UNDERSTANDING AND DEVELOPING SAFER SANITATION AGENTS AND STRATEGIES IN FOOD PRODUCTION ENVIRONMENTS

Pragathi Kamarasu
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UNDERSTANDING AND DEVELOPING SAFER SANITATION AGENTS AND STRATEGIES IN FOOD PRODUCTION ENVIRONMENTS

A Dissertation Presented

by

PRAGATHI KAMARASU

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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The Department of Food Science
UNDERSTANDING AND DEVELOPING SAFER SANITATION AGENTS AND STRATEGIES IN FOOD PRODUCTION ENVIRONMENTS

A Dissertation Presented

by

Pragathi Kamarasu

Approved as to style and content by:

Matthew Moore, Chair

John Gibbons, Member

Lili He, Member

Indu Upadhyaya, Member (External)

Amanda Kinchla, Observer

Lynne A. McLandsborough,
Food Science Department Head
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mates will agree with me when I say we are very lucky to have been guided by you. I wouldn’t have continued with a Ph.D. if it wasn’t for you.

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தமிழில் எழுத்துக்கள்
ABSTRACT

UNDERSTANDING AND DEVELOPING SAFER SANITATION AGENTS AND STRATEGIES IN FOOD PRODUCTION ENVIRONMENTS

SEPTEMBER 2023

PRAGATHI KAMARASU,
B. TECH, ANNA UNIVERSITY

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Matthew D Moore

Food is a primary resource for survival of human beings, and it is also one of the primary resources for spread of infectious diseases. When both these factors come together it does not only cause ill effects on our health but also causes a burden on the food and agricultural industry. Through the help of science, us researchers and scientists have always tried to reduce this burden. And I hope like a drop of water in the ocean in some way my research contributes towards this. According to WHO currently in 2023 there are about 1 in 10 people falling ill due to foodborne diseases and amongst them about 420000 cases result in death and this also includes children. There are various contributing factors towards this. Cross contamination from the equipment’s used during handling and not following good manufacturing or agricultural practices are major contributing factors. Most foodborne outbreaks are caused due to raw food, and this is mainly due to the absence of a kill step such as cooking step. Foodborne illness causing
microorganisms are a major concern in the food industry and this work aimed at mitigating the risks caused by them. Raw produces are commonly consumed without a kill step such as cooking, and their contamination can occur anywhere in the farm to fork production chain. One such practice of concern is one followed by many small-scale and medium-scale farmers specifically in the New England area where they disassemble regular washing machines and convert it into a centrifugation machine for spin drying produce such as leafy greens. Hence, *Listeria innocua* (a leading cause for foodborne deaths) was used as a model to determine the degree of microbial spread in different parts of the retrofitted machine. The work presented here suggests that there is a high possibility of risk as the microbial recovery was in high numbers when enumerated from different parts of the machine. It was also established that there a transfer to fresh produce from contaminated machine, which is a concern for *Listeria* as it is psychrotrophic, able to persist environmentally and forms persistent biofilms. In addition to retrofitted washing machines, this work was also aimed at determining the microbial contamination efficiency of a commercially available spin-drying machine used for drying produce such as leafy greens. The work presented here suggests that the microbial recovery was significant but lower when compared to the retrofitted machine. The main aim of this research is focused on establishing a standard sanitation operating procedure’s (SSOP’s) aimed at informing best practice to local producers. Hence, FDA and EPA recommended food contact surface approved cleaning and sanitation practices were evaluated. The results suggest a microbial reduction to below Limit of Detection (LOD) post cleaning and complete removal of the residual microorganisms post application of food contact surface approved sanitizers such as peroxy acetic acid (110 ppm) and
sodium hypochlorite (200 ppm). However, interestingly, it was not possible to obtain a complete removal of the residual microorganisms post sanitation in the bottom layer of the commercial machine and this was achievable in the retrofitted machine.

Human norovirus is the leading cause for foodborne illness globally accounting for 200,000 deaths annually. One considerable challenge to their sanitation control is their general resistance to many commonly used inactivation agents such as UV radiation, ethanol, and chlorine-based treatments. Researchers are increasingly working towards evaluating the potential for alternative plant-based therapies such as bioactive phytocompounds present in essential oils such as cinnamaldehyde. They are generally recognized as safe (GRAS) and is approved for food use (21 CFR 182.60) by the Food and Drug Administration (FDA) to impart flavor in numerous foods. Hence, this research aimed at investigating an alternative way to evaluate the inactivation of viruses in the environment/foods and especially on food contact surfaces. It was also important to formulate an enhanced delivery system such as cinnamaldehyde incorporated cationic nanoemulsion to ensure the disinfectant particles were able to disrupt the negatively charged viral protein. Previous work had demonstrated that restructuring disinfectants into charged nanoemulsions can enhance inactivation of bacteria and fungi, but their effect on viruses is unknown. A low energy method Phase inversion nanoemulsion formulation method was established. Shelf-life study of the nanoemulsion suggested that the optimum particle size (100nm) and ζ-potential (15nm) was achieved when the disinfectant was stored at 4°C. The virus was treated with different concentrations of nanoemulsion and plain oil (0.5% -3.5%) by suspension assay. Significantly less reduction of MS2 was observed when treated for 1 hour with cationic cinnamaldehyde
nanoemulsion when compared to oil. Human norovirus is a non-enveloped virus; hence, comparative study was conducted to evaluate the efficiency of the disinfectant against enveloped virus human coronavirus-229E (HCoV-229E). Alternatively, significant reduction of coronavirus-229E was observed with treatment of cinnamaldehyde nanoemulsions even when compared to just oil. This result suggests that the efficiency of the essential oil as a disinfectant against coronavirus-229E can be increased when it is incorporated in a nanoemulsion at the appropriate concentration. These data suggest that one potential reason for the reduced efficacy against MS2 bacteriophage in comparison to coronavirus-229E could be that the nanoemulsions are too lipophilic for nonenveloped viruses regardless of the presence of hydrophobic patches on the viral capsid protein, and thus only offer benefit with enveloped viruses. Further work should be continued to determine the efficiency against actual food contact surfaces.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>4</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>7</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>15</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>16</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 2: LITERATURE REVIEW</td>
<td>26</td>
</tr>
<tr>
<td>Abstract</td>
<td>26</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>27</td>
</tr>
<tr>
<td>2.2 Gaseous and Vapor-Based Viral Inactivation Strategies</td>
<td>30</td>
</tr>
<tr>
<td>2.3 Radiation-based Treatments:</td>
<td>33</td>
</tr>
<tr>
<td>2.4 Solution-based Treatments:</td>
<td>38</td>
</tr>
<tr>
<td>2.4.1 Chlorine Inactivation:</td>
<td>38</td>
</tr>
<tr>
<td>2.4.2 Calcium Hydroxide:</td>
<td>40</td>
</tr>
<tr>
<td>2.4.3 Common household sanitizers:</td>
<td>41</td>
</tr>
<tr>
<td>2.5 High Pressure Processing:</td>
<td>43</td>
</tr>
<tr>
<td>2.6 Thermal Inactivation:</td>
<td>48</td>
</tr>
<tr>
<td>2.7 Inactivation During Natural Processes:</td>
<td>49</td>
</tr>
<tr>
<td>2.8 Self-Sanitizing Surfaces:</td>
<td>52</td>
</tr>
<tr>
<td>2.9 Other Inactivation Agents:</td>
<td>54</td>
</tr>
<tr>
<td>CHAPTER 3: EVALUATING THE POTENTIAL FOR CONTAMINATION OF LEAFY GREENS WITH LISTERIA WHEN USING RETROFITTED WASHING MACHINES Pragathi Kamarasu¹, Lynne McLandsborough¹, Matthew D. Moore¹*, Amanda J.Kinchla¹* .................................................................................................................. 58</td>
<td></td>
</tr>
<tr>
<td>3.1 Introduction:</td>
<td>58</td>
</tr>
</tbody>
</table>
3.2 Materials and methods: ................................................................. 61
  3.2.1 Materials: .................................................................................. 61
  3.2.2 Source, procure and build the DIY Converted washing machines: ................................................................. 61
  3.2.3 Bacterial inoculum preparation: .................................................. 63
  3.2.4 Determination of microbial transfer efficiency: ....................... 63
    3.2.4.1 Spin drying of produce: ........................................................ 63
    3.2.4.2 Determination of degree of contamination from inoculated produce to contact points: .......................... 64
    3.2.4.3 Determination of degree of contamination from contaminated washing machine to un-inoculated produce: 65
  3.2.5 Statistical analysis: ................................................................. 66
3.3 RESULTS: ..................................................................................... 67
  3.3.1 Determination of degree of contamination from inoculated produce to contact points: ................................. 67
  3.3.2 Determination of degree of contamination from contaminated washing machine to un-inoculated produce: 69
3.3 DISCUSSION AND CONCLUSION: ............................................. 70
3.4 Supplementary material: .............................................................. 75

CHAPTER 4: Evaluating the influence of different cleaning and sanitation interventions on microbial and ATP recovery in an emerging leafy green processing apparatus........... 76
4.1 INTRODUCTION: ......................................................................... 76
4.2 Materials and methods: ............................................................... 80
  4.2.1 Materials: .................................................................................. 80
  4.2.2 Source, procure and build the DIY Converted washing machines: ................................................................. 81
  4.2.3 Bacterial inoculum preparation: .................................................. 82
  4.2.4 Determination of microbial transfer efficiency: ....................... 82
    4.2.4.1 Spin drying of produce: ........................................................ 82
    4.2.4.2 Determination of degree of contamination from contaminated washing machine to uninoculated produce: 84
  4.2.5 Post spin drying cleaning: .......................................................... 84
  4.2.6 Post spin drying sanitation: ......................................................... 86
    4.2.6.1 Chlorine based sanitizer: ...................................................... 87
    4.2.6.2 Peroxy acetic acid-based sanitizer: ...................................... 87
  4.2.7 ATP Lumitester sanitation indicator: ......................................... 87
  4.2.8 Statistical analysis: ................................................................. 88
4.3 RESULTS: ..................................................................................... 89
  4.3.1 Observed contamination of the modified washing machines by contaminated leafy greens: ................................. 89
  4.3.2 Contamination of clean spinach leaves by contaminated modified washing machines: .................................. 90
4.3.3 Evaluation of the effect of cleaning on Listeria contamination and ATP signal in modified washing machines: ..........................91
4.3.4 Evaluating the influence of different commercial sanitizers after cleaning on microbial load and ATP values of contaminated modified washing machines: ...................................92
4.4 DISCUSSION AND CONCLUSION: ......................................................95

CHAPTER 5: Commercial and retrofitted machine comparative microbial transfer efficiency analysis with *Listeria innocua* .................................................101
5.1 Introduction: .......................................................................................101
5.2 Materials and methods: .................................................................104
5.2.1 Materials: .....................................................................................104
5.2.2 Source, procure and build the DIY Converted washing machines: ........................................................................104
5.2.3 Bacterial inoculum preparation: ..................................................106
5.2.4 Determination of microbial transfer efficiency: .........................106
5.2.4.1 Spin drying of produce: ...............................................................106
5.2.4.2 Determination of degree of contamination from inoculated produce to contact points: .........................................................107
5.2.4.3 Determination of efficiency of risk reduction cleaning and sanitation practices: .................................................................109
5.2.5 Statistical analysis: .................................................................111
5.3 Results: .............................................................................................111
5.4 DISCUSSION AND CONCLUSION: ..................................................115

CHAPTER 6: Inactivation of Foodborne Viruses by Cinnamaldehyde Nanoemulsions Require a Lipid Envelope ..................................................121
6.1 Introduction: .......................................................................................121
6.2 Materials and methods: .................................................................126
6.2.1 Materials: .....................................................................................126
6.2.2 Preparation of cinnamaldehyde essential oil nanoemulsions: ........126
6.2.3 Shelf-life study of the cinnamaldehyde nanoemulsion: ................128
6.2.4 Antimicrobial activity: .................................................................128
6.2.4.1 MS2 Bacteriophage: .................................................................128
6.2.4.2 HUH7 tissue culture for 229E propagation and antiviral studies: ........................................................................129
6.2.4.3 Cytotoxicity assay: .................................................................130
6.2.5 Statistical analysis: .................................................................131
6.2.3 Results: ..........................................................................................131
6.2.3.1 Characteristics of nanoemulsion: ............................................131
6.2.3.2 Antiviral activity against MS2 Bacteriophage: .......................134
Cinnamaldehyde oil treatment: .......................................................134
Cinnamaldehyde nanoemulsion: .....................................................134
6.2.3.3 Antiviral activity against human Coronavirus-229E: ...............135
6.2.4 DISCUSSION AND CONCLUSION: ................................................136
BIBLIOGRAPHY: ..................................................................................................................140
LIST OF TABLES

Table 1: Microbial recovery post inoculation on a range of volume of spinach. ..... 75

Table 2: ATP recovery range from the various contact points post spin drying; post application of cleaning methods and post application of chlorine and peroxy acetic acid sanitizers when measuring with CT and A3T Lumitester machines......................95

Table 3: Comparative study differences between Retrofitted washing machine and Commercial Green machine based on various factors........................................121
LIST OF FIGURES

Figure 3.1: Depiction of the steps involved in building a retrofitted washing machine. A) retrofitting a washing machine and B) adding loading baskets to reduce contact exposure to C) Adding leafy greens to the 3 layered retrofitted machine D) Operating as a dry triple-washed produce (UVM- University of Vermont Extension Agricultural Engineering n.d.)………………………………………62

Figure 3.2: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine; direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3 contact points…………………………………………………………65

Figure 3.3:A) The graph depicts the specific recovery from contact point B2 for a range of inoculation microbial load ($10^2$-$10^6$) post loss of 3 log CFU/ml in wash step (left) ; B) Microbial recovery from the various contact points (right)………………………………………69

Figure 3.4: A) Microbial recovery from the various contact points after inoculation of $10^3$ CFU/ml post wash step; B) Microbial recovery from the various contact points after inoculation of $10^6$ CFU/ml post wash step…………………………………………………………70

Figure 3.5A;5B: Microbial recovery from the various contact points after inoculation of $10^3$ CFU/ml of *Listeria innocua* directly on the B2, L2, R2 contact points; Microbial transfer to produce from the 3 inoculated layers of the machine………………………………71

Figure 4.1: Depiction of the steps involved in building a retrofitted washing machine. A) retrofitting a washing machine and B) adding loading baskets to reduce contact exposure to C) Adding leafy greens to the 3 layered retrofitted machine D) Operating as a dry triple-washed produce………………………………………………79

Figure 4.2: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine; direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3 contact points…………………………………………………84
Figure 4.3: Graphical representation of mode of action of cleaning of food contact surfaces........................................87

Figure 4.4: Graphical representation of mode of action of sanitizing food contact surfaces........................................87

Figure 4.5: Comparative study between two commercially available Lumitester machines and swabs.......................89

Figure 4.6: Microbial recovery from the various contact points; The graph depicts the specific recovery from contact point B2 for a range of inoculation microbial load (10^2-10^6) post loss of 3 log CFU/ml in wash step.................................................................91

Figure 4.7, 4.8 ATP measurement from all the contact points of the machine for an initial microbial load of 10^3 and 10^6 CFU/ml of Listeria innocua.................................................................91

Figure 5.1: Comparative study between commercial green spin-drying machine (CGM) (left) (Electrolux Professional) and retrofitted spin-drying washing machine (RWM) (right) (Speed Queen TC5000) ..........104

Figure 5.3A: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine (RWM); direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3..............................................109

Figure 5.3B: Images of the two different layers and the contact points assigned to each layer in the commercial green spin-drying machine (CGM); direct contact (in built loading basket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points.............109

Figure 5.6A) Microbial recovery from the various contact points in RWM after inoculation of 10^3 CFU/ml post wash step; B) Microbial recovery from the various contact points in RWM after inoculation of 10^6 CFU/ml post wash step. Ns- not significant difference in the means of the individual groups. *-statistically similar individual means of the groups........................................113

Figure 5.7A) Microbial recovery from the various contact points in CGM after inoculation of 10^3 CFU/ml post wash step; B) Microbial recovery from the various contact points after inoculation in CGM
of 10^6 CFU/ml post wash step. *- statistically similar individual means of the groups…………………………………………………114

Figures 5.8: A) Microbial recovery from the contact points B1, B2 and B3 after spin drying, post cleaning and post sanitation in CGM;

B) Microbial recovery from the contact points B1 and B2 after spin drying, post cleaning and post sanitation in RWM…………………………116

Figure 6.1: The low energy emulsification, spontaneous emulsion method is depicted showcasing the spontaneous droplet formation process.

Figure 6.2: The nanoemulsion formation takes place in two stages. Initial discontinuous microemulsion phase and followed by from one phase to another leads to the generation of supersaturated regions that promotes nucleation and droplet growth phenomena….128

Figure 6.3: The nanoemulsion stored at higher temperature was the brightest and there was an increase in the milkiness appearance of the emulsion…………………………………………………133

Figure 6.4;6.5: Nanoemulsion particle size diameter (nm) recorded when stored at 4 °C, 20 °C and 37°C for a period ranging from 0-28 days; Nanoemulsion ζ- Potential recorded when stored at 4 °C, 20 °C and 37°C for a period ranging from 0-28 days…………134

Figure 6.6; Figure 6.7: MS2 Bacteriophage microbial reduction post treatment with a range of cinnamaldehyde oil (0.5%-3.5%); MS2 Bacteriophage microbial reduction post treatment with a range of cinnamaldehyde nanoemulsion (0.5%-3.5%)………………136

Figure 6.8; Figure 6.9: Coronavirus-229E microbial reduction post treatment with a range of cinnamaldehyde oil (0.5%-2.5%); Coronavirus-229E microbial reduction post treatment with a range of cinnamaldehyde nanoemulsion (0.5%-3.5%)………………137
INTRODUCTION

The World Health Organization (WHO) estimates that the consumption of contaminated food results in about 600 million cases of foodborne illness, which are responsible for 420,000 deaths every year (WHO, 2020). The estimated economic impacts of these illnesses range between $51.0 and $77.7 billion annually (Scharff 2012).

