus high heat (15 s at 75 °C) use during food service preparation, (C) Highly contaminated (8 Log GC/L) water used for pesticide application, (D) Highly contaminated (8 Log GC/L) water used for pesticide application plus high heat (15 s at 75 °C) use during food service preparation.

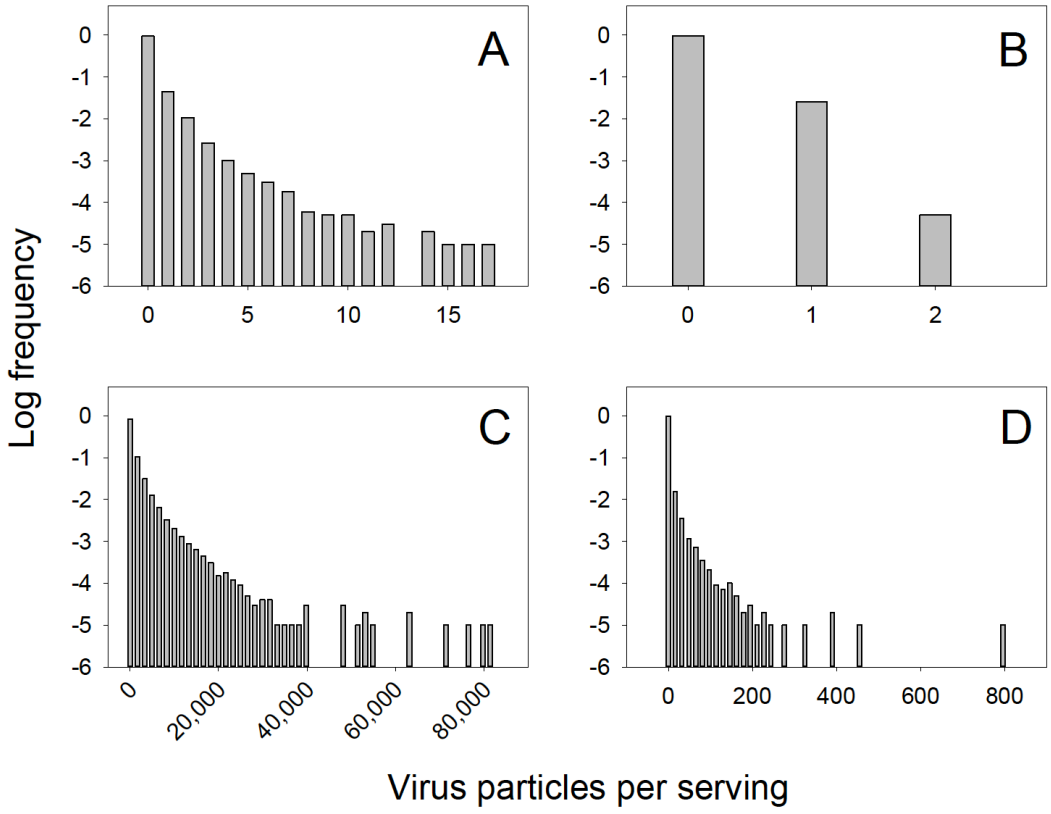

Table 7.1: Norovirus in frozen berries risk model using baseline parameters in @risk for farm to fork quantitative microbial risk assessment

| Cell | Variable | Value/Distribution | Units | Source |
|---|---|---|---|---|
| D2 | **In field** | | | |
| D3 | Starting concentration in water | =RiskUniform (1.2721,4.8428) | Log GC/L | (Maunula et al., 2013; Verhaelen et al., 2013; Lodder and Husman, 2005; Hamza et al., 2009; Kishida et al., 2012; Calgua et al., 2013; van den Berg et al., 2005) |
| D4 | Log reduction of NoV in pesticide solution | =RiskLognorm (0.34575,0.5552, RiskShift (−0.21468)) | Log GC/L | (Verhaelen et al., 2013) |
| D5 | concentration after mixing with pesticide | =D3-D4 | Log GC/L | Calculated |
| D6 | concentration after mixing with pesticide | =10^D5 | GC/L | Calculated |
| D7 | Prevalence of NoV in surface water | =RiskTriang (0.12,0.12,0.95301) | per L | (Hamza et al., 2009; Kishida et al., 2012; Calgua et al., 2013; Aw et al., 2009; Kozyra et al., 2011; Laverick et al., 2004; Lee and Kim, 2008; Lodder et al., 2010; Schets et al., 2008; Skraber et al., 2004; Steyer et al., 2011; Wei and Kniel, 2010) |
| D8 | Number of liters applied | 200 | L/ha | User input |
| D9 | How many liters positive? | =RiskBinomial (D8,D7) | L/ha | Calculated |
| D10 | Effective concentration per liter, considering pos. and neg. liters | =D6*D9/D8 | GC/L | Calculated |
| D11 | Conversion of hectare to acre | 2.47105 | Acre/Ha | User input |
| D12 | Number of berry plants per acre | =ROUND ((9113.22222),0) | Plants/acre | User input |
| D13 | Number of berries per plant | 10 | Berries/plant | User input |
| D14 | Volume of water sprayed per plant | =D8/ (D11*D12) | L/plant | Calculated |
| D15 | Volume of water sprayed per berry | = D14/D13 | L/berry | Calculated |
| D16 | concentration on berry after pesticide treatment | =D10*D15 | GC/berry | Calculated |
| D17 | Log concentration on berry after pesticide treatment | =LOG(D16) | Log GC/berry | Calculated |
| D18 | **Transportation to processing facility** | | | |
| D19 | Temperature, truck | 4 | C | User Input |
| D20 | Time, truck | 1 | Days | User Input |
| D21 | Log reduction at 4 ˚C per day | 0 | Log reduction/day | (Kurdziel et al., 2001) (fresh raspberries - poliovirus, 9 days), (Verhaelen et al., 2012; Dawson et al., 2005) |
| D22 | Log reduction at time of delivery | =D20*D21 | Log reduction | Calculated |
| D23 | concentration on berry at time of delivery | =D17-D22 | Log GC/berry | Calculated |
| D24 | **Washing log reduction (18 ˚C, cold water)** | | | |
| D25 | Mean log reduction on contaminated berries | 0.667 | | (Butot et al., 2008) |
| D26 | SD log red on contaminated berries | 0.332 | | (Butot et al., 2008) |
| D27 | Log reduction on contaminated berries | =RiskNormal (D25,D26) | Log GC/berry | Calculated |
| D28 | concentration on contaminated berries | =D23-D27 | Log GC/berry | Calculated |
| D29 | **Sanitizing log reduction, 200 ppm Chlorine concentration** | | | |
| D30 | Mean log reduction on contaminated berries | 1.35 | | (Butot et al., 2008; Predmore and Li, 2011) |
| D31 | SD log red on contaminated berries | 0.24 | | (Butot et al., 2008; Predmore and Li, 2011) |
| D32 | Log red difference on contaminated berries | =RiskNormal (D30,D31) | Log GC/berry | Calculated |
| D33 | concentration on contaminated berries | =D28-D32 | Log GC/berry | Calculated |
| D34 | **Freezing process** | | | |
| D35 | Log reduction | 0 | Log GC/berry | User input |
| D36 | concentration on berry after freezing | =D33-D35 | Log GC/berry | Calculated |
| D37 | **Transportation from truck to cargo ship, −21C** | | | |
| D38 | Time, transport | =ROUND (RiskUniform(0.5,2),0) | Days | User input |
| D39 | Mean log reduction at frozen storage | 0.4 | Log GC/berry | (Butot et al., 2008) |
| D40 | SD log reduction at frozen storage | 0.18 | | (Butot et al., 2008) |
| D41 | Log reduction, frozen storage after 90 days | =RiskNormal (D39,D40) | | Calculated |
| D42 | Log reduction, frozen storage per day | =D41/90 | | Calculated |
| D43 | Log reduction | =D42*D38 | Log GC/berry | Calculated |
| D44 | concentration on berry at port in China | =D36-D43 | Log GC/berry | Calculated |
| D45 | **Transport via cargo ship, −21C** | | | |
| D46 | Time, transport | =ROUND (RiskUniform(25,30),0) | Days | User input |
| D47 | Log reduction | =D42*D46 | Log GC/berry | Calculated |
| D48 | concentration on berry at port in Germany | =D44-D47 | Log GC/berry | Calculated |