Pathogen contamination of produce can occur at any point in the production chain, with one area of concern being cross contamination of produce from processing equipment (Costa et al. 2006). Food contact surfaces that are contaminated by bacterial pathogens from already contaminated food commodities and the water in which they have been in contact, as well as those contaminated via contact with other external pathogen sources, have been recognized to be one of the significant factors leading to foodborne illnesses (Stuart and Worosz 2012). Previous foodborne outbreaks and recalls of fresh produce have been linked to cross-contamination with *L. monocytogenes* within processing facilities. Specifically, in two of the most lethal produce-related outbreaks, the 2011 cantaloupe and the 2014 caramel apple listeriosis outbreaks, food contact surfaces (FCS) of packing equipment were identified as routes of contamination with *L. monocytogenes* (Angelo et al. 2017). Additionally, a recent recall of fresh-cut fruit was attributed to equipment surfaces that tested positive for *L. monocytogenes* (2020 n.d.).

*Listeria monocytogenes* is a leading cause of foodborne death, often causing illness in
neonates, pregnant women, and immunocompromised adults (CDC). The environment where produce is manipulated, such as food contact surfaces or equipment used in processing, can also play a role in *L. monocytogenes* contamination (Maurel et al. 2016).

This is of particular importance for *Listeria*, as it has been well known to be able to environmentally persist in food processing plants and equipment, often in the form of difficult to remove biofilms.

Regular cleaning and sanitizing of processing equipment plays a major role in mitigating the potential for cross contamination. Additionally, with the recent implementation of the Food Safety Modernization Act in 2011, there are several new regulations that specifically define sanitary regulatory compliance, including the requirement of cleaning and sanitation of the produce processing environment under the Food Safety Modernization Act’s Produce Safety Rule (FDA 2015). However, available trainings and other support only offers basic postharvest practices that help to reduce risk as there is a dearth of research conducted to support the implementation of Sanitation Standard Operating Procedure’s (SSOPs) for standardizing the cleaning and sanitation procedures for many applications. Writing SOPs help outline what areas need to be cleaned, how often, which detergents and sanitizers to use, how to clean and/or sanitize the surface, and who is responsible for completing the task (FDA 2015). Furthermore, such research to support cleaning and sanitation SOPs for practices more uniquely utilized by small and medium-sized processors can especially be lacking.

Producers have increasingly begun retrofitting washing machines by disassembling them and replacing the barrel of the machine with crates to turn the machine into a large salad spinner using its spin cycle. This practice is cost efficient, user friendly, and large
volumes of produce are able to be handled in a single run. The DIY retrofitted washing machine are built with the help of the University of Vermont’s Agricultural Engineering Extension team. A detailed conversion guide is available online (University of Vermont-Retrofitted washing machine conversion guide). Based on the steps mentioned, the different parts of the washing machine such as the panels are taken apart and rewired to only keep the central water collecting drum and the brake system intact. Additionally, a stopwatch timer was attached to the machine to keep the time of each spin-drying cycle while drying the leafy greens, to ensure consistency. Equipment cross contamination does not only occur with adapted processes but there are also instances of microbial contamination spread from the equipment to the produce leading to food recalls due to the usage of well-established equipment’s during any of the farm to fork stages of handling produce. One such produce that is commonly responsible for outbreaks due to microbial contamination are leafy greens. They are regularly colonized by diverse microbiota and can become contaminated with human pathogens and parasites while growing in the field or during harvesting, postharvest handling, processing, and distribution (Beuchat et al. 2003)(Matthews et al. 2012)(Van Boxstael et al. 2013). To control, reduce or eliminate microbial food safety hazards of fresh produce, effective food safety strategies are needed to be implemented throughout production, processing, and distribution (Luning et al. 2008). Microbial food safety hazards and source of contamination varies significantly based on the type of crop, production systems and practices (Warriner n.d.). One main area of focus is during the post-harvest handling of produce as the key characteristic of these operations is that they involve considerable contact between fresh produce and handlers, tools, equipment surfaces, water and the
field environment (Beuchat et al. 2003) (Sapers n.d.). Hence, following strict good agricultural practices (GAP) is a primary priority. During post-harvest handling of leafy greens such as baby spinach they are harvested into bins for transport to the processing facility. Placing the bins directly onto soil can result in contamination. In addition, the bins are also used during washing and drying of the leafy greens and can lead to further contamination from various contributing factors (Matthews et al. 2012).

There are many sanitizers commonly recommended for use on food contact surfaces. It is imperative to ensure the sanitizers used are maintained below the recommended concentration as they are air dried and they come in contact with food (FDA 2015) (FDA-CFR-Title 21 n.d.). However, these sanitizers are not efficient against foodborne illness causing virus such as human norovirus. Human norovirus is the leading cause for foodborne illness globally accounting for 200,000 deaths annually. Noroviruses also account for the majority of foodborne illnesses, as well as the fourth leading cause of foodborne death. It is a single stranded positive sense RNA, non-enveloped virus of the Caliciviridae family. One considerable challenge to their control is their general resistance to many commonly used inactivation agents such as UV radiation, ethanol, and chlorine-based treatments. Norovirus consists of a negatively charged, stable protein capsid concealing an RNA genome. From previous research it can be seen that there are a few efficacious disinfectants for noroviruses, many of which inactivate virus by oxidizing the non-enveloped protein capsid, however, the challenge faced is that none of them are food grade (P. Kamarasu, Hsu, and Moore 2018). Alternative plant-based therapies such as bioactive phytocompounds present in essential oils (EO) are being explored. EO’s are generally recognized as safe (GRAS) and is approved for food use (21 CFR 182.60) by
the Food and Drug Administration (FDA) to impart flavor in numerous foods. Hence, they could be considered an alternative way to impede the proliferation of foodborne pathogens, especially on food contact surfaces. From research conducted by Calo et al (Calo et al. 2015) it was seen that EOs comprise numerous compounds such as aromatic hydrocarbons, terpene (monoterpenes and sesquiterpenes), terpenoids, esters, alcohols, acids, aldehydes and ketones, and their antibacterial activity is not solely contributed by any one compound. A challenge faced by the researchers has been evaluating the most potent antimicrobial compound due to their chemical complexity. To date, most studies have focused on studying the antimicrobial activity of EOs with little discussion on activity of individual components in the EO or their mechanism of action. It was also noted that antimicrobial activity of EOs is not reliant on one specific mode of action; instead, EOs can attack several targets in a cell to inactivate it (Hyldgaard et al. 2015). One such efficient essential oil is acquired from the bark of cinnamon and the primary compound is cinnamaldehyde, which makes up 90% of the essential oil. Cinnamon essential oils have displayed antimicrobial, antitumor, anti-inflammatory and antioxidant activities (Angane et al. 2022). Nanoemulsions have been used increasingly in pharmaceutical, food and cosmetic industries due to their advantage over traditional emulsions. They have the ability to improve the appearance, stability, release properties and bioavailability of the active compounds. One major drawback of incorporating cinnamaldehyde in food systems are their low water solubility and strong odor which can be overcome by incorporating the compound in a nanoemulsion (Ozogul et al. 2022).

Further, some work has demonstrated that incorporation of EOs or their active ingredients in nanoemulsions can enhance their efficacy against bacteria and fungi.
Specifically, research conducted by Paudel SK et al resulted in enhanced inactivation compared to similar concentration of cinnamon essential oil in more than 7.7 and 5.5 log reduction of *Listeria monocytogenes* and *Salmonella enterica* Typhimurium respectively (Paudel, Bhargava, and Kotturi 2019). This increase in inactivation efficiency can be attributed towards the nanomolecular size of the emulsion particles which enhances surface area relative to volume of active ingredient used and makes them easier to reach the target and in turn inactivate target cells, as well as the introduction of charge in the emulsions that can enhance their delivery to the pathogen target. Although the targets mentioned above have a lipid envelope, allowing for a strong attraction of the lipophilic nanoemulsion to the target, the norovirus capsid has hydrophobic protein patches in the capsid protein that are involved in folding and maintaining icosahedral contacts that keep the viral capsid intact. The potential of these nanoemulsions to better deliver inactivation agent to these hydrophobic patches and disrupt capsid stability has not been tested. The long-term goal of this research is focused on developing Sanitation Operating Procedure’s to inform best practices. To reach this goal the overall objectives of this research was achieved by focusing on these specific aims:

1. Evaluating the potential for contamination of leafy greens with *listeria* when using retrofitted washing machines.
2. Evaluating the influence of different cleaning and sanitation interventions on microbial and ATP recovery in an emerging leafy green processing apparatus.
3. Commercial and retrofitted machine comparative microbial transfer efficiency analysis with *listeria innocua*.
4. Inactivation of foodborne viruses by cinnamaldehyde nanoemulsions require a lipid envelop.
CHAPTER 2

2. RESEARCH PROGRESS IN VIRAL INACTIVATION UTILIZING HUMAN NOROVIRUS SURROGATES

KAMARASU, PRAGATHI, HAO-YUAN HSU, AND MATTHEW D MOORE.


Abstract:

Human noroviruses are the leading cause of foodborne illness globally. Numerous challenges in control of these viruses exists due to multiple viral characteristics: the ability to environmentally persist for over a month; relatively low infectious dose; lack of extremely effective, noncorrosive inactivation agents; and considerable inhibitory effects of food matrices and organic loads observed with common use of promising inactivation agents. Although a major breakthrough in the field, recent in vitro human norovirus cultivation systems have been limited in their ability to be widely utilized for identification of promising inactivation agents. Thus, cultivable human norovirus surrogates remain the most readily utilizable models for studying the effect of various inactivation agents on viral infectivity. The purpose of this review is to highlight recent developments and reports related to norovirus surrogate inactivation with a special focus on the various degrees of food matrix-associated inhibition for different inactivation agents.
2.1 Introduction

Human norovirus is the leading cause of foodborne illness globally, accounting for 684 million foodborne illnesses and 212,000 foodborne deaths annually (Kirk et al. 2015). It is a single stranded positive sense RNA, nonenveloped virus of the Caliciviridae family (Alshraiedeh et al. 2013). The viruses have an icosahedral capsid composed of 180 copies of a major capsid protein, VP1, with a few copies of a minor capsid protein, VP2 (Moore, Goulter, and Jaykus 2015). One considerable challenge to their control is their general resistance to many commonly used inactivation agents (Kirsten A. Hirneisen et al. 2010). Further, study and identification of norovirus inactivation has been complicated by the historical lack of an in vitro cultivation system for the virus. Two in vitro cultivation models have been reported (Ettayebi et al. 2016), however their availability and ability to be routinely utilized in the study of viral inactivation is presently limited. Because of this, a number of morphologically similar and cultivable virus surrogates have been used to evaluate the effectiveness of various chemical disinfectants and physical processes. Among them, feline calicivirus (FCV) (Hoover and Kahn 1975) and murine norovirus (MNV) (Karst et al. 2003) have been most widely
used as surrogates for norovirus for inactivation studies (K. A. Hirneisen, MARKLAND, and KNIEL 2011). Feline calicivirus was first reported and chosen as a surrogate because it is in the same family as human norovirus, cultivable, and shared similarities with human norovirus in primary sequence (X jiang n.d.). However, FCV causes respiratory illness in cats, hence it may not be as easily translated to human noroviruses with the study of certain disinfectants (CANNON et al. 2006) (T. L. Cromeans, Kahler, and Hill 2010) (Richards 2012). MNV was subsequently reported, and is more genetically related to human norovirus than FCV, as it is in the norovirus genus (Wobus, Thackray, and Virgin 2006).

But some disadvantages of MNV include the fact it utilizes a different putative receptor than human norovirus and does not display intense diarrhea and vomiting in mice despite being shed fecally (CANNON et al. 2006) (Karst et al. 2003) (Taube, Jiang, and Wobus 2010). More recently, Tulane virus (Farkas et al. 2008) has been utilized and belongs to a newly proposed genus, Recovirus. Like human norovirus, TV binds to histo-blood group antigens and causes diarrhea in rhesus macaques (Farkas et al. 2008) (Zhang et al. 2015). Additionally, other surrogates such as bacteriophage MS2 (Lacombe et al. 2015) and echovirus (Schiff et al. 1984) have also been used to study inactivation. In addition to viral surrogates, infectious human norovirus from human fecal specimens may also be used in combination with RT-qPCR. However, there are a number of challenges in relating reduction of RT-qPCR signal to reduction of infectious virus observed using cultivable surrogates that will be discussed below and has been reviewed elsewhere (Knight et al. 2013) (Serafino et al. 2008) (Moore, Goulter, and Jaykus 2015). Although surrogates provide valuable information related to reduction in viral infectivity, a number of issues related to the ability to extrapolate the results of surrogate studies to human
norovirus exist (Richards 2012). Specifically, notable differences in susceptibility of
different surrogates to different types of inactivation treatments has been a setback, as one
specific surrogate that is the most resistant to a wide variety of inactivation treatments
has not been identified. Similarly, translation of inactivation observed using human
norovirus in fecal specimens has been challenging, as RT-qPCR results tend to
underestimate the degree of inactivation of virus because signal is still obtained from un-
encapsulated RNA, RNA in damaged/nonfunctional capsids, and RNA with fatal
mutations (Knight et al. 2013) (Manuel, Moore, and Jaykus 2018). Although effective
against many vegetative bacteria, most commercially available and commonly used
disinfectants have not displayed ideal efficacy against noroviruses and their
surrogates (Kirsten A. Hirneisen et al. 2010). Although bleach has been demonstrated to be
effective against noroviruses including human noroviruses (T. Cromeans et al. 2014), its
utilization is limited by its unwanted effects on fabrics and stainless steel. Another major
challenge related to foodborne virus inactivation has been to identify disinfectants that are
more resistant to the effects of organic load on inactivation than current agents. Although
frequently discussed, to the authors’ knowledge no review exists with a focus on
summarizing and compiling the effects of organic load on inactivation. While still a
recalcitrant issue, a number of studies reporting novel inactivation agents for norovirus
have been reported since the last comprehensive reviews of foodborne viral
inactivation (DiCaprio 2017). The purpose of this review is to present recent reports of
novel inactivation agents with a focus on the effects of organic load for studies that utilize
norovirus surrogates.
2.2 Gaseous and Vapor-Based Viral Inactivation Strategies

There are various forms of gaseous or vapor treatments employed against microbes in the food industry. Ozone treatment has been reported and evaluated as a novel alternative treatment to traditional methods like chlorine treatment. One advantage of ozone treatment is its limited effects on the environment, as ozone treatments rapidly dissociate into oxygen and do not produce the toxic byproducts that are generated from disinfectants like chlorine (Kirsten A. Hirneisen et al. 2010). Ozone is a strong oxidizer that has been shown to inactivate a wide range of foodborne pathogens (Sharma and Demirci 2002).

It can act as a disinfectant both in the gaseous state as well as dispersed in water. Gaseous ozone is produced by corona discharge from compressed purified oxygen using an ozone generator. The ozone generated is then pumped to the desired location under controlled conditions (Wang, Maryland, and Kniel 2015). Although there have been a lot of studies conducted on assessing the effects of ozone on bacterially contaminated produce, there is a lack of research on the inactivation of viruses by ozone on fresh produce (K. A. Hirneisen, MARKLAND, and KNIEL 2011) investigated the effects of aqueous ozone (not in gaseous form) applied against FCV and MNV on green onions and lettuce and in sterile water. Not surprisingly, the produce matrix was one of the largest variables in the efficacy of the treatment. For instance, to achieve 2-3 log inactivation of FCV with 6.25 ppm ozone, 5 to 10 mins was required with produce samples while only 1 min was required in water. Overall, treatment with 6.25 ppm of ozone for viruses showed promise in buffer (>4 log reduction after 5 min treatment) and to some degree on produce (2-3.8 log reduction after 10 min of 6.25ppm) (Table 1). MNV and FCV showed comparable susceptibility to the treatment on produce samples (K. A. Hirneisen, MARKLAND, and KNIEL 2011). Further, Lim et al (Wolkoff and Nielsen 2017)
investigated the effects of pH and temperature on ozone inactivation of MNV. Interestingly, pH did not appear to have a significant effect on inactivation, but cooler temperatures tended to result in higher reduction of MNV (5°C versus 20°C), although the effect was not statistically significant (Table 1). In an interesting study by Predmore et al (Predmore et al. 2015), the ability of gaseous ozone to inactivate MNV and TV both internalized and deposited on the surface of strawberries and lettuce was investigated. After 40 minutes of treatment, there was a significantly lower log reduction internally (~1.5 log and 1 log for MNV and TV) than externally (3.3 log and >6 log for MNV and TV). This suggests that although gaseous treatments have potential to inactivate virus internalized in produce, one or more unknown factors limit its efficacy. In another study of gaseous ozone on produce, Brie et al (Brié et al. 2018) report promising inactivation of MNV on raspberries (>3 log reduction in 1 min with 3 ppm gaseous ozone); however, hepatitis A virus was also investigated and little inactivation (maximum of <1 log reduction) was observed. Interestingly, higher inactivation of MNV was observed on raspberries (>3.3 log) than in PBS (1.1 log) for the same treatment; suggesting that the raspberry matrix may enhance inactivation of MNV (Table 2). It is possible other components of the raspberries (organic acids, discussed below) could have enhanced inactivation. However, it could be possible that raspberry inactivation results may have been confounded by reduced elution efficiency from the berry as MNV may have bound carbohydrates on the raspberry surface. Given the interesting results observed, further investigation into the mechanisms of enhanced inactivation on raspberries observed would be of value. Overall, the results observed for ozone suggest that ozone inactivation of viruses is variable and depends largely on factors such as the nature of the matrix and virus being studied (Brié et al. 2018). Another gaseous treatment for viral
inactivation is atmospheric cold plasma. It is an emerging non-thermal technology that is chemical- and water-free. Typically, the cold plasma can be generated at atmospheric pressure using corona discharge, dielectric barrier discharge, radiofrequency plasma or gliding arc discharge (Aboubakr et al. 2015). Atmospheric plasma had shown promise in inactivation of bacteria, yeasts, molds and biofilms that are generally very difficult to inactivate (Lacombe et al. 2015) (Alshraiedeh et al. 2013) studied the effect of atmospheric pressure nonthermal plasma on bacteriophage MS2 with different percentages of oxygen gas added in the generation of the plasma (0-1% oxygen with the rest being helium carrier gas). Overall, the most reduction was observed for treatments with 0.75% oxygen, as nearly a 4 log (3.90) reduction was observed after 5 min, and ~7 log reduction was observed after 9 min.