| Cell | Variable | Value/Distribution | Units | Source |
|---|---|---|---|---|
| D49 | **Transport to distribution center, −21C** | | | |
| D50 | Time, transport | =ROUND (RiskUniform(0.5,5),0) | Days | User input |
| D51 | Log reduction | =D42*D50 | Log GC/berry | Calculated |
| D52 | concentration on berry upon arrival at distribution center | =D48-(D51) | Log GC/berry | Calculated |
| D53 | **Frozen storage at distribution center, −21C** | | | |
| D54 | Time, distribution center | =ROUND (RiskUniform(0.5,90),0) | Days | User input |
| D55 | Log reduction | =D42*D54 | Log GC/berry | Calculated |
| D56 | concentration on berry | =D52-D55 | Log GC/berry | Calculated |
| D57 | **Transport, distribution center to catering facility, −21C** | | | |
| D58 | Time, transport | =ROUND (RiskUniform(0.5,5),0) | Days | User input |
| D59 | Log reduction | =D42*D58 | Log GC/berry | Calculated |
| D60 | concentration on berry | =D56-D59 | Log GC/berry | Calculated |
| D61 | **Foodservice preparation and consumption** | | | |
| D62 | concentration on berry (antilog) | =10^D60 | GC/berry | |
| D63 | **Serving and dose response** | | | |
| D64 | Serving size | 4 | Berries | User input |
| D65 | concentration (non log) | =D62 | GC/berry | Calculated |
| D66 | concentration per serving | =D64*D65 | GC/berry | Calculated |
| D67 | Dose-response η | =2.55*10^−3 | No units | (Teunis et al., 2008) |
| D68 | Dose-response r | 0.086 | No units | (Teunis et al., 2008) |
| D69 | Probability of illness | =1-(1 + (D66*(0.00255)))^−0.086 | | Calculated |
| D70 | **Illnesses** | | | |
| D71 | Number of servings to consider per iteration | 100,000 | Servings | User input |
| D72 | Illness per number of servings per iteration | =RiskBinomial (D71,D69) | Illnesses | Calculated |
| D73 | Was there illness? | =IF(D72 > 0,1,0) | No units | Calculated |
| D74 | Log number ill | =IF(D73 = 1,LOG(D72),−5) | Calculated | Calculated |

Table 7.2: A comparison of baseline conditions to other scenarios showing minimum, mean and maximum number of illnesses as well as factor sensitivities for different scenarios.