The higher reduction with less oxygen (0.75% > 1% oxygen) was suspected to be due to the increasing oxygen concentration influencing the plasma production itself (Alshraiedeh et al. 2013). Another study by Aboubakr et al (Aboubakr et al. 2015) reported >6 log reduction of FCV when plasma generated with 1% oxygen was applied for 90 seconds, a reduction that is considerably larger than many other norovirus inactivation methods. Lacombe et al (Lacombe et al. 2017) further investigated the ability of cold plasma to non-thermally inactivate TV and MNV on the surface of blueberries (keeping temperature-time treatments below that required to kill enteric viruses), which achieved a maximum reduction of about 3.5 log and >5 log PFU/g reduction of TV and MNV on blueberries with a maximum treatment time of 2 mins when ambient air was introduced (Table 1). Although it has demonstrated considerable promise as a norovirus control agent, further
study of atmospheric cold plasma is warranted to further characterize and elucidate any discrepancy in results between different surrogates and on different matrices.

2.3 Radiation-based Treatments:

Irradiation has become a standard process used to sterilize many consumers and medical products, including some application in the food industry (Predmore et al. 2015) (Tauxe 2001). Radiation-based techniques for treatment of foods have numerous advantages such as the generally high efficacy against bacteria and maintenance of the treated food quality. The safety of consuming irradiated foods have been evaluated in large scale trials in animals with no ill effects observed, and, no teratogenic effects (Samuel 2007). The most extensively investigated radiation-based treatments include gamma radiation, electron beam and X-rays. High energy X-rays are produced when an electron beam is applied to metal foil. Like gamma rays, a beam of X-rays can penetrate foods to a much greater depth than electron beams (Tauxe 2001). Gamma radiation is commonly produced using cobalt 60 and cesium 137, with rays that are generated during the isotopic negative beta decay of radioactive isotopes. Gamma radiation also has the powerful advantage of being completely able penetrate through the food, thereby killing both microbes on the surface as well as those internalized without substantially affecting the quality of the treated food (De Roda Husman et al. 2004). Electron beams are produced by passing electrons through high voltage electrostatic fields prior to application to the target food or surface. Unlike gamma radiation, electron beam radiation has shown more promise to be utilized for inactivation of surfaces, as it is not capable of completely penetrating
foods. The efficacy of both electron beam and gamma radiation in both buffer and on whole strawberries was evaluated for TV by DiCaprio et al (DiCaprio 2017). Overall, electron beam treatment did not appear as effective as gamma irradiation. For electron beam inactivation, a fairly high dose of exposure (about 11 kGy) was required to obtain a 4-log reduction in buffer (PBS/DMEM), with an even higher amount required on strawberries (~15 kGy) (Table 2). A lower dose of gamma radiation was required to achieve the same level of inactivation in buffer (~9 kGy). DiCaprio et al and Park et al studied the effect of gamma irradiation on reduction of MNV inoculated onto edible green and brown algae, fulvescens (Capsosiphon fulvescens) and fusiforme (Hizikia fusiforme) (DiCaprio et al. 2016) (S. Y. Park, Kang, and Ha 2016). Generally, minor reductions of viral titer were observed, as there was 1.2-2.5 log reduction in fulvescens and 0.4-2.2 log reduction in fusiforme after electron beam treatments of 3 to 10 kGy. Additionally, the sensory characteristics such as the color and appearance of the seaweed were analyzed, with no significant difference noted. Similar results were observed for electron bean reduction of FCV on lettuce, with only a maximum of 2 log reduction observed (ZHOU et al. 2011). The FDA has approved a maximum dose of 4 kGy of gamma irradiation for treatment of fresh produce, which has been effective for bacteria but appears not to be as effective for norovirus surrogates, including novel surrogates like vesicular stomatitis virus (Feng et al. 2011) (Table 1). UV light treatment has historically been widely utilized by water treatment plants, has also been used for seawater disinfection in depuration tanks (Garcia, Nascimento, and Barardi 2014). Depuration is a process where specific organisms (in this case bivalve mollusks) are placed into a large area or tank of clean water to allow for expulsion and diffusion/removal of any
contaminants that may have been present on the organism (in this case viruses) to be removed. This is particularly relevant, as molluscan shellfish are a leading food implicated in human norovirus illness. Garcia et al investigated the ability of UV to reduce MNV and recombinant human adenovirus artificially seeded into depuration water (Garcia, Nascimento, and Barardi 2014). Water samples were taken after the treatment and prior to further chemical disinfection (without residual free chlorine). More reduction of MNV was observed than adenovirus after 24 and 48 hours of treatment, however the treatment was quite effective overall. There was a 4-log reduction of adenovirus and MNV had reached undetectable limits (>6 log reduction) after 24 hours, with adenovirus being reduced to the same levels after 48 hours.

Garcia et al investigated the effects of different variables of UV wavelength and photocatalysis on inactivation of MNV (Garcia, Nascimento, and Barardi 2014) (S. Y. Park, Kang, and Ha 2016). The experiment was conducted on a solidified agar matrix (SAM) used to replicate the matrix of a blueberry. There are multiple types of UV treatment based on the wavelength of the radiation chosen (UV-A, UV-B, UV-C). Additionally, differences in efficacy of UV lamps had been reported for lamps coated and not coated with TiO2. Both of these variables and the effects on reduction of MNV were investigated. UV-C treatment caused a significantly higher reduction of virus than UV-A and UV-B. Additionally, application of TiO2 further enhanced viral inactivation. The mechanism of inactivation was also investigated, and the UV treatment was found to inactivate virus by causing damage to both the capsid protein and genomic RNA (S. Y. Park, Kang, and Ha 2016). Similar observations are reported by Lee and Ko when applying different UV wavelengths to inactivate MS2 with and without TiO2, with UV-A
showing no significant reduction without enhancement of inactivation by including TiO$_2$ (J. E. Lee and Ko 2013). In the case of UV-B, a 4-log reduction was obtained even without the presence of TiO$_2$. UV-C was not investigated (J. E. Lee and Ko 2013). As with other inactivation methods, differences in the susceptibility of different surrogates have also been observed for UV treatment. Park et al compared the UV inactivation of FCV, echovirus 12, and bacteriophage MS2 in suspension. The order of UV resistance from highest to lowest was MS2>echovirus 12>MNV>FCV. The UV dose required to obtain 4 log reduction was 70, 30, 29, and 25 mJ cm$^{-2}$ for MS2, echovirus 12, MNV, and FCV, respectively. Interestingly, viral association with host cells was found to have a protective effect against UV, as cell-associated echovirus 12 was three times more resistant than free virus (G. W. Park, Linden, and Sobsey 2011). Ronnqvist et al also obtained promising results for reduction of MNV on dry glass, observing over a 4 log reduction at 60 mJ/cm$^2$ (Rönnqvist et al. 2014) (Table 1). Because UV treatment alone in some cases has not traditionally achieved the desired levels of viral reduction, Li et al. (2011) investigated the potential use of vapor and liquid hydrogen peroxide in addition to UV treatment for reduction of MNV and bacteriophages X174 and B40-8 (Kitajima et al. 2010). The results obtained suggested that liquid hydrogen peroxide was more effective than the vapor when used without UV treatment, as a 4-log reduction of virus on stainless steel disks was observed at 2% liquid hydrogen peroxide and less than 1 log reduction was observed for vapor hydrogen peroxide treatments for MNV and bacteriophage respectively. However, when used in conjunction with UV there was faster inactivation, as a 4-log reduction of X174 and B40-8 and 3 log reduction for MNV in the case of vapor hydrogen peroxide with UV treatment in about half the time. Additionally, the ability
of the combined hydrogen peroxide-UV treatments to reduce virus in shredded iceberg lettuce was also investigated, with similar trends to liquid versus vapor hydrogen peroxide and with added efficacy for UV- hydrogen peroxide used in combination (Table 1). However, the overall degree of inactivation of MNV and bacteriophages reduced, with 2 log and 1 log maximum reductions observed, respectively (Kitajima et al. 2010) (Table 2). Another radiation-based method is pulsed light treatment, which has been shown to be effective against a broad spectrum of bacteria, viruses, and spores, and is approved by the U.S. Food and Drug Administration for use on food and food contact surfaces (Rowan et al. 1999). Pulsed light inactivation of TV and MNV was recently assessed in PBS and on blueberries and strawberries by Huang et al (Li and Chen 2015). TV displayed higher resistance to treatment than MNV, with maximum reductions of 4.9 log reduction and 5.8 log reduction achieved after 8 pulses for TV and MNV, respectively in PBS. As with other studies, higher inactivation of MNV was seen in PBS than on the surface of the berries when treated with 6 pulses of pulsed light, with 1.5 less log reduction in blueberries and 4 log less reduction in strawberries (Huang et al. 2017) (Table 2). Steam ultrasound is another alternative technology for disinfection of microorganisms on food and food contact surfaces. This technology combines pressurized steam and high-power ultrasound to efficiently remove the boundary air present on the surface of the treated sample by intensifying the air into oscillation. This allows the steam to penetrate into the microstructures and cavities on the surfaces of the treated sample more easily, resulting in a reduction of microorganisms within seconds. Schultz et al. (2012) tested this method against MS2, FCV, and MNV on the surface of raspberries as well as plastic. The highest reduction of >8 log was observed for MS2 on plastic surface
after 3 seconds of treatment. Similarly promising reductions on plastic surfaces were observed for FCV and MNV: 4.5 log and 4 log reductions, respectively. However, a reduction of about 1 log of the MS2 was observed on the surface of the raspberries. Although displaying a promising degree of inactivation on the plastic surface, the treatment was not effective on the surface of the raspberries while also damaging the appearance of the berry (SCHULTZ et al. 2012).

2.4 Solution-based Treatments

2.4.1 Chlorine Inactivation:

Chlorine is a commonly used solution-based disinfectant in the food industry as it is affordable, and generally broadly effective as a biocide. Chlorine in water is used in many forms amongst which hypochlorous acid has been found to be effective against numerous microorganisms (Xu et al. 2014). The main disadvantage in using chlorine-based disinfectants is their ability to form potentially toxic byproducts upon reacting with various matrix-based substances. As it does not react with matrix-based substances to form halogenated byproducts, chlorine dioxide has been widely investigated as a solution-based disinfectant. Lim et al investigated the ability of chlorine and chlorine dioxide to inactivate MNV and MS2 at two different temperatures (5 and 20°C) in oxygen demand free buffer. In the case of chlorine, it took 2 mins to obtain a maximum 2 log reduction in MNV at 5°C, and 1 min to obtain a maximum 2.5 log reduction at 20°C. For MS2 it took 4 mins at 5°C and 1.5 min at 20°C to attain a maximum
4 log reduction. For chlorine dioxide, a 3.5 log reduction was observed for MNV after 1 min at 5°C and 0.5 min at 20°C. For MS2, it took 2 min at 5°C and 0.7 min at 20°C to observe the same reduction (Wolkoff and Nielsen 2017) (Table 1). In addition to MNV and MS2, poliovirus has been used as a cultivable non-enveloped enteric virus used in the study of inactivation. In a study investigating the ability of chlorine to inactivate nonenveloped viruses in water, Kitajima et al inoculated water with poliovirus and MNV prior to chlorination and studied the resulting viral reduction using plaque assay (Kitajima et al. 2010). Two free chlorine concentrations of 0.5 mg l⁻¹ and 0.1 mg l⁻¹ were used. At 0.1 mg l⁻¹, there was more inactivation with MNV (4 log reduction) than poliovirus (3.8 log reduction) after 120 min. Whereas in the case of 0.5 mg l⁻¹ there was 0.5 more log reduction of poliovirus than MNV after 30 minutes.

In an interesting study by de Abreu Corrêa et al, chlorine inactivation of MNV in relatively clean artificial seawater and natural seawater was investigated. More reduction in viral titer in the artificial seawater was observed, suggesting that the presence of various other matrix-associated components in natural seawater reduced the ability of chlorine to inactivate MNV (de Abreu Corrêa et al. 2012) (Table 2). Sodium hypochlorite (bleach) is another form of chlorine that is a recommended disinfectant for human norovirus. In an interesting study by Takahashi et al, the degree to which food residue aids MNV persistence and reduces the efficacy of sodium hypochlorite on stainless steel surfaces was investigated. The food samples used were lettuce, cabbage and ground pork which were applied to stainless steel coupons. In positive control stainless steel coupons without any food residue, complete inactivation with 1,000 ppm (6.2 log MPN/ml) was observed after 30 days whereas in the steel surfaces with food residues, a maximum
1.4 log MPN/ml reduction was observed with a higher concentration of bleach (2,000 ppm). These drastic differences in viral reduction underscore the importance of proper cleaning protocols prior to disinfection in food processing and preparation settings (Takahashi et al. 2011).

### 2.4.2 Calcium Hydroxide:

In addition to chlorine-based treatments, calcium hydroxide is another alkaline microbial inactivation agent, and is available in powder, solution, and suspension forms. It has also been demonstrated to inactivate bacteria even in the presence of organic compounds. Sangsriratanakul et al investigated the degree to which the food grade calcium hydroxide obtained in powder, solution, and suspension forms could inactivate MNV in solution and in the presence of organic material using TCID$_{50}$. Calcium hydroxide in a solution form (0.17%) was highly effective with and without organic load, obtaining over a 4-log reduction in 30 seconds, including the inclusion of 5% fetal bovine serum. Similarly, the 0.17% solution was effective on rayon sheets with 5% fetal bovine serum, achieving over a 4-log reduction in 5 minutes. For suspension assay, 1%, 2.5% and 5% suspensions of calcium hydroxide were applied to MNV spiked into mouse feces, and there were less dramatic reductions of 1.3, 2.3 and 1.5 log reductions within 15 mins, respectively. Calcium hydroxide applied directly in powdered form 10% or 20% (weight/weight) to feces and litter resulted in $\geq$3 log reduction in 30 minutes. These results are promising given the ability of calcium hydroxide to reduce virus in high organic load samples; however, the extremely alkaline nature of calcium hydroxide (pH 13 at 0.17% solution) may limit its practical application (Sangsriratanakul et al. 2018).
2.4.3 Common household sanitizers:

Investigation of the effectiveness of common sanitizers added to products that are used on an everyday basis on norovirus surrogates has also been conducted. In an interesting study by Whitehead and McCue, common household disinfectants including alcohol, acid, quaternary ammonium compounds, and phenols both alone and in various combinations as formulated in common disinfectants were tested on FCV with an initial titer of 4 log dried on polystyrene surfaces for 1 minute. The dried virus was treated with the active test substance in both aerosol and trigger spray forms and virus enumerated using TCID\textsubscript{50}. Sodium hypochlorite at a concentration of 1,000 ppm demonstrated greater than 3 log reduction, similarly organic and inorganic acid at a pH of 2 and alkali at pH of 12 demonstrated a greater than 3 log reduction. However, the results for organic acids should be taken with caution given FCV’s higher susceptibility to low pH. Alternatively, purified alcohol and quaternary compound solutions and phenol and pine oil at common disinfectant use concentrations were not as effective. Both ethanol and isopropanol at 60% concentration and alkyl dimethyl benzyl ammonium chloride at 0.3% active at pH 8.0 provided a 1 log reduction, while a phenol-based disinfectant at 500 ppm active ingredient demonstrated a 2-log reduction. pH was found to be an important factor in the efficacy of some treatments. An alkali, monomethylamine, was not considerably effective at a pH of 10.8, but was at pH 12 (Whitehead and McCue 2010). In a similar survey study by Zonta et al, the antiviral efficacy of seven different disinfectant formulations (alcohol, halogens, peracetic acid/hydrogen peroxide, quaternary ammonium compounds/alcohol/aldehyde, and alcohol/biguanide) when applied to MNV and FCV in suspension and on stainless steel and
gloves was investigated. Several treatments showed promising reductions for both surrogates, with nearly all treatments exceeding a 3-log reduction. These generally higher numbers of complete formulations of disinfectants observed by Zonta et al compared to those reductions observed with the active ingredient by Whitehead and McCue (2010) could be due to the longer contact times studied by Zonta et al. (2016) (5-15 min versus 1 min) or could also suggest that disinfectant formulation/combination can enhance viral inactivation (Elaissi et al. 2012)(Whitehead and McCue 2010). In an interesting study by Predmore et al, the addition of surfactants to a chlorine solution (200 ppm chlorine) demonstrated significantly higher removal and reduction of MNV from different produce samples. Interestingly, the most inactivation tended to be observed on either gloves or in suspension while the least inactivation was observed on stainless steel surfaces for MNV and FCV; however, determination of the potential mechanism for this was beyond the scope of the study and could be an avenue of future investigation. Additionally, the mechanisms and effects of disinfectant combination and formulation on enhancing inactivation could also be of future study (Predmore et al. 2015). Levulinic acid with sodium dodecyl sulfate (SDS) has been demonstrated to be rapidly effective against bacterial foodborne pathogens, including in the presence of samples with high organic load. Cannon et al. (2012) investigated the effectiveness of levulinic acid and SDS both alone and in combination against MNV and FCV in suspension and on stainless steel surfaces at pH 2-5. Generally, lower reduction for both MNV and FCV was observed when levulinic acid and SDS were used individually (<0.5 log reduction). However, the combination of 0.5% levulinic acid with 0.5% SDS inactivated both surrogates by 3 to 4.21 log PFU/ml after 1 min of exposure in suspension for MNV and
FCV, respectively, suggesting a synergistic effect. There was significantly more inactivation at lower pH for the combination for both MNV and FCV. Overall this study suggested that addition of detergents to organic acids can produce a synergistic effect and enhance viral inactivation (Cannon et al. 2012).

In another study investigating the antiviral ability of organic acids and alcohols, Rabenau et al tested the efficacy of five disinfectants against a number of non-enveloped model viruses on stainless steel surfaces and in suspension, specifically a model adenovirus (Adenovirus 5) and different animal parvoviruses (bovine, murine, canine, and porcine). Five compounds were investigated: glutaraldehyde (125-2,500 ppm), peracetic acid (50-1,500 ppm), ethanol (40-60%), 1-propanol (10-60%) and 2-propanol (20-60%). Interestingly, the general stability of porcine parvovirus to these disinfectants was similar to previous reports for MNV. Specifically, the concentrations of 1,500 ppm for PAA and 2,500 ppm for GDA to achieve a 4-log inactivation within 5 mins previously observed for MNV were similar to the 4.27-5.61 log reduction for 1,500 ppm peracetic acid and 3.65-3.8 log reduction for 2,500 ppm glutaraldehyde observed. Further, the alcohols tested had generally limited activity against porcine parvovirus, suggesting it should be investigated further as a potential cultivable surrogate for human noroviruses (Rabenau et al. 2014).