| Parameter | Variations | Simulated illnesses | | | | | Factor sensitivity |
|---|---|---|---|---|---|---|---|
| | | Minimum | 5th percentile | Mean | 95th percentile | Maximum | (Log relative change) |
| Baseline conditions [a] | | 0 | 0 | 1.89 | 9 | 487 | 0.000 |
| Concentration in surface water | 8 Log GC/L | 0 | 646 | 7,694 | 18,306 | 36,056 | 3.601 |
| | 5 Log GC/L | 0 | 0 | 22 | 83 | 1,348 | 1.057 |
| | 2 Log GC/L | 0 | 0 | 0.023 | 0 | 3 | −1.924 |
| Pesticide application | 2000 L/ha | 0 | 0 | 19 | 91 | 3,762 | 0.993 |
| | 20 L/ha | 0 | 0 | 0.193 | 1 | 83 | −1.000 |
| NoV prevalence in surface water | Spring 57% | 0 | 0 | 2.68 | 13 | 521 | 0.143 |
| | Summer 33% | 0 | 0 | 1.56 | 8 | 238 | −0.092 |
| | Fall 14% | 0 | 0 | 0.684 | 4 | 263 | −0.451 |
| | Winter 70% | 0 | 0 | 3.4 | 17 | 808 | 0.246 |
| Pesticide strength | Strong | 0 | 0 | 0.023 | 0 | 7 | −1.930 |
| | Weak | 0 | 0 | 1.71 | 9 | 242 | 0.009 |
| | No pesticide effect | 0 | 0 | 1.97 | 1 | 398 | −0.053 |
| Truck temperature | 10 °C | 0 | 0 | 1.75 | 8 | 359 | −0.043 |
| | 21 °C | 0 | 0 | 1.57 | 8 | 415 | −0.090 |
| Washing step | Warm water | 0 | 0 | 1.06 | 6 | 166 | −0.260 |
| | No wash | 0 | 0 | 6.5 | 34 | 1,119 | 0.527 |
| Sanitizing step | 5 ppm ClO2 | 0 | 0 | 8.28 | 41 | 1,165 | 0.633 |
| | 10 ppm ClO2 | 0 | 0 | 6.67 | 33 | 814 | 0.539 |
| | No sanitizer | 0 | 0 | 35.8 | 180 | 4,270 | 1.268 |
| Foodservice preparation | Mild heat (30 s at 65 °C) | 0 | 0 | 0.026 | 0 | 9 | −1.871 |
| | High heat (15 s at 75 °C) | 0 | 0 | 0.00001 [b] | 0 | 0 | −5.300 |

[a] Baseline conditions: Concentration of NoV in water as a uniform distribution, volume of liquid to apply pesticides 200 L/ha, seasonality for prevalence of NoV in surface water as a triangular distribution, the effect of pesticide on reduction in NoV concentration as a lognormal distribution, truck temperature for transport from the field by 4 °C, berries washed in 18 °C water, sanitized using 200 ppm chlorine, and no heating step during foodservice preparation.

[b] Since no illnesses were predicted under high heat conditions, and it is not possible to calculate the logarithm of zero, we assumed one iteration resulted in one illnesses for purposes of calculating factor sensitivity (log relative change)

## 7.5 Bibliography

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# Chapter 8: Development of an Agent-Based Model for Norovirus Contamination to Berries from Infected Workers on Farm

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## 8.1 Abstract

Many quantitative microbial risk assessments have historically used a linear structure to represent the "farm to fork" continuum. While such structures are relatively straightforward to implement in commonly used modeling programs, they cannot easily handle less linear transmission paths. One example where these traditional models are less useful would be in the simulation of microbe transmission from the hands of a symptomatic worker who is hand-harvesting delicate fruits like strawberries or raspberries. The microbe concentration on the workers hands changes through the day or week depending upon resolution of symptoms, bathroom use, handwashing and number of fruits picked. One solution to these complex modeling problems involves the use of agent-based models. In an agent-based modeling environment, individual workers, virus particles and berries all constitute "agents" whose behaviors can be simulated. The objective of this risk assessment was to develop an agent-based model to simulate the spread of human norovirus on a farm, starting from a single ill fruit picker. The model predicts prevalence and concentration of norovirus particles on berries picked over one week as the disease spreads to other pickers on the farm and the impact of prevention strategies, such as handwashing.

## 8.2 Introduction

Norovirus (NoV) is the leading cause of foodborne illness worldwide, resulting in approximately 19-21 million cases in the United States (U.S.) annually *(6)*. Fruits and vegetables, specifically berries, have been frequently linked to foodborne viral disease outbreaks, most commonly arising from NoV or hepatitis A virus (HAV) *(3, 17, 23, 25, 26, 36, 37, 39)*. These viruses may be transmitted through water, food, contact with contaminated surfaces or directly from person to person *(5)*. The largest NoV outbreak on record occurred in 2012 in Germany was linked to frozen strawberries imported from China; these berries were served to children in hundreds of schools resulting in over 11,000 illnesses *(3)*.

Fruit harvesting may be done by hand depending on the size of the farm and fragility of the fruit. Hand harvesting can increase the opportunity for ill employees to contaminate the product as hands are a means of foodborne disease transmission. The role food handlers play in the contamination of food with enteric viruses is well established *(1, 4, 41)*. Infected people can excrete high quantities of enteric viruses in their feces (i.e. $10^5$ –$10^9$ virus particles/g feces) and shedding at this level has been shown to continue for several weeks in symptomatic and asymptomatic patients *(40)*.

Agent-based modeling (ABM) is a bottom-up modeling approach that simulates interactions between computer-generated units known as “agents”. Individual agents can be assigned a variety of attributes, states, behavioral properties and interactions. An ABM featuring individual agents can use personalized behaviors taken directly from the published literature and can be used to build complex models based on simple components *(24)*. ABM has been used in healthcare to simulate epidemiological

occurrences, as well as medical innovations or interventions *(11, 32, 35)*. Agent-based methods simulating the transmission of human viral and bacterial diseases, such as influenza and *Listeria* spp., has been conducted in the food supply chain *(2, 21, 29)*. Simulation offers a potential to help understand how an aspect of the world operates, considering real-world variability and complexity without time consuming, expensive and sometimes impossible experiments. Such simulations can help identify areas for improvement and minimize the risk of errors when implementing changes.

Our model is used to study the transmission dynamics of NoV on farm at an individual level, with respect to timing and dispersal of secondary infections from a single infected person. This model includes many parameters such as infection rate, shedding rate, infection period, immune period and the effectiveness of hand hygiene compliance. The model simulates the stochastic contact events from the individual level including berry picking, allowing us to predict prevalence and concentration at the level of the individual berry. This model is designed for two reasons: (1) to determine if an outbreak the size of the one which occurred in Germany in 2012, which was traced back to a 22-ton lot of strawberries imported from China can occur through increased contact rates and improper hand hygiene and (2) to influence policy and prevention strategies in regards to the impact of handwashing on the outcome of contaminated berries *(19)*.