**2.5 High Pressure Processing:**

High pressure processing (HPP) has been an alternative non-thermal inactivation method growing in popularity commercially for specific foods and has been well-studied for viruses in foods (Chen, Hoover, and Kingsley 2005) (Brié et al. 2018). In a study by Sanchez et al, the effectiveness of HPP against MNV and norovirus GII.4 in solution at
different temperatures were investigated. Specifically, the effect of HPP at 200-500 MPa after 15 min treatment at two temperatures (25°C and 45°C) was tested. For MNV, HPP was able to inactivate at least 6.5 log of infectious viruses at a pressure of 450 MPa independently of the temperature applied when analyzed by TCID$_{50}$. However, when 10 mM CaCl$_2$ was added, less than 1 log reduction was observed, suggesting that certain matrix-based components or foods treated with HPP may require more intense treatment.

MNV genomes were detected even after 15 min treatment at 500 MPa, with $<2.5 \log_{10}$ reduction demonstrating that RT-qPCR underestimates the extent of viral inactivation as mentioned above (Manuel, Moore, and Jaykus 2018)(Sánchez et al. 2011). Thus, the observation by the authors that HPP did not reduce GII.4 genomes by more than 1 log$_{10}$ for any treatment may be potentially misleading. On the other hand, a subsequent study by Lou et al did support the observation that GII.4 norovirus may be more resistant than MNV, as the effectiveness of HPP against GII.4 VLPs as a surrogate was analyzed.

Interestingly, the study found that treatment that was reported to completely inactivate MNV and FCV (500-600 MPa for 2 minutes) did not have a considerable effect on the reduction of VLP HBGA (receptor) binding ability. However, when the pressure was increased above 800 MPa complete reduction of VLP integrity was observed in 15 minutes or less. Interestingly, further human norovirus work for which infectivity was determined using a gnotobiotic pig model demonstrated the efficacy of HPP with treatments as low as 350 MPa for 2 min so long as treatments were at 0°C (Lou et al. 2012). Further HPP work on noroviruses evaluated the effectiveness of HPP on virus in different food matrices beyond those investigated previously. Huang et al. (2014) investigated the degree to which the effectiveness of HPP on MNV in strawberries could
be enhanced using different additional treatment factors. Reducing the treatment temperature generally ended up enhancing MNV inactivation, as reducing the temperature from 20°C to 0°C increased viral reduction by nearly 4 \( \log_{10} \) when a 350 MPa 2 min treatment was applied (Lou et al. 2012) similar to the observations for human norovirus strains (Lou et al. 2012). Additionally, introducing water to the samples prior to treatment also significantly enhanced reduction, achieving additional 1-2 \( \log \) reduction over the “dry” sample. Cycling pressure treatments did not appear to contribute considerable additional reduction further investigated the effect including water in HPP treatment has on inactivation of MNV in green onions at different temperatures. As observed, inclusion of water significantly enhanced MNV inactivation after treatment at 350 MPa for 2 min at 4°C (>4 \( \log \) reduction compared to 1.5 \( \log \) reduction). However, no effect was observed at 20°C (both about 1.5 \( \log \) reduction). As has been observed in other reports, decreasing temperature resulted in higher reductions of MNV, as 30 MPa treatment for 2 min at 1°C resulted in a higher reduction than similar treatments at 4°C and 10°C (Sido et al. 2017) (Table 1). HPP was tested in addition to other treatment strategies to reduce MNV, hepatitis A, and adenovirus 41 in an interesting study by Hirneisen and Kniel. In it, the ability of UV (240 mJ/s cm²), ozone (6.25 ppm, 10 min), calcium hypochlorite (150 ppm, 4°C), and HPP (500 MPa, 5 min, 20°C) to inactivate viruses both internalized and on the surface of green onions was evaluated. Not surprisingly, lower reductions were observed for internalized viruses compared to surface-associated viruses. Of the treatments, the HPP treatment achieved the highest reductions relative to the other three treatments. For instance, for MNV, reductions of >4.7 \( \log \) and >6.4 \( \log \) virus were observed for internalized and surface viruses, respectively. The
next most effective treatment was ozone, with 1.5 and 2.5 log reductions for internalized and surface viruses, respectively (Kirsten A. Hirneisen et al. 2010). This could suggest that the physical nature of the inactivation caused by HPP also makes it less effected by organic load than many chemical-based treatments and allows for comparably better efficacy for internalized pathogens. Another study by Hirneisen et al compared the ability of HPP to inactivate different picornaviruses (hepatitis A virus and Aichi virus) and caliciviruses (MNV and FCV) in salsa. Samples were treated at 9°C with 250-500 MPa for 1-10 min in salsa. FCV and hepatitis A were both rapidly inactivated, as >5 log reduction was observed in less than 1 minute at 400 MPa; whereas MNV and Aichi virus were more resilient. For MNV, 5 min of 400 MPa treatment was needed to achieve a 4 log reduction, while <1 log reduction was observed after 10 min with Aichi virus (Table 1). As seen above for MNV, a similar generally similar results between buffer and salsa for Aichi virus reduction were observed (Kirsten A. Hirneisen et al. 2010) (Table 2).
HPP has also been used in conjunction with the development of alternative molecular infectivity estimation methods for noroviruses due to the inability of RT-qPCR to reflect the infectious virus as discussed above. Li and Chen (2015) investigated the ability of a porcine gastric mucin binding assay followed by RT-qPCR to estimate the inactivation of MNV and TV in buffer by HPP compared to inactivation observed using plaque assay. MNV and TV were treated for 2 min at 250-550 MPa in both neutral and acidic buffer (pH 4) at refrigeration and room temperatures. Generally, the PGM-RT-qPCR method displayed similar reduction to plaque assays for the first 2 log of viral inactivation but then began to underestimate the degree of inactivation at higher inactivation levels. Overall, TV was more susceptible to HPP treatment than MNV, generally displaying 2 logs more reduction than MNV for a given treatment under all temperature conditions. Further, reduction of pH at room temperature (21°C) decreased the efficacy of HPP treatment for both viruses (Li and Chen, 2015), making consideration of the food being treated important in application of HPP despite the technology’s previously demonstrated general resistance to organic load-related effects. Similar trends were observed for HPP treatment of TV in buffer in a follow-up study by Li et al, which additionally evaluated the ability of direct RT-qPCR and propidium monoazide (PMA) pretreatment followed by RT-qPCR to estimate infectivity. Both RT-qPCR and PMA/RT-qPCR methods tended to further underestimate TV reductions relative to PGM-RT-qPCR and plaque assay methods for HPP treatment at refrigeration and room temperatures. A similar trend for the methods was observed with heat treatment in another study(Li and Chen 2015). Interested readers are referred to a recent review on

*in vitro* molecular infectivity methods(Manuel, Moore, and Jaykus 2018).
2.6 Thermal Inactivation:

Numerous studies have been conducted evaluating the thermal susceptibility and inactivation kinetics of human norovirus surrogates in foods, as understanding the degree to which cooking can reduce the risk of foodborne norovirus transmission is of importance. As thermal inactivation of human norovirus surrogates has been excellently reviewed relatively recently, only a few more recent selected studies will be discussed (Brié et al. 2018) (Bozkurt, D'Souza, and Davidson 2015). One interesting study by Takahashi et al examined the persistence and thermal stability of MNV in breads and dough in response to a large outbreak associated with bread in Japan in 2014. Not surprisingly, MNV was able to persist for at least 5 days with little reduction on both the crust and inside all three types of breads investigated: butter-rich rolls, French bread, and white bread. Further, the ability of MNV to survive on the surface of toasted white bread was investigated, with results suggesting that mere warming/ slight toasting may not be sufficient to reduce >4 log of virus—though it should be noted that generally contamination at that high of a level of virus is uncommon. The ability of MNV to survive in dough when baked (130-190°C, 0-12 min) was tested, with some virus persisting in lightly baked/under-baked breads. Once the internal temperature of the bread reached around 70°C (~2-9 min of baking) generally >5.0 log of MNV was reduced (Takahashi et al. 2016). The thermal inactivation kinetics of MNV and TV in strawberries (specifically strawberry puree), a food more commonly associated with norovirus outbreaks, was investigated by Bartsch et al. Generally, MNV was slightly more resistant to heat than TV, usually with 0.5-1 log less reduction at more intense heat treatments, though the difference was not statistically significant. Overall, treatment of
the puree at 80°C for 8 s was sufficient to remove all virus tested for both viruses (>7 log); with over a 4-log reduction achieved after 4 s. Higher TV inactivation (~1 log more reduction at 60°C versus 63°C) in buffer was observed in another study primarily focused on evaluating molecular assays for estimating infectivity of heat and HPP treated viruses (Li et al., 2017), suggesting that some potential degree of strawberry matrix-associated protection of virus against heat treatment could occur. However, considerably more study of different treatments and additional controls on the internal temperature and its distribution in the food matrix would need to be conducted to confirm this. In another study in oysters, a similar phenomenon of a protective effect of oyster matrices against heat treatment of MNV and TV was observed, but as with the strawberry puree, the mechanism of the protective effect was beyond the scope of the study. One potential mechanism of a protective effect could be viral binding to carbohydrates present in the strawberry and oyster matrices, as such binding has been shown to protect the virus in the case of binding to bacterial cell surface carbohydrates (E. J. Kim et al. 2015). Future study into the mechanisms of the potential protective effects of different food matrices—especially those associated with norovirus outbreaks—would likely be of value.

2.7 Inactivation During Natural Processes:

There are many natural processes in the production of food products that have the potential to protect the food from spoilage while producing sensory and nutritional benefits. Traditionally, these foods have been produced without consideration of their antiviral effect. For example, fermentation is one such process in which favorable bacteria are
grown that suppresses the growth of unfavorable microbes though competitive exclusion and the production of antimicrobial compounds. This has led to investigation of the effects of fermentation on viral inactivation. Lee et al. (2012) evaluated the antiviral effect of fermentation of Dongchimi, a type of kimchi, on MNV and FCV.

The Dongchimi was initially fermented in a traditional manner at room temperature overnight, stored at 4°C, and spiked with MNV and FCV. Along with viral titer, the levels of lactic acid bacteria, pH, and acidity were also tested over the course of 20 days. In the case of FCV, there was about a 1.5 log reduction after 10 days, followed by a more intense decrease to get over a 4-log reduction by the end of the 20th day.

Comparatively, there was less inactivation in the case of MNV, with a maximum reduction of 1.5 log after 20 days. Increases in lactic acid bacteria and acidity in both samples proceeded as would be expected for the fermentation. Overall, FCV was much more susceptible than MNV, which is consistent with previous reports demonstrating the high acid sensitivity of FCV (J. E. Lee and Ko 2013). In another interesting study involving kimchi, Bae et al tested the potential of black raspberry extract to enhance reduction of MNV and FCV in the development of kimchi over the period of 50 days. FCV titers were completely removed (>5 log) after 30 days, likely due to both the acidity and extract, while MNV titers were reduced by a maximum of 2.1 log after 50 days. Interestingly, the black raspberry extract seemed to have a more pronounced effect on enhancing FCV inactivation beyond the reduction observed with the kimchi fermentation alone than was observed for MNV, where inclusion of the extract had little additional effect (Baert et al. 2010). Molluscan shellfish are among one of the most common foods associated with human norovirus outbreaks (Kirk et al. 2015).
There have been many cases of human norovirus outbreaks due to the consumption of raw oysters; however, they have not been as associated with fermented oysters, a popular Korean dish. Thus, Seo et al conducted a study to determine if the fermentation process potentially has an antiviral effect on FCV and MNV. Oysters spiked with virus and two spontaneous fermentations based upon the addition of 5% and 10% NaCl were conducted at 18°C. The viral levels, pH, enzymatic activity, acidity, and levels of lactic acid bacteria were measured until the 15th day post fermentation. In both the fermentations, there was more reduction for FCV than MNV after 15 days. There was a 3-log reduction for FCV and a 1.6 log reduction for MNV in the 5% NaCl fermentation, and a 1.1 log reduction of FCV and 0.9 log reduction for MNV in the 10% NaCl fermentation. The higher reduction observed in the 5% fermentation compared to the 10% also corresponded to higher levels of lactic acid bacteria, acidity, enzymatic activity, and lower pH for the 5% fermentation compared to the 10%. The specific composition of the lactic acid bacteria communities in the two fermentations was not measured nor the degree of bacteriocin production, some of which have shown effectiveness against viruses (Seo et al. 2014)(Elaissi et al. 2012). Potential differences in the effect of temperature on viral reduction during fermentation were investigated by Baert et al, who investigated the inactivation of MNV and Bacteroides fragilis phage B40-8 in mesophilic (37°C) and thermophilic (52°C) anaerobic digestion of pig slurry, which is commonly used as a fertilizer in agricultural and livestock production and thus a potential source of viral contamination of produce. B. fragilis phage B40-8 had been reported to be more prevalent in sewage and is more resistant than other surrogate phages and has been used as an alternative model for viral persistence in
sewage. At the mesophilic conditions, 4 and 3 log reductions of MNV and phage B40-8 were observed after 13 minutes respectively. Overall, more reduction under thermophilic conditions was observed, with 4 and 2.5 log reductions for MNV and B40-8 after 7 minutes, respectively. In order to study the effect of live bacteria on inactivation, the experiment was also conducted with autoclaved slurry. In both thermophilic and mesophilic digestion, in MNV the level of inactivation was similar to the previous experiments, suggesting live bacteria do not play a significant role in MNV reduction. However, there was more reduction observed for B40-8, suggesting that phage replication in the slurry that was not autoclaved may have occurred, confounding those results (Baert et al. 2010). These applications demonstrate a potential protective, antiviral effect of fermentations in some conditions to reduce levels of norovirus surrogates. Future work to potentially identify the specific components and/or mechanisms involved in these reductions may be useful in the development of novel disinfectant formulations.

2.8 Self-Sanitizing Surfaces:

The use of antimicrobial surfaces in high-risk environments may help to prevent the spread of many infectious agents, including noroviruses, that can retain infectivity on surfaces for prolonged periods of time. Copper and copper alloys have been shown to be effective at rapidly killing a range of bacterial, fungal, and viral pathogens in laboratory studies under various temperatures and humidity conditions. This led to investigation of their effectiveness against human norovirus and its surrogates. An initial study by Warnes et al found a range of copper alloy surfaces (60-100% copper) were effective at inactivating MNV in both wet and dry contamination conditions.
Specifically, a larger volume of MNV that would take longer to dry was applied to the surface to represent wet contamination conditions, whereas a very small volume (1 µl) that would very rapidly dry was applied to simulate dry touch contamination. Dry touch conditions resulted in the most rapid reduction, with a 4-log reduction of virus in less than 5 minutes for pure copper compared to a similar reduction on the wet contamination surface taking a little over 20 minutes. Further, Warnes et al also investigated the relative role of different ionic forms of copper on inactivation of MNV; finding that Cu$^{+1}$ was extremely effective while Cu$^{+2}$ had some but less dramatic effect. Additionally, inclusion of molecules capable of quenching reactive oxygen species generated with Fenton chemistry did not prevent viral reduction, suggesting that copper inactivation of MNV did not involve Fenton generation of reactive oxygen species (Warnes, Summersgill, and Keevil 2015). A follow-up study by Warnes et al investigated the ability of a larger range of copper alloys to inactivate MNV. MNV reduction was measured with plaque assay and the morphology of the virus studied by using TEM. MNV-1 was significantly inactivated on alloys containing 79 to 89% copper (~4 log reduction in 80 min), but a considerable amount of antiviral efficacy was lost at 70% copper and below (<2 log reduction over 120 min), suggesting that a small difference in copper content, 70 to 79% can have a large effect on antiviral efficacy. Further, alloys containing zinc (copper brasses) were generally more effective than copper-nickel alloys, suggesting the inclusion of zinc in a copper-based alloy may have a synergistic effect for viral reduction. In both studies, stainless steel did not have any antiviral activity (Warnes, Summersgill, and Keevil 2015).
2.9 Other Inactivation Agents:

Some components found in foods like plant antimicrobials have been investigated as potential food-grade inactivation agents for noroviruses. These natural disinfectants have been reviewed relatively recently, but there have been some subsequent reports of the use of essential oils for inactivation of human norovirus surrogates. One such plant antimicrobial compound is curcumin. Curcumin is identified as an excellent natural photosensitizer, as the exposure of curcumin to light causes the generation of reactive oxygen species that can damage organic molecules. Randazzo et al tested the antiviral efficacy of different concentrations (5-50 µg/ml) of curcumin subjected to photoactivation by intense LED blue light against FCV and MNV in suspension with media.

Additionally, the experiment was conducted at two different temperatures, room temperature and 37°C. A promising degree of reduction was observed for FCV at 37°C (>4 log reduction), with less reduction observed at room temperature (1.7-3.3 log reduction). Although promising results were observed for FCV, there was little inactivation against MNV (0.6-0.9 log reduction), suggesting that additional treatment or more optimization of this method for viral inactivation may be warranted(Ryu et al. 2015) (Randazzo, Aznar, and Sánchez 2016). In another study, Gilling et al investigated the antiviral properties of oregano oil and its active ingredient, carvacrol, against MNV in buffer and on surfaces. Overall, carvacrol was much more effective at inactivating MNV, as the tested concentration of oregano oil (4%) produced about a 1 log reduction, while 0.5% carvacrol produced up to a 3.87 log reduction after 1 hour of treatment and over 4.5 log reduction after 24 hours at room temperature. This is interesting, as carvacrol is the major component of oregano oil, sometimes comprising as much as 85% of
oregano oil—thus the disproportionally higher inactivation observed with carvacrol suggests other components in oregano oil could act in a way to reduce carvacrol’s antiviral efficacy. Additional use of cell binding, RNase protection, and electron microscopy suggested that carvacrol acts to disrupt/destroy the viral capsid, with capsids expanding in size substantially after treatment prior to degradation (Gilling et al. 2014). Another study by Gilling et al investigated the efficacy of multiple other essential oils and one active ingredient (allspice oil, lemongrass oil, and citral) in buffer, all with modest reductions observed (<1 log-2 log) after 6 hours of treatment, and more substantial reductions (2-3 log) after 24 hours. Interestingly, the oils seemed to have different inactivation mechanisms. Allspice oil appeared to directly damage the capsid and genome of the virus with capsids enlarging to over twice their size, while lemongrass oil and its active component citral appeared to indirectly inactivate MNV by coating the viral capsid and preventing effective binding and uncoating of the virus in host cells (Gilling et al. 2014).

In a subsequent study on antiviral efficacy of lemongrass oil, Kim et al investigated the ability of lemongrass essential oil to inactivate MNV both in suspension and therapeutically in vivo in a mouse model. Using plaque assay, a maximum reduction in plaque formation of less than 1 log (75%) was observed. Next, GC-MS was further used to identify individual components responsible for viral reduction. Of them, 32 were selected for screening and citral was still found to be the most effective, achieving a similar maximum reduction (75.5%) as the oil. For the in vivo studies, pretreatment of MNV with lemongrass oil for 72 hours at 4°C prior to oral administration resulted in significant reduction in viral RNA shedding over seven days, and a somewhat similar reduction in viral shedding of lemongrass oil-treated virus to that observed in vitro (~1
Another biological component with previously reported antimicrobial activity is heat denatured lysozyme. Lysozyme is an enzyme that hydrolyses the cell walls of gram-positive bacteria, specifically targeting peptidoglycans. Although it had been tested and used for control of bacteria, its effectiveness for noroviruses had not been tested, likely because of the lack of a viral lipid envelope. Takahashi et al tested the ability of heat denatured lysozyme to inactivate MNV in 4 types of salads (coleslaw, thousand island salad, vinaigrette salad, and egg salad) stored for 5 days at 4°C. The addition of the lysozyme reduced persistence of MNV >4 log for vinaigrette and thousand island salads, by a little less than 3 log for coleslaw, and by 0.3 log in egg salad. Interestingly, this trend was related to the acidity of the salads, as egg salad (pH 6.27) and coleslaw (pH 4.33) were the two least acidic of the salads tested (Takahashi et al. 2016). In a recent, subsequent study, the application of heat denatured lysozyme to reduce levels of HAV and MNV on the surface of fresh berries was investigated. Specifically, Takahashi et al demonstrated the ability of heat denatured lysozyme to inactivate HAV in addition to MNV in solution, and then tested its ability to reduce infectious virus on the surface of blueberries and mixed berries (strawberry and raspberry). Exposure of a 1% solution of heat denatured lysozyme to the berry surface for 1 min resulted in reductions comparable to those observed for 100 ppm sodium hypochlorite for HAV (~2-3 log) and MNV (~2-2.5 log) relative to a distilled water control (Takahashi et al. 2016)(Table 1). These findings are interesting, as heat denatured lysozyme lacks enzymatic activity. Further work identifying the anti noroviral mechanism of heat denatured lysozyme and its higher efficacy at lower pH would be valuable for this promising edible inactivation agent.
CHAPTER 3

3. EVALUATING THE POTENTIAL FOR CONTAMINATION OF LEAFY GREENS WITH LISTERIA WHEN USING RETROFITTED WASHING MACHINES

PRAGATHI KAMARASU\textsuperscript{1}, LYNNE MCLANDSBOROUGH\textsuperscript{1}, MATTHEW D. MOORE\textsuperscript{1*}, AMANDA J. KINCHLA\textsuperscript{1*}

\textsuperscript{1}Department of Food Science, University of Massachusetts Amherst, MA, USA

*Corresponding Authors: Matthew D. Moore, mdmoore@umass.edu; Amanda J. Kinchla, amanda.kinchla@foodsci.umass.edu

3.1 Introduction:

The CDC has reported that 16\% of identified foodborne outbreaks are associated with produce, and amongst those vegetable row crops such as leafy greens comprise 46\% of the illnesses. In part, the prevalence of produce-associated outbreaks is due to the fact that produce is often consumed raw, and is often not processed with a thermal inactivation step that can remove microbial contamination; therefore, it is imperative that
Good Agricultural Practices (GAP) are used on-farm to reduce the produce safety risk. Microbial contamination of fresh produce can occur at multiple stages throughout the farm-to-fork chain (Matthews et al. 2012); during the cultivation of fresh produce, at harvest, during preparation/washing, within distribution chains and transport to shops, and during preparation (Machado-Moreira et al. 2019).

*Listeria monocytogenes* is a leading cause of foodborne death, often causing illness in neonates, pregnant women, and immunocompromised adults (CDC), and has also been associated with produce outbreaks (Lane et al. 2020), (CDC 2022- Dole Packaged Salads n.d.), (CDC 2022- Fresh Express Packaged Salads n.d.). The environment where produce is manipulated, such as food contact surfaces or equipment used in processing, can also play a role in *L. monocytogenes* contamination (Maurel et al. 2016). For example, reusable crates have been shown to be a potential source of cross-contamination among different batches of leafy greens (Murray et al. 2017).

With the recent implementation of the Food Safety Modernization Act in 2011, there are several new regulations that specifically define sanitary regulatory compliance yet there are few tools available, and the available trainings and other support only offers basic postharvest practices that help to reduce risk (Title, Project, and Partner 2022). One such practice of concern is the use of Do-It-Yourself (DIY) washing machines to dry washed leafy greens, a practice increasingly being used by some of the nearly 1,000 small scale and medium scale farmers (Chimelis 2020). Post-harvest handling of leafy greens in large scale facilities includes a series of steps whereby the produce is initially triple-washed to remove field heat and debris, followed by drying in a controlled environment where the
air temperature, air speed and humidity levels are constantly monitored. With larger commercial operations, the drying is conducted in constantly moving belts or in commercial machines that utilize centrifugal forced spin cycle rotational force to dry the leafy greens. However, the equipment and cost to obtain and utilize this equipment for small and medium scale producers is often prohibitive. Alternatively, these producers have increasingly begun retrofitting washing machines by disassembling them and replacing the barrel of the machine with crates to turn the machine into a large salad spinner using its spin cycle (Figure 1). Retrofitted washing machines also have the additional benefit of a brake system which helps with having controlled spinning movements at regular intervals while drying the leafy greens. Utilizing retrofitted washing machines is cost efficient and has an effective production speed for drying leafy greens. However, the potential for the use of these retrofitted washing machines to contribute to contamination and/or cross-contamination of leafy greens has not been investigated. Although the recently implemented Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) does not prohibit the use of these retrofitted machines for drying leaves, there is a need to conduct a science-based risk hazard identification that helps in development of specific best practices to inform guidance for small and medium-sized producers to stay in compliance with the new rule (Machado-Moreira et al. 2019). The purpose of this study was to investigate the potential of retrofitted washing machines to result in contamination and cross-contamination of leafy greens with *Listeria*, as well as characterize the nature of such contamination if it occurs.
3.2 Materials and methods:

3.2.1 Materials:

The washing machine Speed Queen TC5000, Classic Clean™ were purchased from US Appliances (Alliance Laundry Systems LLC, Ripon, WI). *Listeria innocua* (ATCC 33090) was obtained from ATCC and cultivated per manufacturer instructions (American Type Culture Collection, Manassas, Virginia. 3M Quick microbial swabs were purchased from 3M, (Maplewood, Minnesota, USA). Listeria selective media Oxford agar and Oxford agar supplements were purchased from Fisher Scientific USA, Waltham, MA. In addition, nonselective media such as tryptic soy agar (TSA) was purchased from Fisher Scientific USA, Waltham, MA. Baby spinach was obtained from The University of Massachusetts Amherst Dining Services, Amherst, MA.

3.2.2 Source, procure and build the DIY Converted washing machines:

The DIY retrofitted washing machine was built with the help of the University of Vermont’s Agricultural Engineering Extension team. A detailed conversion guide is available online (University of Vermont- Retrofitted washing machine conversion guide). Based on the steps mentioned, the different parts of the washing machine such as the panels were taken apart and rewired to only keep the central water collecting drum and the brake system intact. Additionally, a stopwatch timer was attached to the machine to keep the time of each spin-drying cycle while drying the leafy greens, to ensure consistency. The series of steps involved in building the retrofitted machines are depicted in Figure 1. Traditional perforated fishing baskets were inserted into the machine that
were used for loading and unloading the spinach. The machine was placed at the pilot plant in Chenoweth laboratory, University of Massachusetts Amherst, MA. A protective enclosure was built with polyethylene sheets and PVC pipes (Lowe’s, Mooresville, North Carolina) to ensure that there was no spread of the aerosolized microorganisms during spin drying experimental analysis to meet the Environmental Health and Safety Standards.

Figure 3.1: Depiction of the steps involved in building a retrofitted washing machine. A) retrofitting a washing machine and B) adding loading baskets to reduce contact exposure to C) Adding leafy greens to the 3 layered retrofitted machine D) Operating as a dry triple-washed produce (UVM- University of Vermont Extension Agricultural Engineering n.d.).
3.2.3 Bacterial inoculum preparation:

*Listeria innocua*, a surrogate for *Listeria monocytogenes*, was inoculated onto spinach and food contact points with initial inoculation loads (10⁶ and 10⁹ log_{10} CFU/ml) to evaluate microbial transfer. *L. innocua* was cultured in tryptic soy broth (TSB) at 37°C for 18 hours with continuous shaking at 130 rpm to obtain an inoculum level of 9 log_{10} CFU/ml and that was further diluted with TSB to obtain a range of inoculation load. The growth curve of the microbe was determined by measuring the Optical Density (OD600) at regular intervals in the Spectrophotometer machine followed by plating, and OD_{600} used to normalize inoculum level. Prior to the analysis, individual spinach leaves were marked, placed in empty petri plates, inoculated with *L. innocua*, and dried for 1 hour.

3.2.4 Determination of microbial transfer efficiency:

3.2.4.1 Spin drying of produce:

The initial spin-drying assay was conducted by analyzing a range of volumes of spinach added in each spin-drying cycle as well as with a low microbial load and a high microbial load (10⁶ and 10⁹ log_{10} CFU/ml). The inoculated spinach leaves, which were pre-marked to allow recovery of inoculated leaves, were mixed with regular spinach of varying volumes, ranging from 100-500 grams per spin drying cycle. A washing step was then conducted, where the batch of spinach was dipped in clean water, after which water was discarded accordingly. The washed spinach was added to the external perforated orange load basket, placed inside the retrofitted washing machine, and spun 6 times for 10 seconds each to conduct spin cycle drying to remove the water from the spinach. Pre-
marked inoculated spinach leaves sample were removed from the retrofitted washing machine post spin drying and added into stomacher bags with 250 ml of 0.1% peptone water. The bags were stomached for 10 minutes, serially diluted, and enumerated by plating on Oxford agar plates after incubation at 37°C for 48 hours. All assays were repeated on separate days in triplicate.

3.2.4.2: Determination of degree of contamination from inoculated produce to contact points:

Three different layers in the retrofitted machine and 3 contact points were assigned to each layer as seen in Figure 2. 3M Quick microbial swabs (Minnesota, USA) were used to swab the contact points and recovered *Listeria* was enumerated by serial dilution and plating on Oxford agar plates and TSB plates at 37°C for 48 hours. The microbial recovery experiments were conducted in triplicate along with positive and negative controls such as swabbing the machine prior to spin drying with inoculated spinach, non-swabbed microbial swab plating and plating direct *Listeria innocua* culture.

Figure 3.2: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine; direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3 contact points.
3.2.4.3 Determination of degree of contamination from contaminated washing machine to un-inoculated produce:

The potential for the retrofitted washing machine environmentally seeded with *L. innocua* to contaminate uninoculated spinach was evaluated. First, 3.77+/−0.29 log CFU of *L. innocua* was inoculated onto direct contact points (Layer 1) as well as the indirect contact points (Layer 2 and 3) of the washing machine followed by a spin-drying run of the fresh spinach as described above, except without inoculation of leaves of spinach. After each dry run, 25g of spinach including the inoculated and labelled leaves were stomached in 250 ml of 0.1% peptone water for 10 mins. The stomached media was then serially diluted and enumerated on Oxford agar and TSA plates as described above. The retrofitted washing machines were cleaned and sanitized post spin drying analysis of the experimental conditions. Cleaning detergent such as Dawn® Heavyduty Degreaser (Procter & Gamble, Cincinnati, Ohio, USA) in the concentration of 7% Sodium cumenosulfonate surfactant was used for a contact time of 15 minutes in volume of 300 ml along with water to physically remove soil and residue from the retrofitted washing machine. Scotch-Brite™ Scotch-Brite Non-Scratch Scrub Sponges Individually-Sealed (3M, St. Paul, MN, USA) was used to scrub the surface of the different layers of the washing machine. Surfaces were scrubbed and rinsed to remove visible soil and debris. This was followed by scrubbing using a clean scrub brush (Holikme, Amazon USA) and finally rinsed with clean water to remove all residue and suds. It is important to ensure all
the residual detergent suds are completely removed to guarantee complete efficiency of the following application of the sanitizers. Machines were allowed to air dry prior to application of the next step. The cleaning step was followed by application and investigation of two commonly used food contact surface approved sanitizers, SaniDate 15 (which is a 15% peroxy acetic acid-based sanitizer) (Biosafe Systems, Hartford, Connecticut, USA) and Clorox germicidal bleach (7.03% sodium hypochlorite chlorine-based sanitizer) (The Clorox Company, Oakland, California) in the volume of 300 ml and in the food contact surface approved concentrations such as 100 ppm free peroxyacetic acid and 200 ppm free chlorine respectively. The efficacy of the cleaning and sanitation steps were confirmed by swabbing the different contact points of the machine and enumerating them on both Oxford and TSA plates prior to the next experimental replicate. Additionally, Hygiena Ultrasnap ATP swabs and Hygiena ATP Lumitester machine (Hygiena, Camarillo, California, USA) were used to ensure the machine was cleaned thoroughly after each spin-drying cycle.

3.2.5 Statistical analysis:

The experimental results from triplicate measurements were expressed as mean values and standard deviation (mean ± SD). Graphpad prism was used to conduct Turkey-test and One way- ANOVA and multiple comparison Turkey-test was performed using GraphPad Prism version 9.3.1 (350) for Mac OS X, GraphPad Software LLC, San Diego, California, USA, (www.graphpad.com). A P value less than 0.05 was considered significant.
3.3 RESULTS:

3.3.1 Determination of degree of contamination from inoculated produce to contact points:

The purpose of this study was to evaluate the potential of retrofitted washing machines to result in contamination of leafy greens with *Listeria*. There are a series of steps followed during post-harvest handling of spinach and the wash step is very important as it is imperative for the removal of debris and dirt from the soil. Hence, it was also important to include a wash step during microbiological analysis to evaluate the degree of microbial contamination in the washing machine. From the results, it was seen there was a loss of 3.19+/−0.38 log CFU/ml of *Listeria innocua* on initially inoculated leaves prior to loading into the retrofitted machines after the wash step was observed irrespective of the initial microbial load.

Initial preliminary studies were conducted varying the amount of spinach used in the machine, ranging from 100-500 grams, however, the initial amount of spinach added did not influence the degree of microbial spread in the different parts of the machine for the lower volume. When higher volume of spinach (500g) was added in the machine for spin drying it resulted in reduced microbial recovery (Supplementary table 1). Hence, a consistent volume of 250 g of spinach was chosen for conducting consequent experimental trials. There are three different layers of the retrofitted machine and three specific sampling points assigned to each layer for swabbing. as depicted in Figure 3B. The highest level of contamination when using inoculated spinach leaves was observed
on the three bottom most contact points of the machine, such as B1, B2, B3 as well as from the immediate contact points L1 and R1 (Figure 3A). The degree of microbial recovery was on a higher level irrespective of the logarithmic load of the initial \( L.\) *innocua* microbial culture inoculated onto the spinach surface. There was also a consistent microbial loss of around 3 log CFU/ml of *Listeria* after the single wash step and prior to adding the inoculated spinach leaves in the retrofitted machine for spin drying and there was a nearly complete recovery of 98% and 99% from 10\(^3\) and 10\(^6\) of initial \( L.\) *innocua* on washed leaves loaded into the machine, respectively.

![Diagram](image)

**Figure 3.3:** A) The graph depicts the specific recovery from contact point B2 for a range of inoculation microbial load (10\(^2\)-10\(^6\)) post loss of 3 log CFU/ml in wash step (left); B) Microbial recovery from the various contact points (right)

Nearly complete recovery of \( L.\) *innocua* was observed for both 10\(^3\) and 10\(^6\) CFU/ml initial inoculum loads respectively (Figures 4A,4B). From all the different contact points it can be inferred that the highest level of contamination was observed in the B1, B2 and B3 contact points.
3.3.2 Determination of degree of contamination from contaminated washing machine to un-inoculated produce:

In addition to introducing contaminated spinach into the washing machine, the potential for *L. innocua* from an environmentally contaminated washing machine to contaminate uninoculated fresh spinach and its degree of spread to other parts of the machine from the initial inoculated contact point was also evaluated. In order to conduct this analysis, the contact points pertaining to the 3 different layers of the machine were inoculated separately in each experimental analysis. The results from this have shown there was no microbial spread from the contact points that were initially inoculated (B2, L2, R2) to the other non-inoculated contact points of the machine as seen in Figure 5A. Further, no cross-contamination of the uninoculated spinach was observed from the non-direct contact points such as B2, L2, R2 and B3, L3, R3. However, 1 log CFU/ml of transfer to the spinach was observed when the orange load basket was contaminated with 3.77+/−0.29 log CFU of *L. innocua* as seen in Figure 5B. This suggests that there are lower
possibilities for contamination of greens from the lower layers of the machine, but the potential for cross-contamination from direct contact points of the machine does exist.