## 8.3 Methods

### AnyLogic: Simulation Environment

AnyLogic is a Java-based programming and simulation tool. The software allows the user to combine different modeling methods including differential equations, discrete events and agent-based systems. While the software offers a simple visual interface to

enable fast prototyping, Java code is always available "under the hood" to allow the programmer to address specific customization needs. Our model has two types of agents: fruit pickers and the individual berries to be picked. These agents interact with their simulated farm environment.

Fruit Picker Agent

The fruit pickers in the simulation can have different health statuses. The health status of the 'fruit picker' agent is controlled by a state-chart which defines the state-transitions and actions for these transitions, as shown in Figure 1. A modified Kermack-McKendrick SIR model (susceptible, infected, recovered) was used to describe disease spread within a population *(20)*. Our modified SEIR (susceptible, exposed, infected, recovered) model allows the inclusion of many features and characteristics of epidemic diseases by using parameters specific to NoV. The system uses parameters that contain a variety of influences that essentially affect the progress or prevent an epidemic.

Figure 1 shows each 'fruit picker' as immune, susceptible, exposed, infectious, shedding or recovered. The first branch determines whether the fruit picker is immune or susceptible to NoV infection. It has been shown that a portion of the population is immune to NoV which is determined by the presence or absence of a functional FUT2 gene. Approximately 20% of the Caucasian population does not encode a functional FUT2 gene and are considered "non-secretors" and are therefore believed to be immune to NoV *(9)*. The model has been set so that there is 1 initially infected 'fruit picker' at time 0, and the model runs for 10 days. The arrows represent various transitions from state to state as described by the parameter shown in Table 1. When running the

simulation, each fruit picker in a particular disease state is represented by a different color to help visualize the infection states on farm.

Berry Agent

The berry agent, a single berry, is represented by a small red oval in the graphical interface. The concentration on berries was collected in a data set to determine how many berries and at what level were contaminated by each individual fruit picker. All of the berries were assumed to be free of NoV contamination prior to harvesting.

Baseline Scenario

The baseline scenario assumes that there are 20 fruit pickers on the farm, that one fruit picker is initially infected, and all fruit pickers wash their hands 1 time/day. On day 1, the initial ill employee is able to contact 10 other fruit pickers, putting those picker agents into the susceptible state. Depending on their state, handwashing will result in a 1 log reduction in viruses on the hands. Studies have found that a standard handwashing practice typically results in 1-2 log reductions of NoV from hands *(10, 12, 22, 38)*. The parameters for the baseline scenario are in Table 1.

Scenario Analysis

The model simulated the behavior of fruit pickers and how this effects contamination of berries that are harvested. Different scenarios are described in Table 2. The scenarios vary factors such as handwashing behavior, contact rate between employees and exclusion of an ill worker with the replacement of a healthy employee, to see how each affects final NoV prevalence and concentration on berries. Contact rate per day was varied to see the impact of person to person transmission and its outcome of berry contamination. Simulations were run to see the effect of contact rate of 5 people per

day versus 15 people per day (with 10 people per day being the baseline). Handwashing compliance was also varied. Simulations were run to see the effect of handwashing 0 times per day versus 2 times per day (with 1 time per day being the baseline). The final scenario of interest was the exclusion of an ill worker and replacement by a healthy employee midweek (end of day 5).

## 8.4 Simulation modeling

Our previously published quantitative microbial risk assessment *(30)* was combined with the outputs from the AnyLogic simulation to look at final effect on risk. The published risk assessment followed the farm to fork continuum of NoV contamination on strawberries, assuming pesticide application water was the source of virus contamination. The new risk assessment assumed that contamination occurred from berries harvested by infected fruit pickers where the prevalence and concentration on these berries was calculated by AnyLogic. The Excel add-in @Risk (Palisade Corporation) was used to perform the Monte Carlo simulations of 100,000 iterations for the AnyLogic baseline and each scenario evaluated.

## 8.5 Results

### Baseline

The baseline scenario of our published risk assessment *(30)* resulted in a mean of ~2 illnesses from 100,000 servings. Substituting baseline inputs from our AnyLogic simulation presented here, resulted in the same ~2 mean illnesses, with a maximum simulated 92 illnesses from 100,000 servings. The baseline number of infected workers at the end of 10 days was normally distributed with a mean of 7 ± 5 infected workers and a median of 7 infected workers, where workers were considered infected when in the

infectious and shedding state. The baseline prevalence of NoV on berries at harvest was approximately 3.3% and the concentration was normally distributed with mean log of 3.1 ± 1.3 virus particles per berry. The scenarios of decreased contact rate (5 people per day) and improved handwashing compliance (2 times/day) resulted in 1 mean illness, as compared to the baseline that resulted in 2 mean illnesses as described below and in Table 3.

Handwashing scenarios

The effect of reduced handwashing compliance, (no fruit pickers washed their hands) increased the mean (15) and maximum (1,306) illnesses from 100,000 servings. The reduced handwashing compliance scenario yielded 6.8% prevalence and mean log of 5.8 ± 1.6 virus particles per contaminated berry. The number of infected workers at the end of 10 days in the reduced handwashing compliance scenario was normally distributed with a mean of 11 ± 4 infected workers and a median of 10 infected workers. The improved handwashing compliance scenario (2 washes/day), resulted in a mean of 1 mean and a maximum of 89 illnesses from 100,000 servings. Improved handwashing compliance yielded a 1.7% prevalence of NoV on berries with a mean log of 1.7 ± 1.1 virus particles per berry beat described by a Weibull distribution. The number of infected workers at the end of 10 days was normally distributed with a mean of 7 ± 3 infected workers and a median of 6 infected workers.