3.3 DISCUSSION AND CONCLUSION:

Cross contamination has been estimated to be responsible for about 26% of all food safety loss incidents (Costa et al. 2006), and processing operations of produce have been demonstrated to contain multiple points in which cross-contamination can occur (Axelsson et al.2013). Foodborne illness outbreaks caused by Listeria monocytogenes have occurred via food sources such as raw fruits and vegetables, raw milk, soft cheeses, deli meat and mushrooms etc. The cause for a significant number of these have occurred due to transfer from contaminated food contact surfaces (CDC 2022- Dole Packaged Salads n.d.) (CDC- Bidart Prepackaged caramel apples n.d.) (CDC-Jensen Farms n.d.)
Cantaloupes n.d.). Additionally, Smolinski et al. (2018) and Buchholz et al. (2012) et al have reported microbial transfer and redistribution during pilot scale processing of baby spinach, cilantro, and romaine lettuce. The results from the research findings have shown the highest level of inoculum transfer to the wash water and the second highest product inoculum was lost during centrifugal drying (Smolinski et al. 2018), (Buchholz et al. 2012). The increasing use of retrofitted washing machines to dry leafy greens, primarily by small and medium sized processors, may introduce another point of potential risk in produce processing; however, the degree and potential of this practice to result in contamination had hitherto not been explored. The results of the work reported here do suggest there is potential for both cross-contamination of equipment and greens to occur, using L. innocua as a surrogate.

About a 98-99% of microbial recovery was observed after initial inoculation of \(10^6\) and \(10^9\) log CFU/ml respectively. It should also be noted that there was a consistent loss of 3 log of Listeria innocua post the wash step irrespective of the level of initial inoculation load. The wash step conducted in this work does not likely mimic that commonly used in industry (i.e. use of only water for only one wash), however the purpose of this work was to focus on the potential of contaminated produce after the wash step to result in transfer of pathogen to the machine and/or other produce. The results of this work suggest that if inoculated produce is loaded into these machines, the risk of contamination of the machine as well as other produce exists. Further, the data reported here suggest that microbial spread throughout the different parts of the machine was more evenly distributed with a lower initial inoculation load (\(10^3\) log CFU/ml). However, higher contamination was observed on direct contact points (B1, L1, R1) of the
machine was observed when the inoculum was higher (10^6 log CFU/ml). It should be noted that often pathogen contamination of leafy greens occurs at lower levels than that observed for this load. Thus, the degree to which these direct contact points pose a higher risk relative to other parts of the machine requires further investigation. Regardless of initial inoculum load, the potential for pathogen contamination of the washing machines if *Listeria* is present on washed leaves being loaded into the machine exists. This is of particular importance for *Listeria*, as it has been well known to be able to environmentally persist in food processing plants and equipment, often in the form of difficult to remove biofilms.

In addition, a study was also conducted to evaluate the potential for *Listeria* transfer from an environmentally contaminated washing machine to uninoculated fresh produce. The results presented here suggest that although cross-contamination was not observed at high levels or from the indirect contact points of the machine, some transfer of about 1 log CFU/ml was observed from the direct contact points of the machine to spinach, which is a potential concern for *Listeria* as it has potential to grow in the refrigeration conditions in which the leafy greens are commonly stored. However, only detectable signal was observed when direct food contact surfaces of the machine were contaminated, and not from lower levels of the machine. The results here underscore the need to maintain strict sanitation and testing regimes for food contact surfaces in produce processing.

Although the purpose of this study was exploratory, there were a number of limitations. Food contact surfaces are considered high risk even in conditions where the concentration of microorganisms is on the lower side, as the risk is evaluated based on the presence or absence of contamination and not based on the concentration. Hence, natural
contamination conditions result in much lower concentration of microbial enumeration when compared to the concentration used for conducting experimental trails.

The washing of fresh produce is an important step for removing soil and debris and some studies have reported a 1-6 log CFU/ml microbial load reduction on the surface of produce (Jensen et al. 2015). However, the use of untreated wash water can increase the risk of spreading contamination as it can be inferred from research conducted by Buchholz et al and Jensen et al where the result showed a 90% transfer of inoculum to the wash water and a transfer of 90-99% of the bacteria to the wash water irrespective of the wash time respectively (Buchholz et al. 2012) (Jensen et al. 2015). Similarly, in this work, we conducted a single wash step prior to spin drying the inoculated spinach leaves in the machine and observed a loss of about 3.19+/-0.38 log CFU/ml of *Listeria* after the wash for all the initial concentrations evaluated. In our research, treated wash water with an antibacterial component already present was not used. Hence, changing the frequency of water, use of treated water, cleaning and the use of food contact surface approved sanitizers are the best practices to reduce the risk of contamination (Gombas et al. 2017). However, given the numerous outbreaks of triple washed lettuce in the past, the potential for contaminated leaves to be loaded into these machines still exists. Further, the levels of *Listeria* used may not mimic some instances of contamination of lettuce. Higher levels were evaluated in this study based on the limit of detection of the plating technique we used. It is possible that transfer with initial inoculum levels below those used here may not result in the same degree of contamination of the machine. However, *Listeria* is notably capable of growing and persisting in the environment, often in the form of biofilms, thus even low levels of contamination in a processing environment present a
potential concern. Additionally, the inoculated spinach leaves were allowed to dry for about 2 hours as the focus of the research was on estimating the extent of microbial spread in the machine and not focused on the contamination of the spinach leaves. Incubating for longer times may have provided additional unnecessary variability, as the focus of the study was to evaluate the potential of leaves with residual *Listeria* after washing to contribute to contamination of the machine. As mentioned, *Listeria* has the tendency to form biofilms and microbial adhesion and subsequent biofilm formation could have influence the degree to which contamination occurs. Future work investigating the potential of spinach to contaminate the machine after a longer inoculation time would be of value given the results presented here. Additionally, future studies evaluating if these same experimental conditions result in risk with a gram-negative bacterium like *Salmonella enterica* would also be of value.

The results from the experimental analysis suggest that the utilization of these machines pose a potential risk for cross-contamination with *Listeria* during leafy green processing with these machines. A logical next step of this research should focus on evaluating the efficiency of various risk reduction mitigation strategies commonly followed for food contact surfaces to reduce the contamination observed here. The results of this work will also help inform areas of concern for producers to stay in compliance with the recently implemented Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) that states specific sanitary regulatory conditions and there are specific regulations that discuss postharvest handling and sanitation.
3.4 Supplementary material:

<table>
<thead>
<tr>
<th>Volume of spinach (g)</th>
<th>Microbial recovery after inoculation of 10^6 CFU/ml of <em>Listeria innocua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>100 g</td>
<td>5.31 +/- 0.74 CFU</td>
</tr>
<tr>
<td>250 g</td>
<td>5.66 +/- 0.18 CFU</td>
</tr>
<tr>
<td>350 g</td>
<td>5.7 +/- 0.24 CFU</td>
</tr>
<tr>
<td>500 g</td>
<td>3.57 +/- 0.11 CFU</td>
</tr>
</tbody>
</table>

Table 1: Microbial recovery post inoculation on a range of volume of spinach.
CHAPTER 4:
4. EVALUATING THE INFLUENCE OF DIFFERENT CLEANING AND SANITATION INTERVENTIONS ON MICROBIAL AND ATP RECOVERY IN AN EMERGING LEAFY GREEN PROCESSING APPARATUS

4.1 INTRODUCTION:

The World Health Organization (WHO) estimates that the consumption of contaminated food results in about 600 million cases of foodborne illness, which are responsible for 420,000 deaths every year (WHO, 2020). The estimated economic impacts of these illnesses range between $51.0 and $77.7 billion annually (Scharff 2012). Advances in innovative agricultural practices, packaging, and temperature control, combined with the need for fresh locally grown produce, have resulted in increased demand and consumption of fresh fruits and vegetables (USDA n.d.). However, there has been a significant increase in the number of foodborne outbreaks associated with fresh produce, as it is often consumed raw without a cooking kill step. Pathogen contamination of produce can occur at any point in the production chain, with one area of concern being cross contamination of produce from processing equipment (Costa et al. 2006). Previous foodborne outbreaks and recalls of fresh produce have been linked to cross-contamination with L. monocytogenes within processing facilities. Specifically, in two of the most lethal produce-related outbreaks, the 2011 cantaloupe and the 2014 caramel
apple listeriosis outbreaks, food contact surfaces (FCS) of packing equipment were identified as routes of contamination with *L. monocytogenes* (Angelo et al. 2017). Additionally, a recent recall of fresh-cut fruit was attributed to equipment surfaces that tested positive for *L. monocytogenes* (2020 n.d.). *Listeria monocytogenes* is a leading cause of foodborne death, often causing illness in neonates, pregnant women, and immunocompromised adults (CDC). The environment where produce is manipulated, such as food contact surfaces or equipment used in processing, can also play a role in *L. monocytogenes* contamination (Maurel et al. 2016). This is of particular importance for *Listeria*, as it has been well known to be able to environmentally persist in food processing plants and equipment, often in the form of difficult to remove biofilms.

Regular cleaning and sanitizing of processing equipment plays a major role in mitigating the potential for cross contamination. Additionally, with the recent implementation of the Food Safety Modernization Act in 2011, there are several new regulations that specifically define sanitary regulatory compliance, including the requirement of cleaning and sanitation of the produce processing environment under the Food Safety Modernization Act’s Produce Safety Rule (FDA 2015). However, available trainings and other support only offers basic postharvest practices that help to reduce risk as there is a dearth of research conducted to support the implementation of Sanitation Standard Operating Procedure’s (SSOPs) for standardizing the cleaning and sanitation procedures for many applications. Writing SOPs help outline what areas need to be cleaned, how often, which detergents and sanitizers to use, how to clean and/or sanitize the surface, and who is responsible for completing the task (FDA 2015). Furthermore, such research to
support cleaning and sanitation SOPs for practices more uniquely utilized by small and medium-sized processors can especially be lacking.

Producers have increasingly begun retrofitting washing machines by disassembling them and replacing the barrel of the machine with crates to turn the machine into a large salad spinner using its spin cycle (Figure 1). Previous work by the authors has demonstrated the potential for use of these machines to result in contamination with *Listeria*.

Specifically, contamination of multiple parts of the machine occurred when spinach containing *Listeria innocua* at both low and high levels was observed, especially near the basin of the machine. Further, contamination of clean spinach leaves by the contaminated machine was also observed (Kamarasu et al., Under Review). However, the efficacy of different cleaning and sanitation interventions on these machines to mitigate the risk for contamination has not been evaluated.

![Figure 4.1: Depiction of the steps involved in building a retrofitted washing machine. A) retrofitting a washing machine and B) adding loading baskets to reduce contact exposure to C) Adding leafy greens to the 3 layered retrofitted machine D) Operating as a dry triple-washed produce.](image-url)

*University of Vermont Extension Ag Engineering*
As a clear presence of risk was observed, the next goal of the research was focused on analyzing the efficiency of various methods of risk reduction to recommend mitigation strategies. Chlorine based sanitizers are commonly approved to be used for food contact surfaces during post-harvest handling of produce. The germ-killing effect in a solution of chlorine bleach and water is due to available chlorine, present as hypochlorite and hypochlorous acid. Federal regulations permit the use of sanitizing solutions containing sodium hypochlorite on food processing equipment and food contact articles with provisions that equipment or articles sanitized with the solution must be allowed to drain adequately before contact with food and that the solutions used for sanitizing equipment shall not exceed 200 parts per million (ppm) available chlorine (McGlynn 2004). If higher concentrations are used, the surface must be rinsed with potable water after sanitizing. Another commonly used sanitizer is peroxyacetic acid-based sanitizing agents. It is diluted to obtain 110 ppm free peroxyacetic acid as it is the federally allowed limit for food contact surfaces (FDA- CFR- Title 21 n.d.). One objective of this study was to determine the efficiency of different cleaning methods and sanitizing agents, including chlorine and PAA, to reduce microbial contamination risk post spin drying leafy greens.

In addition to cleaning and sanitizing surfaces, many food processing operations utilize the measurement of adenosine triphosphate (ATP) as an indicator for sanitation in addition to environmental microbial swabbing, the latter of which can take 24-72 hours for results. There are numerous commercial ATP swab technologies available; however, one commercial technology, the A3 Lumitester (A3T), claims to provide higher sensitivity than traditional swabbing technologies, as it detects ATP, ADP, and AMP with one swab (Bakke 2022). Another major objective of this work was to evaluate and
compare the performance of this new A3T with Conventional Lumitester machine (CT) ATP swabbing technology to serve as a tool for sanitation in modified washing machine processing of leafy greens.

4.2 Materials and methods:

4.2.1 Materials:

The washing machine Speed Queen TC5000, Classic Clean™ were purchased from US Appliances (Alliance Laundry Systems LLC, Ripon, WI). Listeria innocua (ATCC 33090) was obtained from ATCC and cultivated per manufacturer instructions (American Type Culture Collection, Manassas, Virginia. 3M Quick microbial swabs were purchased from 3M, (Maplewood, Minnesota, USA). Listeria selective media Oxford agar and Oxford agar supplements were purchased from Fisher Scientific USA, Waltham, MA. In addition, nonselective media, tryptic soy agar (TSA) was purchased from Fisher Scientific USA, Waltham, MA. Baby spinach was obtained from The University of Massachusetts Amherst Dining Services, Amherst, MA. Cleaning materials included Scotch-Brite™ Non-Scratch Scrub Sponges Individually Sealed (3M, St. Paul, MN, USA), scrub brush (Holikme, Amazon USA), garden hose, Dawn® Heavyduty Degreaser (Procter & Gamble, Cincinnati, Ohio, USA) and Clorox germicidal bleach (a 7.03% chlorine-based sanitizer) (The Clorox Company, Oakland, California) purchased from Amazon, USA. Spray bottles were used to apply the sanitizing agents. SaniDate 15 (which is a 15% peroxy acetic acid-based sanitizer) was purchased from Biosafe Systems (Hartford, Connecticut, USA). Hydrion Peracetic acid (Hydrion, Vista, California) test
strips and Lamotte chlorine test strips (Chestertown, Maryland) were purchased from Thomas scientific (Swedesboro, New Jersey). Kikkoman Lumitester A3 machine, Kikkoman A3 swabs, Hygiena Lumitester machine and Hygiena ATP swabs were provided by Kikkoman Biochemifa (Tokyo, Japan).

4.2.2 Source, procure and build the DIY Converted washing machines:

The DIY retrofitted washing machine was built with the help of the University of Vermont’s Agricultural Engineering Extension team and followed the detailed conversion guide available online (University of Vermont - Retrofitted washing machine conversion guide). Based on the steps mentioned, the different parts of the washing machine such as the panels were taken apart and rewired to only keep the central water collecting drum and the brake system intact. Additionally, a stopwatch timer was attached to the machine to keep the time of each spin-drying cycle while drying the leafy greens, to ensure consistency. The series of steps involved in building the retrofitted machines are depicted in Figure 1. Perforated fishing baskets were inserted into the machine that were used for loading and unloading the spinach. The machine was placed at the pilot plant in Chenoweth laboratory, University of Massachusetts Amherst, MA. A protective enclosure was built with polyethylene sheets and PVC pipes (Lowe’s, Mooresville, North Carolina) to ensure that there was no spread of the aerosolized microorganisms during
spin drying experimental analysis to meet the Environmental Health and Safety Standards.

4.2.3 Bacterial inoculum preparation:

Listeria innocua, a surrogate for Listeria monocytogenes, was inoculated onto spinach and food contact points with initial inoculation loads (10^6 and 10^9 log_{10} CFU/ml) to evaluate microbial transfer. L. innocua was cultured in tryptic soy broth (TSB) at 37°C for 18 hours with continuous shaking at 130 rpm to obtain an inoculum level of 9 log_{10} CFU/ml and that was further diluted with TSB to obtain a range of inoculation load. The growth curve of the microbe was determined by measuring the Optical Density (OD600) at regular intervals in the VWR V-1200 Spectrophotometer machine (Chester, Connecticut) followed by plating, and OD_{600} used to normalize inoculum level. Prior to the analysis, individual spinach leaves were marked, placed in empty petri plates, inoculated with L. innocua, and dried for 1 hour before trials.

4.2.4 Determination of microbial transfer efficiency:

4.2.4.1 Spin drying of produce:

An initial spin-drying assay was conducted by analyzing a range of volumes of spinach added in each spin-drying cycle as well as with a range of initial inoculation load (10^2-10^9 CFU/ml). The inoculated spinach leaves, which were pre-marked to allow recovery of inoculated leaves, were mixed with regular spinach of varying volumes, ranging from 100-500 grams per spin drying cycle. A washing step was then conducted, where the batch of spinach was dipped in clean water, after which water was enumerated.
for microbial recovery and discarded accordingly. The washed spinach was added to the perforated orange load basket, placed inside the retrofitted washing machine, and spun 6 times for 10 seconds each to conduct spin cycle drying to remove the water from the spinach. Three different layers in the retrofitted machine and 3 contact points were assigned to each layer as seen in Figure 2. The contact points of the machine were swabbed with both the types of ATP swabs and the microbial swabs, serially diluted, and enumerated by plating on Oxford agar plates after incubation at 37°C for 48 hours and APC plates using TSA after incubation at 37°C for 24 hours. Pre-marked inoculated spinach leaves sample (25g) were removed from the retrofitted washing machine post spin drying and added into stomacher bags with 250 ml of 0.1% peptone water. The bags were stomached using a stomach lab lender (Thermo scientific) for 10 minutes, serially diluted, and enumerated by plating on Oxford agar plates after incubation at 37°C for 48 hours and APC plates using TSA after incubation at 37°C for 24 hours. The microbial recovery experiments were conducted in triplicate along with positive and negative controls by swabbing the machine prior to spin drying with inoculated spinach, non-swabbed microbial swab plating and plating direct *Listeria innocua* culture.

![Figure 4.2: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine; direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3 contact points](image-url)
4.2.4.2 Determination of degree of contamination from contaminated washing machine to uninoculated produce:

The potential for the retrofitted washing machine environmentally seeded with *L. innocua* to contaminate uninoculated spinach was evaluated. First, $3.77 \pm 0.29$ log CFU of *L. innocua* was inoculated onto direct contact points (Layer 1) as well as the indirect contact points (Layer 2 and 3) of the washing machine followed by a spin-drying run of the fresh spinach as described above, except without inoculation of leaves of spinach. Post spin drying the contact points of the machine were swabbed with both the types of ATP swabs and the microbial swabs, serially diluted, and enumerated by plating on Oxford agar plates after incubation at 37°C for 48 hours and APC plates such as TSA after incubation at 37°C for 24 hours. After each spin drying cycle, 25g of spinach including the inoculated and labelled leaves together were stomached in 250 ml of 0.1% peptone water for 10 mins. The stomached media was then serially diluted and enumerated on Oxford agar and TSA plates as described above.

4.2.5 Post spin drying cleaning:

The retrofitted washing machines were cleaned and sanitized post spin drying analysis of the experimental conditions. The cleaning steps were conducted in a 3-step process as shown in figure 3. Dawn® Heavyduty Degreaser was used as the cleaning detergent in the quantity of 300 ml and concentration of 7% Sodium cumenosulfonate surfactant was sprayed on all the layers of the machine for a contact time of 15 minutes followed by
lightly spraying potable water to physically remove soil and residue from the retrofitted washing machine. Three different methods of application of detergent were applied. The methods of application analyzed were spraying, dipping, combination of both. Non-scratch scrub sponge (Scotch Brite, Amazon USA) (Holikme, Amazon USA) was used to scrub the surface of the different layers of the washing machine. Surfaces were scrubbed and rinsed to remove visible soil and debris. This was followed by scrubbing using a clean scrub brush (Holikme, Amazon USA) and finally rinsed with clean water to remove all residue and suds. It is important to ensure all the residual detergent suds are completely removed to guarantee complete efficiency of the following application of the sanitizers. The water collected from conducting the cleaning practices were sanitized by addition of 10% Bleach solution and then discarded as per the recommendation by EH&S. The efficacy of the cleaning practices was confirmed by swabbing the different contact points of the machine and enumerating them on both Oxford and TSA plates prior to the next experimental replicate. Additionally, CT ATP swabs, CT ATP Lumitester machine, A3T swabs and A3T Lumitester machines were used to evaluate the RLU recovered post cleaning. Spin drying machines were allowed to air dry prior to application of the next step.
4.2.6 Post spin drying sanitation:

The cleaning method that provided the best result was chosen and it was followed by conducting comparative study between two common approved sanitizers (SaniDate 15 (15% available PAA at 110ppm) and chlorine-based like Clorox at 200ppm (7.03% available Chlorine)) for use during post-harvest handling of produce as represented in Figure 4. The efficacy of the sanitation step was confirmed as outlined above.
4.2.6.1 Chlorine based sanitizer:

A mixture of Clorox (7.03% available Chlorine) and distilled water were prepared by mixing one tablespoon (1/2 fluid ounce, 15 ml) of chlorine bleach per gallon of water to obtain 200 ppm free chlorine. Confirmation of free chlorine concentration was confirmed using Lamotte chlorine test strips. A volume of about 300 ml of the prepared sanitizer solution was sprayed all over the three different layers of the machine. The spray used was trigger based which is more ideal for decontamination as the spray was more targeted and softer in comparison to fine mist spray. The latter aerosolizes the sanitizer and leads to spreading of the contaminant particles in the air. It was ensured the food contact surfaces stayed wet for 5 minutes and it was allowed to air dry.

4.2.6.2 Peroxy acetic acid-based sanitizer:

SaniDate solution was prepared by diluting 0.41 fl oz in 5 gallons of distilled water to obtain a concentration of 109 ppm of free peroxyacetic acid. The concentration of the sanitizers was confirmed using Hydrion Peracetic acid test strips as per manufacturer instructions. A volume of 300 ml of the diluted solution was applied using a spray and the surface was maintained wet for 10 minutes and it was allowed to air dry. No subsequent rinsing after application was performed.

4.2.7 ATP Lumitester sanitation indicator:

The CT and A3T ATP swabs were used to swab the three different layers of the retrofitted machine and 3 specific contact points were assigned to each layer for swabbing (as described in Figure 2). Namely, B1 (Bottom), L1(Left) and R1(Right)
contact points in the 1\textsuperscript{st} layer (direct contact load basket); B2, L2 and R2 contact points in the 2\textsuperscript{nd} layer (secondary basket indirect contact point) and finally B3, L3 and R3 contact points in the 3\textsuperscript{rd} layer (indirect water collecting chamber). Each contact point was swabbed (100 cm\textsuperscript{2}) 2 times per replicate with 2 different types of swabs. Comparative study was conducted between A3T Lumitester machine vs CT Lumitester machine as shown in Figure 5.

![Figure 4.5: Comparative study between two commercially available Lumitester machines and swabs](image)

\begin{center}
\textit{Left- A3T Lumitester machine and A3 swab; Right- CT Lumitester machine and ATP}\n\end{center}

\textbf{4.2.8 Statistical analysis:}

The experimental results from triplicate measurements were expressed as mean values and standard deviation (mean ± SD). Graphpad prism was used to conduct Turkey-test and One way- ANOVA and multiple comparison Turkey-test was performed using GraphPad Prism version 9.3.1 (350) for Mac OS X, GraphPad Software LLC, San Diego,
California, USA, (www.graphpad.com). A P value less than 0.05 was considered significant.

4.3. RESULTS:

4.3.1 Observed contamination of the modified washing machines by contaminated leafy greens:

The results from previous research reported by the authors were also observed in this work, as nearly complete recovery from the contact points irrespective of the initial \textit{L. innocua} inoculation load was observed (Figure 6). It is important to note that there was a consistent loss of about 3 log CFU/ml of \textit{Listeria innocua} during the leafy green single wash step prior to spin drying and addition to the retrofitted washing machine. The results from measuring the presence of ATP from the non-porous washing machine contact points displayed high values from the indirect 2nd layer contact points of the machine as seen in Figures 7 and 8 showcasing the ATP recovery for $10^3$ and $10^6$ initial microbial inoculation levels, respectively. From conducting comparative studies between two different Lumitester machines, the A3 Lumitester displayed higher RLU values than the CT (Figures 7,8). For a lower inoculation load of $10^3$ CFU/ml the ATP measured from the contact points were $20K\pm28K$ RLU and $3550\pm4880$ RLU when measured with A3T and CT respectively. Additionally, for a higher inoculation load of $10^6$ CFU/ml the ATP measured from the contact points were $48K\pm45K$ RLU and $6300\pm8061$ RLU when measured with A3T and CT, respectively.
3.2 Contamination of clean spinach leaves by contaminated modified washing machines

In addition to evaluating the potential of spinach containing *L. innocua* to contaminate the machine, the ability of inoculated washing machine contact points to contaminate uninoculated fresh spinach was investigated. As expected, high levels of ATP were observed on the contact points that were directly inoculated (B2, L2, R2) as seen in Figure 9. Additionally, a high level of ATP was observed with contact point B1, likely due to transfer of ATP from the uninoculated spinach. Similar to the trend...
observed above, the A3T device registered higher RLU values (51K±70K RLU) compared to the CL device (11K±13K RLU; Figure 9).

![Graph showing ATP (RLU) levels across different washing machine contact points.]

Figure 4.9: ATP measurement to showcase the transfer from inoculated non-contact bottom layer to non-inoculated spinach and immediate contact surface

### 4.3.3 Evaluation of the effect of cleaning on Listeria contamination and ATP signal in modified washing machines:

The ability of different methods of application of commercial food-grade cleaning detergents was only conducted for the outermost orange basket (immediate food contact point) of the retrofitted washing machine. The different methods of application analyzed; specifically dipping the basket into detergent solution versus and hose spraying of detergent solution. No significant difference in both the methods when looking into the efficiency of removal of soil and debris and thereby reduction of microbial and ATP recovery rates. However, during the analysis it was observed that process of spraying with a gentle nozzle spray was more user friendly and quick when compared to dipping. Detergent such as Dawn® was applied onto the surface for a contact time of 15 minutes. This was followed by the application of potable water by using a garden hose. To
efficiently remove the soil from the surface scratch free food contact surface approved
scrubs were used, followed by a final rinse to remove the detergent suds and water
(Figure 3). The different contact points of the retrofitted machine were swabbed using CT
and A3T ATP swabs. The A3T generated higher RLU values compared to CL.
Specifically, the ATP values recovered post spin drying and pre cleaning was in the range
of 45K±49K RLU and 8000K±7071K RLU when measured with A3T and CL devices,
respectively. The different contact points of the machine were swabbed to enumerate the
ATP values post application of the cleaning methods. The results from this have shown a
drastic decrease in the ATP values using both devices (Figures 10 and 11). The ATP
signal was reduced to values between 305±418 RLU and 25±35 RLU when measured
with the A3T and CL devices, respectively.

4.3.4 Evaluating the influence of different commercial sanitizers after cleaning on
microbial load and ATP values of contaminated modified washing
machines:

After evaluating the influence of a cleaning step alone, the effect of application of two
different sanitizers, chlorine bleach and a commercial PAA/H₂O₂ product, on reducing
Listeria contamination and ATP values was tested. Figure 12 shows the microbial
recovery obtained from spin drying spinach leaves inoculated with 10⁶ log CFU/ml of
Listeria innocua before cleaning or sanitizing the machine, while Figure 13 shows that
microbial recovery was reduced to lower than limit of detection (LOD) when the food
contact surfaces were cleaned and/or sanitized. It should be noted that some level of
microbial recovery was observed after cleaning alone, but it was below the level of
quantification (<25 CFU/plate).
The ATP recovery once again followed a similar trend to the previous analysis as the A3T Lumitester machine displayed higher RLU values compared to CT. Interestingly, the ATP values were not significantly reduced after application of the sanitizers as seen in Table 1. This is expected as sanitizers are efficient at microbial reduction which was established in the results obtained in this research and cleaning influences the ATP values recovered. This can be seen from the results obtained shown in figure 14;15. There was
only a small difference in the ATP recovery values after the application of the sanitizing agents. After the application of chlorine sanitizer, CT and A3T measured 31±44 RLU and 231±316 RLU, respectively while after the application of the PAA-based sanitizer, CT and A3T measured 10±14 RLU and 310±410 RLU, respectively.

<table>
<thead>
<tr>
<th>Type of sanitizer</th>
<th>ATP recovered post spin drying (RLU)</th>
<th>ATP recovered post cleaning (RLU)</th>
<th>ATP recovered post sanitizing (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>• 8000±8500 RLU</td>
<td>25±35 RLU</td>
<td>• Chlorine-31±44 RLU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Peroxy acetic acid-10±14 RLU</td>
</tr>
<tr>
<td>A3T</td>
<td>• 18000-80000 RLU</td>
<td>9-600 RLU</td>
<td>• Chlorine-231±317 RLU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Peroxy acetic acid-310±410 RLU</td>
</tr>
</tbody>
</table>

TABLE 2: ATP recovery range from the various contact points post spin drying; post application of cleaning methods and post application of chlorine and peroxy acetic acid sanitizers when measuring with CT and A3T Lumineter machines.

Figure 4.14; 4.15 ATP recovery from the various contact points post application of chlorine sanitizer (left); ATP recovery from the various contact points post application of PAA sanitizer. (right)
4.4 DISCUSSION AND CONCLUSION:

Food contact surfaces that are contaminated by bacterial pathogens from already contaminated food commodities and the water in which they have been in contact, as well as those contaminated via contact with other external pathogen sources, have been recognized to be one of the significant factors leading to foodborne illnesses (Stuart and Worosz 2012). In this work, the efficacy of multiple interventions to mitigate risk of cross-contamination of modified washing machines commonly used in drying of leafy greens by small and medium-sized processors was investigated. Further, comparison and evaluation of the ability of two different commercial ATP-based indicator technologies in this leafy green process to aid sanitation was conducted.

Similar to previous work conducted by the authors (P. Kamarasu et al.), nearly complete microbial recovery from the washing machine was observed post spin drying with spinach leaves inoculated with *Listeria innocua*. In this work, the use of cleaning with detergent removed the observed contamination of *L. innocua* below the limit of quantification; and following this cleaning step with application of sanitizing agents, either chlorine (Clorox- 200 ppm free chlorine) or a commercial peroxy acetic acid-based product (SaniDate15-109 ppm of free peroxyacetic acid), completely removed residual microorganisms. It should be noted that the microbial recovery was evaluated after enumerating the microbial swabs on both APC (TSA plates) and oxford agar plates.
(Listeria selective media) and the results were similar. It should be noted that the presence of cleaning detergent can sequester the efficiency of sanitizers hence it should be completely washed off and the suds should be removed prior to application of the sanitizer, as was performed in this work. It is possible that this additional rinsing also contributed to the removal of residual microorganisms. Concentration and contact times of the detergent and sanitizers applied were based on common industry practice and maximum allowed concentrations according to the Environmental Protection Agency (EPA) for food contact surfaces (FDA- CFR- Title 21 n.d.). It is possible, that such steps may be less efficacious if lower concentrations of detergent were applied, or if no detergent was applied prior to sanitization. As has been reported elsewhere, the efficacy of sanitizers can be dramatically reduced when residual organic load is present (FDA 2015). It is also possible that these steps may have been less effective if pre-existing biofilms of Listeria existed on the machine, as the timing utilized in this experiment likely did not allow for adhesion and growth of the biofilms. Especially as Listeria is well known to form recalcitrant biofilms in processing settings, future work evaluating the potential of cross-contamination by biofilms existing in this processing apparatus, as well as the effect of the interventions presented here should be conducted. Nevertheless, the results observed here underscore the importance of implementation and observance of proper cleaning and sanitation practices have resulted in significant mitigation of the potential for Listeria contamination when using retrofitted washing machines for drying of leafy greens. In this research, higher dose of microbial concentration was used to evaluate the degree of microbial spread in the different parts of the machine. However, it is important to remind oneself that in a real world on-farm environment the CFU/area of
contamination present will most likely be present in lower concentrations. However, *Listeria* has a low infectious dose that can lead to possible foodborne illness in humans.

In addition to evaluating the efficacy of cleaning and sanitizing interventions on *Listeria* contamination in the modified washing machines, the potential of two different commercial ATP-based technologies to serve as a tool in maintenance of the aforementioned sanitary practices was evaluated. Although it cannot be used in isolation as a sanitation indicator, ATP monitoring devices are commonly used as sanitation tools used to complement microbial plate counts in kitchens, retail food establishments, dairy processing plants, and many other food processing settings. The device gives sanitation results in real time by detecting the residual amount of ATP on a food contact surface, which indirectly serves as an indicator of the presence of biological matter (Lane et al. 2020)(Costa et al. 2006). The two machines tested were the Kikkoman A3 Lumitester machine (A3T) and the Hygiena commercial machine (CT) commercial technology. The A3T machine claims to provide higher sensitivity than traditional swabbing technologies, as it detects ATP, ADP, and AMP with one swab (Bakke 2022). The former machine comes along with an application that can be downloaded on the phone and it is useful for visualizing the data. The results from measuring the presence of ATP from the non-porous washing machine contact points and conducting comparative studies between two different Lumitester machines (CT VS A3T) it was seen that the A3T demonstrated high level of sensitivity as the RLU measured was a lot higher than when measured with CT. When measured with the former there was a recovery of 49K±44K RLU post spin drying which was drastically reduced to 304±418 RLU after cleaning the machine. When measured with the latter the ATP recovery was in the range 8000±8485 RLU and it was
reduced to 25±35 RLU post cleaning. Cleaning alone may not kill microbial cells completely but can decrease their number and decrease the amount of biological, chemical and soil materials. Hence, when the surface bound ATP is reduced below a defined value, the surface can be considered clean (i.e.), biological, chemical and soil materials have been removed (Bakke 2022). The results from this research have proven the same as the application of the sanitizers have not influenced the ATP recovery.

Sanitizer application has completely killed the residual microorganisms when measured with microbial swabs as mentioned above. When measured with A3T, chlorine sanitizer application resulted in 231±317 RLU and PAA application resulted in 310±410 RLU. Additionally, when measured with CT, chlorine sanitizer application resulted in 0-62 RLU and PAA application resulted in 10±14 RLU recovery. Food contact surfaces are commonly considered clean and meet the requirements for a clean food contact surface when the ATP recovered is below 150 RLU and it is considered fail when the ATP recovered is above 300 RLU (FDA-READER n.d.) This was achieved when measured with CT Lumitester machine. However, this pass limit was only met in the contact points R2, L3, and R3 when measured with A3T machine. Additionally, the ATP recovery was in very high numbers in the contact points B1, B2, and B3 for the latter machine. Hence, it is important to be aware of the level of sensitivity and type of Lumitester machine being used when evaluating the sanitation efficiency of a food contact surface. The high levels of sensitivity of A3T machines are due to its ability to measure ATP and its broken down components such as ADP and AMP. Achieving an acceptable RLU recovery when using machines such as the one made by A3T can solidify the confidence that the producers have followed proper cleaning practices; however, given the significantly
higher sensitivity observed here, new relative acceptable RLU levels are needed to be established. There are certain advantages of using microbial swabs over ATP swabs for estimating the potential for risk on food contact surfaces. While using the former, after swabbing a certain surface the microbial swab is inserted into a tube containing 1 ml of neutralizing buffer, and generally will not significantly degrade over the normal processing time used when testing. However, ATP and its broken-down components such as ADP and AMP are highly sensitive and not as stable in the environment, and multiple other nonmicrobial biological materials (like food residues) also contribute to the observed signal. Hence, the RLU measured can be inconsistent depending on various contributing factors. This work supports that, as variation in ATP values was observed within both machines. However, overall, a higher degree of variability in the data obtained from using A3T Lumitester machine, likely due to its higher sensitivity compared to CT. A main limitation of this study is the inability to conduct analysis with lower concentration of *Listeria innocua* due to the limit of detection. In addition, future studies can also focus on other pathogenic microorganisms such as *Escherichia coliophage* and *Salmonella*. Further studies can also be conducted to evaluate the efficiency of other sanitizers, quaternary ammonium compounds and/or GRAS certified food contact surface approved plant derived phytocompound sanitizers. In sum, the work performed here informs the development of strategies to mitigate the risk of *Listeria* contamination when utilizing these modified washing machines for leafy greens, suggesting that proper cleaning and sanitation SSOPs show potential to mitigate risk, as well as the use of ATP-based swabbing technologies as an effective tool for monitoring sanitation in this
emerging processing apparatus commonly employed by small and medium sized produce producers.
CHAPTER 5:
5. COMMERCIAL AND RETROFITTED MACHINE COMPARATIVE MICROBIAL TRANSFER EFFICIENCY ANALYSIS WITH *LISTERIA INNOCUA*

5.1 Introduction:

Equipment cross contamination does not only occur with adapted processes but there are also instances of microbial contamination spread from the equipment to the produce leading to food recalls due to the usage of well-established equipment’s during any of the farm to fork stages of handling produce. One such produce that is commonly responsible for outbreaks due to microbial contamination are leafy greens. They are regularly colonized by diverse microbiota and can become contaminated with human pathogens and parasites while growing in the field or during harvesting, postharvest handling, processing, and distribution (Beuchat et al. 2003) (Matthews et al. 2012) (Van Boxstael et al. 2013). Pathogens such as *Listeria monocytogenes* are psychotropic, hence they also have the ability to grow when stored post-harvest at refrigeration temperatures. To control, reduce or eliminate microbial food safety hazards of fresh produce, effective food safety strategies are needed to be implemented throughout production, processing, and distribution (Luning et al. 2008). Microbial food safety hazards and source of contamination varies significantly based on the type of crop, production systems and practices (Warriner n.d.). One main area of focus is during the post-harvest handling of
produce as the key characteristic of these operations is that they involve considerable contact between fresh produce and handlers, tools, equipment surfaces, water and the field environment (Beuchat et al. 2003) (Sapers n.d.). Hence, following strict good agricultural practices (GAP) is a primary priority.