Contact rate scenarios

The scenario with the largest increase in NoV contamination resulted from a contact rate increased from 10 people per day to 15 people per day when 20 people were working, resulting in a mean of 21 illnesses and a maximum of 1,409 illnesses from

100,000 servings. Berries in the increased contact rate scenario had a NoV prevalence of 9.5% prevalence and concentration with mean log of 6.3 ± 1.9 virus particles per berry, best described by a triangle distribution. The number of infected workers at the end of 10 days was normally distributed with a mean of 12 ± 8 infected workers and a median of 12 infected workers. Decreasing the contact rate per day from 10 to 5 people resulted in an outcome of approximately 1 mean illness and a maximum of 87 illnesses from 100,000 servings. This scenario resulted in a NoV prevalence of 2.9% where the contaminated berries had a concentration of mean log of 3.6 ± 2.1 virus particles per berry, best described by a log normal distribution. The number of infected workers at the end of 10 days was normally distributed with a mean of 6 ± 5 infected workers and a median of 5 infected workers.

Exclusion of ill employee

When an ill employee was excluded from work on day 5 and replaced with a healthy employee, the outcome was approximately 2 mean illnesses and a maximum of 162 illnesses from 100,000 servings. This scenario resulted in a NoV prevalence of 2.0% and the concentration on berries was represented by a log normal distribution with a mean log of 3.7 ± 2.8 virus particles per berry. The number of infected workers at the end of 10 days was normally distributed with a mean of 3 ± 4 infected workers and a median of 6 infected workers.

## 8.6 Discussion

A recent U.K. study tested lettuce and fresh and frozen raspberries sold at retail for NoV over a 13-month period. This study found that 5.3% of lettuce, 2.3% of fresh raspberries and 3.6% of frozen raspberries were positive for NoV RNA *(8)*. A recent

study in China on retail frozen and fresh berries found the prevalence of NoV to be 9% and 12.1%, respectively. This Chinese surveillance study included samples of fresh and frozen strawberry, raspberry, blueberry, cranberry, blackberry and blackcurrant *(14)*. The data from these previously published surveillance studies of viral contamination on fresh fruits and vegetables in real-time are similar to the prevalence of NoV contamination produced from our AnyLogic simulation. Though these studies do not report on the concentration of NoV on the berries, the prevalence of the virus is evident and appropriate risk management strategies should be put in place to decrease NoV prevalence on fresh and frozen berries. The FDA has recently begun surveillance (November 2018) on frozen berries (raspberries, strawberries, blackberries) for NoV and hepatitis A virus (HAV) to better protect consumers and is expected to last for 18 months *(13)*. The surveillance thus far has resulted in several recalls of frozen fruit products, such as frozen blackberries, that tested positive for NoV *(13)*.

Government agencies recommend prevention strategies to decrease the risk of foodborne illness from NoV *(7)*. It is important to understand which factors minimize the spread of NoV in the exposed population. While large experiments would be the ideal way to determine which prevention strategies work best, they are both prohibitively expensive and ethically challenging. The use of simulations to predict outcomes have become increasingly popular in healthcare settings *(11, 32, 35)*.

Quantitative microbials risk assessments have often been used to model bacterial transmission, and many risk assessments involving NoV have been completed in recent years. The most recent risk assessment representing the transmission of NoV in a food establishment has been published by the U.S. Food and Drug Administration (FDA) *(10)*.

The objective of FDAs risk assessment was to observe the risks associated with NoV transmission to consumers from food contaminated by ill employees in a retail food setting. Conclusions from this study showed that exclusion of a symptomatic employee from work is key in decreasing the risk of NoV transmission to food being served to the consumer. Other factors that influenced infection to consumers included handwashing efficiency and frequency, as well as limiting contact between hands with objects such as door and faucet handles *(10)*. There have been additional exposure assessments and risk assessments focused on NoV transmission by ill food employees, and those authors have also highlighted the importance of hand hygiene in NoV spread *(15, 18, 28, 31, 34)*.

ABM has been viewed as a promising approach to affecting policy change *(2, 21, 29)*. An ABM project has been completed to represent NoV transmission in a school setting to evaluate school policies *(16)*. This ABM observed daily activities that would expose students to NoV and how two policies derived from the Centers for Disease Control and Prevention (CDC), limiting student-to-student interaction, had an effect on illness outcome. The study concluded that implementing either policy developed under the CDC helped in reducing student illnesses and that if the policy was implemented quicker, the outbreak duration would be shortened *(16)*.

Though it is a relatively new concept to the food industry, several studies have implemented ABM to look specifically at the behavior of *Listeria* in a food processing facility, as well as the complexity of the food supply chain and food safety inspections at an individual inspector and consumer level *(27, 42, 43)*. Zoellner et al. (2019) observed the behavior of *Listeria* spp. in the environment and equipment surfaces in a cold-smoked salmon facility. The authors developed EnABLe, “Environmental monitoring with an

Agent-Based model of *Listeria*" and determined sanitary design to be a large predictor of *Listeria* spp. contamination. This ABM used two agent types, employees and equipment surfaces, that were able to interact with one another and the environment *(43)*. The outcome of ABM will help researchers better understand the complexity of processes such as the spread of pathogenic bacteria and viruses, as well as interactions between human behavior. Our results show the importance and limitations of interventions such as handwashing, and how they impact the safety of hand-harvested berries.

Figure 8.1: Infection statechart

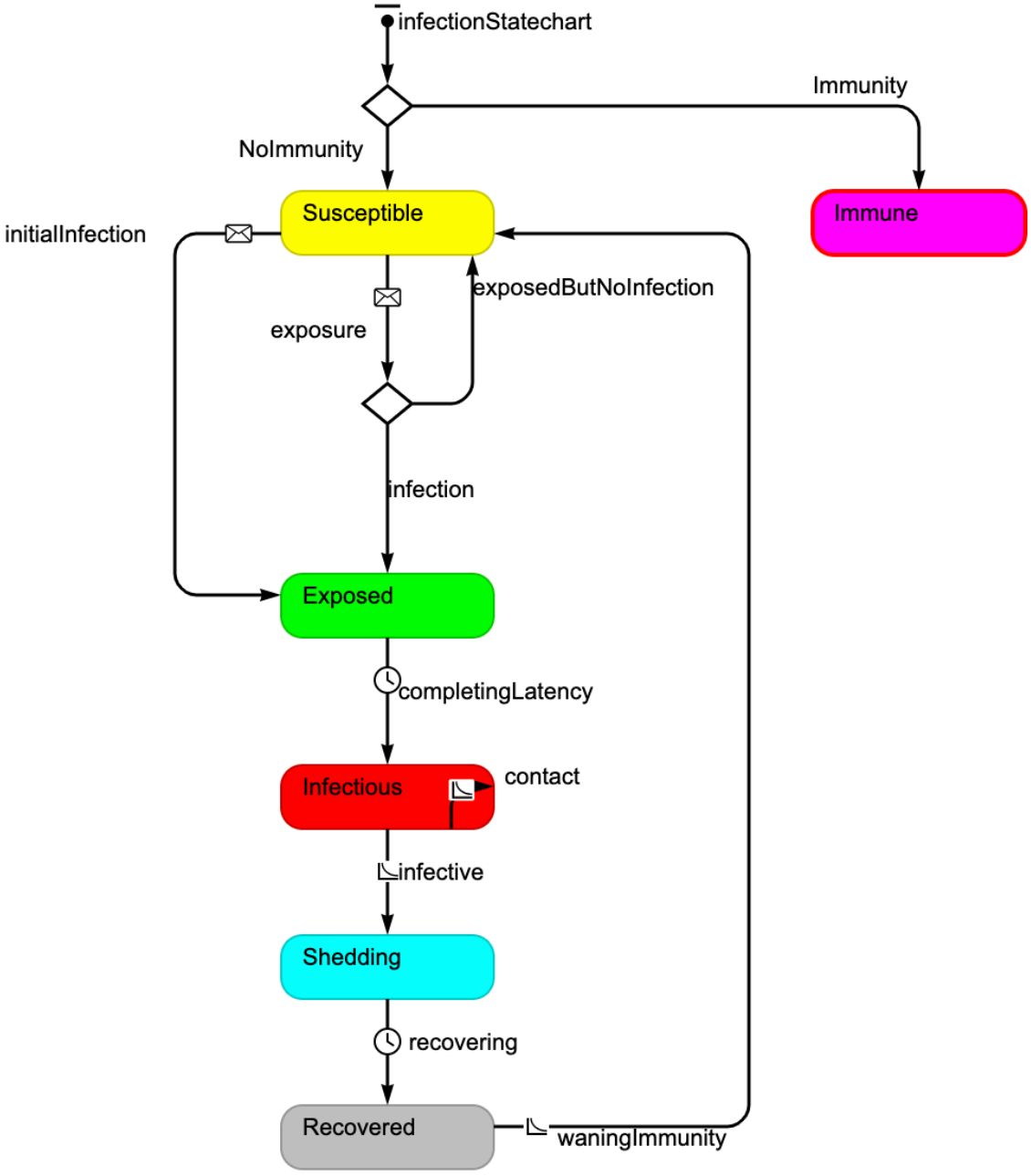

Figure 8.2: Disease state simulation over a 10-day period

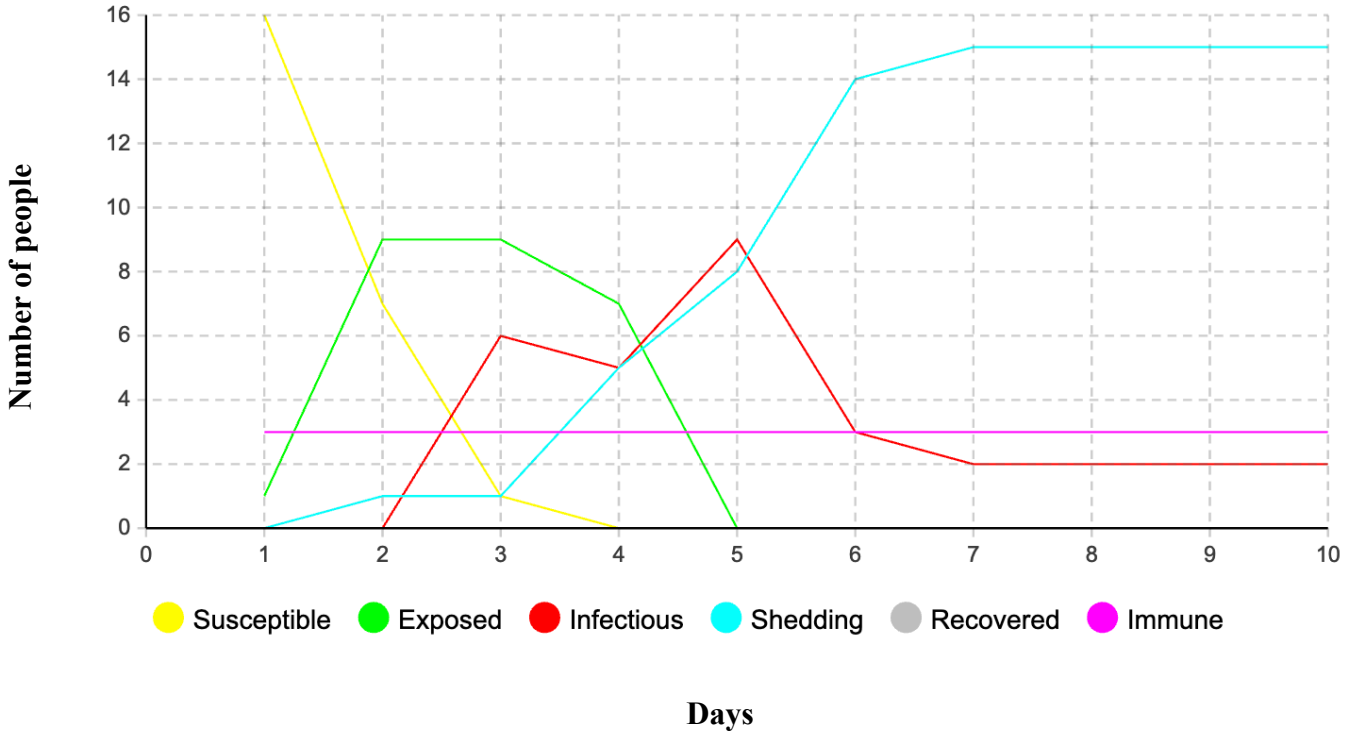

Figure 8.3: Comparison of distribution of simulated virus particles per berry from 100,000 iterations of quantitative microbial risk assessment for norovirus on frozen strawberries. (A) baseline scenario, (B) excluded ill employee, (C) high contact rate, (D) low contact rate, (E) 0% handwashing, (F) 100% handwashing

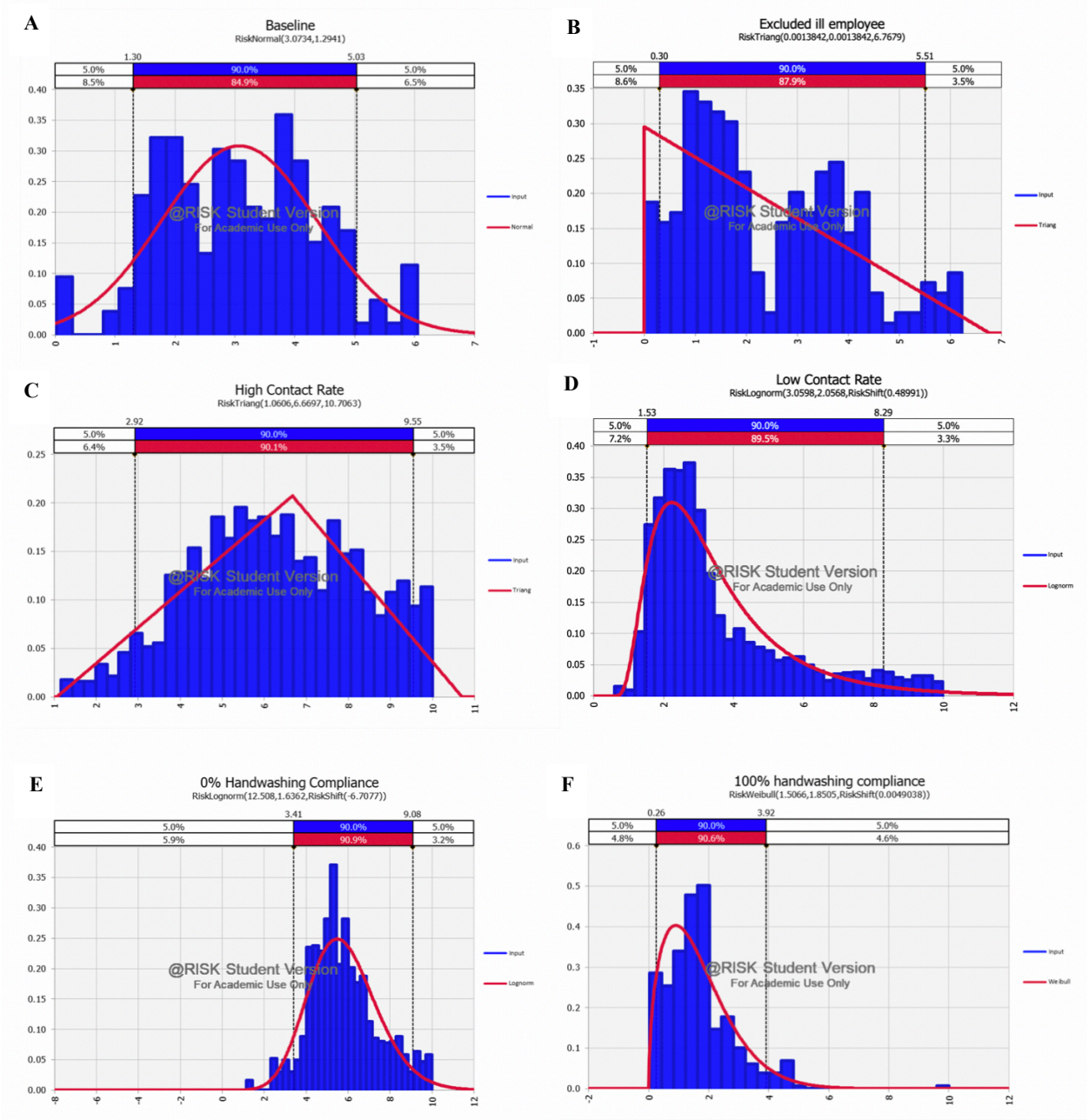

Figure 8.4: Comparison of distribution of predicted illnesses from 100,000 iterations of quantitative microbial risk assessment for norovirus on frozen strawberries. (A) baseline scenario, (B) excluded ill employee, (C) high contact rate, (D) low contact rate, (E) 0% handwashing, (F) 100% handwashing

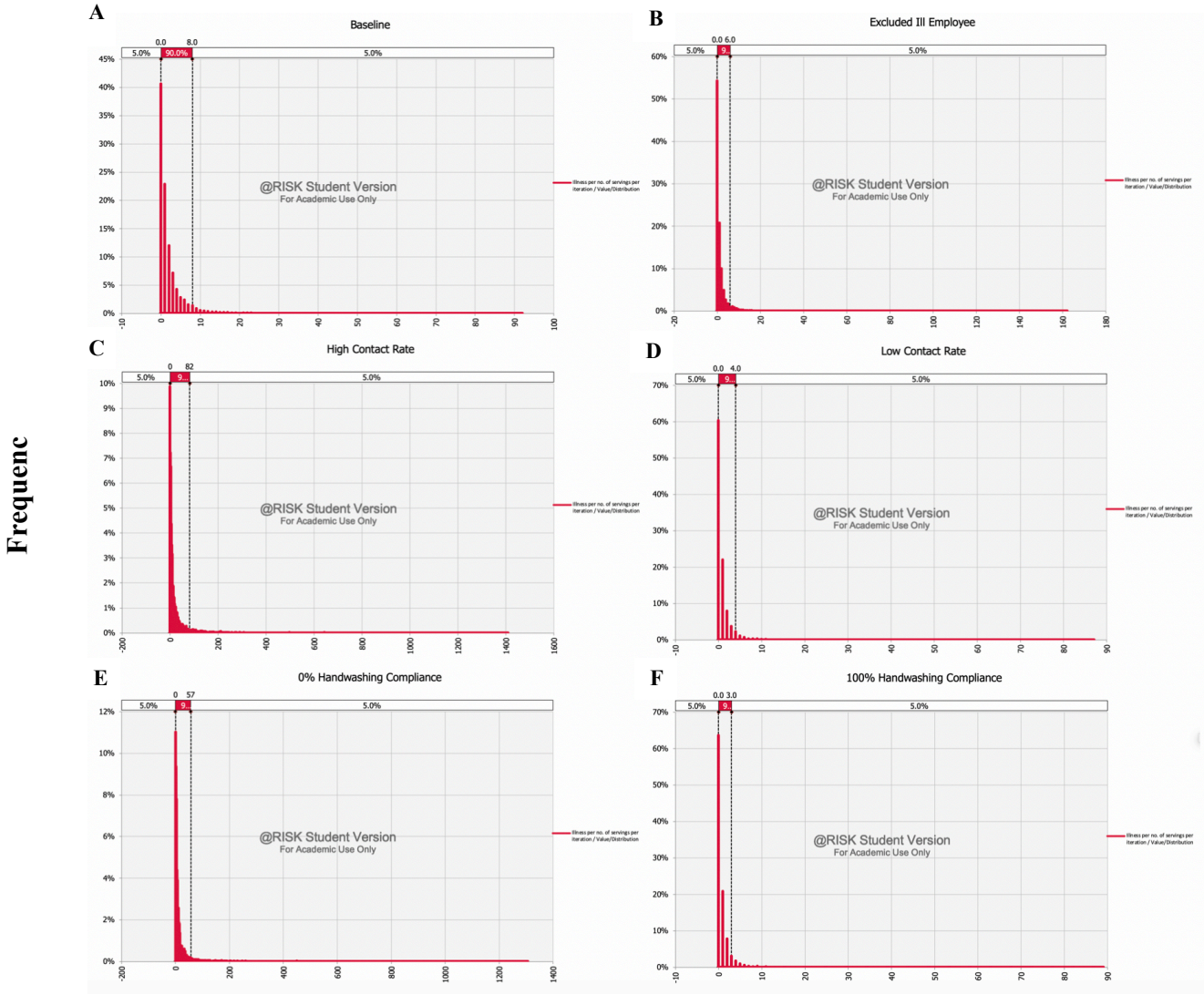

Table 8.1: AnyLogic baseline input

| **Variable** | **Input** | **Unit** | **Reference** |
|---|---|---|---|
| Contact rate | 10 | People/day | Input |
| Transmission Probability (person to person) | Uniform (0.12,0.46) | % | Matthews et al 2012 |
| Transmission Probability (foodborne) | Uniform (0.31,0.71) | % | Matthews et al 2012 |
| Duration of Latency | Uniform (0.5, 2) | Days | https://www.cdc.gov/hai/pdfs/norovirus/229110-ANoroCaseFactSheet508.pdf |
| Duration of Infectiousness | Normal (1,2) | Days | Lee et al 2013 |
| Duration of Waning Immunity | Uniform (180, 730) | Days | Simmons et al 2013 |
| Duration of Shedding | Uniform (13, 56) | Days | Atmar et al 2008 |
| Handwashing | 1 | Day | Input |

Table 8.2: AnyLogic Scenarios

| Scenario | Variables changed | Contact rate (contacts/infected picker/day) | Normal Handwashing event/day |
|---|---|---|---|
| Baseline | Inputs from references | 10 | 1 |
| 1 | High contact rate | 15 | 1 |
| 2 | Low contact rate | 5 | 1 |
| 3 | 100% handwashing compliance | 10 | 2 |
| 4 | 0% handwashing compliance | 10 | 0 |
| 5 | Exclusion of ill worker with replacement of healthy employee | 10 | 1 |

Table 8.3: Outputs from AnyLogic to determine the resulting illnesses following consumption of frozen strawberries.

| Scenario | Variables changed | Mean infected workers after 10 days | Prevalence of NoV on berries (%) | Concentration of NoV on berries (log virus particles/berry) | Mean illnesses | Maximum illnesses |
|---|---|---|---|---|---|---|
| Baseline | Inputs from references | 7 ± 5 | 3.3 | 3.1 ± 1.3 | 2 | 92 |
| 1 | High contact rate | 12 ± 8 | 9.5 | 6.3 ± 1.9 | 21 | 1,409 |
| 2 | Low contact rate | 6 ± 5 | 2.9 | 3.6 ± 2.1 | 1 | 87 |
| 3 | 100% handwashing compliance | 7 ± 3 | 1.7 | 1.7 ± 1.1 | 1 | 89 |
| 4 | 0% handwashing compliance | 11 ± 4 | 6.8 | 5.8 ± 1.6 | 15 | 1,306 |
| 5 | Exclusion of ill worker with replacement of healthy employee | 3 ± 4 | 2.0 | 3.7 ± 2.8 | 2 | 162 |

## 8.7 Bibliography

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