During post-harvest handling of leafy greens such as baby spinach they are harvested into bins for transport to the processing facility. Placing the bins directly onto soil can result in contamination. In addition, the bins are also used during washing and drying of the leafy greens and can lead to further contamination from various contributing factors (Matthews et al. 2012). Post-harvest handling of leafy greens such as spinach has various steps involved. Initially the produce is triple washed which is followed by drying process. One such practice of concern is a Do-It-Yourself (DIY) adapted practice followed by small scale and medium scale farmers where they use modified retrofitted washing machines for drying their leafy greens during post-harvest handling of produce. This practice is prevalent among small- and medium-sized producers, especially in New England. Previous work by the authors has demonstrated the potential for use of these machines that resulted in cross contamination of high number with a nearly complete recovery in multiple parts of the machine when spinach containing *Listeria innocua* at both low and high levels (10³ and 10⁶ CFU/ml) was observed, especially near the basin of the machine (Chapter 3). Further, contamination of clean spinach leaves by transfer from the contaminated machine was also observed. A clear presence of risk was observed based on the research findings.

In traditional medium and larger scale produce processing environment with higher resources, produce drying equipment such as Electrolux Professional VP2 Greens
machine spin dryer (CGM) is being used. The usage of 2 layered green machine provides benefits such as built-in basket with perforations for ease of loading and unloading of produce. In addition, it also has a built-in water collecting reservoir that increases the ease of discarding wastewater. Hence, investigating the possibility of the degree of microbial spread in two machines that are drastically different from one another will provide to better inform the best strategies required to conduct risk reduction mitigation strategies specific towards the individual machines.

This led the researchers to develop an objective to address the research question “What will be the degree of microbial spread in the different parts of the machines after spin drying spinach inoculated with *Listeria innocua* in a commercially available spin-drying machine (CGM) and conduct comparative studies with retrofitted spin-drying washing machine (RWM)”).

![Figure 5.1: Comparative study between commercial green spin-drying machine (CGM) (left) (Electrolux Professional) and retrofitted spin-drying washing machine (RWM) (right) (Speed Queen TC5000).](image)
5.2 Materials and methods:

5.2.1 Materials:

The washing machine Speed Queen TC5000, Classic Clean™ were purchased from US Appliances (Alliance Laundry Systems LLC, Ripon, WI). The VP2 Greens machine spin dryer was purchased from Electrolux Professional, Charlotte, NC. *Listeria innocua* (ATCC 33090) was obtained from ATCC and cultivated per manufacturer instructions (American Type Culture Collection, Manassas, Virginia). 3M Quick microbial swabs were purchased from 3M, (Maplewood, Minnesota, USA). Listeria selective media Oxford agar and Oxford agar supplements were purchased from Fisher Scientific USA, Waltham, MA. In addition, nonselective media such as tryptic soy agar (TSA) was purchased from Fisher Scientific USA, Waltham, MA. Baby spinach was obtained from The University of Massachusetts Amherst Dining Services, Amherst, MA. Scotch-Brite™ Non-Scratch Scrub Sponges Individually Sealed (3M, St. Paul, MN, USA), scrub brush (Holikme, Amazon USA) garden hose, Dawn® Heavyduty Degreaser (Procter & Gamble, Cincinnati, Ohio, USA) and Clorox germicidal bleach (a 7.03% chlorine-based sanitizer) (The Clorox Company, Oakland, California) were purchased from Amazon, USA.

5.2.2 Source, procure and build the DIY Converted washing machines:

The DIY retrofitted washing machine was built with the help of the University of Vermont’s Agricultural Engineering Extension team. A detailed conversion guide is available online (University of Vermont- Retrofitted washing machine conversion guide).

Based on the steps mentioned, the different parts of the washing machine such as the
panels were taken apart and rewired to only keep the central water collecting drum and the brake system intact. Additionally, a stopwatch timer was attached to the machine to keep the time of each spin-drying cycle while drying the leafy greens, to ensure consistency. The series of steps involved in building the retrofitted machines are depicted in Figure 2. Traditional perforated fishing baskets were inserted into the machine that were used for loading and unloading the spinach. The machine was placed at the pilot plant in Chenoweth laboratory, University of Massachusetts Amherst, MA. A protective enclosure was built with polyethylene sheets and PVC pipes (Lowe’s, Mooresville, North Carolina) to ensure that there was no spread of the aerosolized microorganisms during spin drying experimental analysis to meet the Environmental Health and Safety Standards.

Figure 2: Depiction of the steps involved in building a retrofitted washing machine. A) retrofitting a washing machine and B) adding loading baskets to reduce contact exposure to C) Adding leafy greens to the 3 layered retrofitted machine D) Operating as a dry triple-washed produce (UVM- University of Vermont Extension Agricultural Engineering n.d.).
5.2.3 Bacterial inoculum preparation:

*Listeria innocua*, a surrogate for *Listeria monocytogenes*, was inoculated onto spinach and food contact points with initial inoculation loads ($10^6$ and $10^9 \log_{10} \text{CFU/ml}$) to evaluate microbial transfer. *L. innocua* was cultured in tryptic soy broth (TSB) at $37^\circ C$ for 18 hours with continuous shaking at 130 rpm to obtain an inoculum level of $9 \log_{10}$ CFU/ml and that was further diluted with TSB to obtain a range of inoculation load. The growth curve of the microbe was determined by measuring the Optical Density (OD600) at regular intervals in the Spectrophotometer machine followed by plating, and OD$_{600}$ used to normalize inoculum level. Prior to the analysis, individual spinach leaves were marked, placed in empty petri plates, inoculated with *L. innocua*, and dried for 1 hour.

5.2.4 Determination of microbial transfer efficiency:

5.2.4.1 Spin drying of produce:

The microbial contamination spread was analyzed in both the RWM and CGM machines by spin-drying spinach inoculated with *Listeria innocua* in a low microbial load and a high microbial load ($10^6$ and $10^9 \log_{10} \text{CFU/ml}$). The inoculated spinach leaves, which were pre-marked to allow recovery of inoculated leaves, were mixed with regular spinach to make up to a total volume of 250 grams per spin drying cycle. A washing step was then conducted, where the batch of spinach was dipped in clean distilled water, after
which the microbial recovery was evaluated from the wash water by enumerating it on APC and Oxford agar plates and it was discarded accordingly. The washed inoculated spinach was added to the external perforated load basket, placed inside the RWM, and spun 6 times for 10 seconds each to conduct spin cycle drying to remove the water from the spinach. Similarly, the washed inoculated spinach was added to the built-in load basket, placed inside the CGM, and spun 6 times for 10 seconds each to conduct spin cycle drying to remove the water from the spinach. After each dry run, 25g of spinach including the inoculated and labelled leaves were taken from RMW and CGM machines and stomached in 250 ml of 0.1% peptone water for 10 mins. The stomached media was then serially diluted and enumerated on Oxford agar and TSA plates after incubation at 37°C for 48 hours and 24 hours respectively. All assays were repeated on separate days in triplicates.

5.2.4.2: Determination of degree of contamination from inoculated produce to contact points:

Three different layers in the RWM and 3 contact points were assigned to each layer as seen in Figure 3A. Additionally, there are two different layers in the CGM ad 3 contact points were assigned to each layer as seen in Figure 3B. 3M Quick microbial swabs (Minnesota, USA) were used to swab the contact points with 2 swabs per point of both the machines and recovered Listeria was enumerated by serial dilution and plating on Oxford agar plates and TSB plates at 37°C for 48 hours and 24 hours respectively. The microbial recovery experiments were conducted in triplicate along with positive and
negative controls such as swabbing the machine prior to spin drying with inoculated spinach, non-swabbed microbial swab plating and plating direct *Listeria innocua* culture.

![Diagram of layers and contact points](image1)

**Figure 5.3A**: Images of the three different layers and the contact points assigned to each layer in the retrofitted washing machine (RWM); direct contact (orange load bucket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points; 3rd layer (indirect water collecting layer) has B3, L3 and R3 contact points.

![Diagram of layers and contact points](image2)

**Figure 5.3B**: Images of the two different layers and the contact points assigned to each layer in the commercial green spin-drying machine (CGM); direct contact (in built loading basket) has B1, L1 and R1 contact points; the 2nd layer (secondary basket indirect contact point) has B2, L2 and R2 contact points.
5.2.4.3 Determination of efficiency of risk reduction cleaning and sanitation practices:

The RWM and CGM were cleaned and sanitized post spin drying analysis of the experimental conditions. The cleaning steps were conducted in a 3-step process as shown in figure 4. Cleaning detergent such as Dawn® Heavyduty Degreaser in the quantity of 300 ml and concentration of 7% Sodium cumenosulfonate surfactant was sprayed on all the layers of the machine for a contact time of 15 minutes followed by lightly spraying potable water to physically remove soil and residue from the retrofitted washing machine. Non-scratch scrub sponge (Scotch Brite, Amazon USA) (Holikme, Amazon USA) was used to scrub the surface of the different layers of the machines. Surfaces were scrubbed and rinsed to remove visible soil and debris. This was followed by scrubbing using a clean scrub brush (Holikme, Amazon USA) and finally rinsed with clean water to remove all residue and suds. It is important to ensure all the residual detergent suds are completely removed to guarantee complete efficiency of the following application of the sanitizers. The water collected from conducting the cleaning practices were sanitized by addition of 10% Bleach solution and discarded accordingly. The efficacy of the cleaning practices was confirmed by swabbing the different contact points of the machines and enumerating them on both Oxford and TSA plates.
The cleaning step was followed by application of food contact surface approved sanitizer such as peroxy acetic acid-based sanitizer SaniDate 15 (15% available PAA) solution was prepared by diluting 0.41 fl oz in 5 gallons of distilled water to obtain a concentration of 109 ppm of free peroxyacetic acid. The concentration of the sanitizers was confirmed using Hydrion Peracetic acid test strips as per manufacturer instructions. A volume of 300 ml of the diluted solution was applied using a spray and the surface was maintained wet for 10 minutes and it was allowed to air dry as seen in Figure 5. No subsequent rinsing after application was performed. The efficacy of sanitation step was confirmed by swabbing the different contact points of both the machines and enumerated.
on both Oxford and APC such as TSA plates. The microbial recovery experiments were conducted in triplicates.

5.2.5 Statistical analysis:

The experimental results from triplicate measurements were expressed as mean values and standard deviation (mean ± SD). Graphpad prism was used to conduct Turkey-test and One way- ANOVA and multiple comparison Turkey-test was performed using GraphPad Prism version 9.3.1 (350) for Mac OS X, GraphPad Software LLC, San Diego, California, USA, (www.graphpad.com). A P value less than 0.05 was considered significant.

5.3 Results:

The purpose of this study was to evaluate and conduct comparative study to determine the degree of microbial contamination spread in the different parts of both RWM and CGM machines. This was determined by spin drying baby spinach inoculated with *Listeria innocua* (gram positive bacterium, non-pathogenic surrogate for *Listeria monocytogenes*) with initial inoculation loads (10^6 and 10^9 log_{10} CFU/ml). There are a series of steps followed during post-harvest handling of spinach and the wash step is very important as it is imperative for the removal of debris and dirt from the soil. Hence, it was also important to include a wash step during microbiological analysis to evaluate the degree of microbial contamination in the washing machine. From the results, it was seen there was a loss of 3.23+/−0.56 log CFU/ml of *Listeria innocua* on initially inoculated
leaves into the wash water prior to loading into both the machines irrespective of the initial microbial load.

There are three different layers of RWM, and three specific sampling points assigned to each layer for swabbing as depicted in Figure 3A. There was a nearly complete recovery from all contact points except L3 and R3 irrespective of the initial microbial load. For the lower initial inoculation load there was lower than limit of detection (LOD<25 CFU) recovery from the contact points L3 and R3. The highest level of contamination when using inoculated spinach leaves was observed on the three bottom most contact points of the machine, such as B1, B2, B3 as well as from the immediate contact points L1 and R1 (Figure 3). The degree of microbial recovery was on a higher level irrespective of the logarithmic load of the initial L. innocua microbial culture inoculated onto the spinach surface as seen in Figure 6A and 6B.

![RWM Microbial recovery](image)

Figure 5.6: A) Microbial recovery from the various contact points in RWM after inoculation of $10^3$ CFU/ml post wash step; B) Microbial recovery from the various contact points in RWM after inoculation of $10^6$ CFU/ml post wash step. Ns- not significant difference in the means of the individual groups. *-statistically similar individual means of the groups.

The primary difference between the RWM and CGM is the number of layers in each machine. The RWM is a three-layered machine including the external loading basket and
the CGM is a two layered machine where the first layer is an in-built loading basket. The layers of both the machines and the contact points are depicted in Figures 3A and 3B. The microbial recovery for the CGM was highest in the contact points B1 and B2 and the contact points that came in direct contact with the inoculated spinach such as L1 and R1 for both low and high initial inoculation loads as seen in Figures 7A and 7B. It can also be seen that the microbial recovery rates were significantly higher in the specific contact point B2 for both low and high initial inoculation loads. Additionally, there was a higher degree of variability in the microbial recovery when the spinach inoculated with a lower initial microbial load was spin dried in the machine as seen in Figure 7A. Interestingly, when comparative study was conducted between the RWM and CGM it can be inferred that the degree of microbial recovery was higher from the former machine that the latter.

Figure 5.7: A) Microbial recovery from the various contact points in CGM after inoculation of $10^3$ CFU/ml post wash step; B) Microbial recovery from the various contact points after inoculation in CGM of $10^6$ CFU/ml post wash step. *- statistically similar individual means of the groups.
Cleaning and sanitation were conducted for both the machines after spin drying the inoculated spinach and microbially swabbing the various contact points in each machine. Cleaning detergent such as Dawn® Heavyduty Degreaser (Procter & Gamble, Cincinnati, Ohio, USA) in the concentration of 7% Sodium cumenosulfonate surfactant in the volume of 300 ml was used for a contact time of 15 minutes along with water to physically remove soil and residue from the machines. The contact points in both the machines were microbially swabbed with two swabs per contact points and the swabbing analysis was conducted as triplicates on different days. The results after application of cleaning practices show that the microbial recovery was reduced to lower than LOD (<25 CFU) in both RWN and CGM as seen in Figures 8A and 8B respectively. The cleaning practice was followed by application of food contact surface approved sanitizer such as peroxy acetic acid-based sanitizer SaniDate 15 (15% available PAA) prepared by diluting 0.41 fl oz in 5 gallons of distilled water to obtain a concentration of 109 ppm of free peroxyacetic acid. A volume of 300 ml of the diluted solution was applied using a spray and the surface was maintained wet for 10 minutes and it was allowed to air dry. No subsequent rinsing after application was performed. The contact points in both the machines were microbially swabbed with two swabs per contact points and the swabbing analysis was conducted as triplicates on different days. It was mentioned above that the degree of microbial recovery was higher when enumerated from the RWM when compared to CGM. However, from the results post sanitation it was seen that it was not possible to obtain a complete removal of the residual microorganisms specifically in the contact point B2 in CGM even after following the FDA and EPA recommended post-
harvest food contact surface approved sanitation procedures as seen in Figures 8A and 8B.

5.4 DISCUSSION AND CONCLUSION:

Food contact surfaces that are contaminated by bacterial pathogens from already contaminated food commodities and the water which they have been in contact, as well as those contaminated via contact with other external pathogen sources, have been recognized to be one of the significant factors leading to foodborne illnesses and has caused as much as 28.6% of all food safety loss incidents (Soon 2020) (CDC 2017). Moreover, processing operations of crops such as washing/sanitizing, cutting, drying, packaging and storing are known to provide opportunities for cross contamination (Axelsson et al. 2013) (Buchholz et al. 2012). From previous researches, it was seen that cross contamination of pathogenic bacteria between plant cops has been demonstrated to
occur on conveyer belts and stainless-steel bench surfaces. In a study conducted by Lehto et al it was seen that highest microbial contamination was observed on a centrifuge basket used in the washing of lettuce leaves that had 100 CFU/cm². Additionally, it was seen that even after cleaning, lifting conveyers, floors, gloves and peeling machines remained heavily contaminated with about 50 to 72 CFU/cm² microbial recovery (Lehto 2011). It is to be noted that the use of centrifuge machines during post-harvest handling of produce can highly contribute towards further microbial spread throughout the different parts of the machine due to the high force of action conducted. The previous research conducted by Kamarasu et al has demonstrated exactly that as there was high levels from microbial recovery from all the contact points in the different layers of a retrofitted centrifuge spin drying machine used during post-harvest handling of leafy greens (Pragathi Kamarasu n.d.). From further investigation it was seen that following FDA and EPA recommended cleaning and sanitation practices can efficiently reduce the risk involved in following such practices by completely removing the residual microorganisms present in the food contact surfaces (FDA 2015)(FDA- CFR- Title 21 n.d.). With the recent implementation of the Food Safety Modernization Act in 2011, there are several new regulations that specifically define sanitary regulatory compliance, including the requirement of cleaning and sanitation of the produce processing environment under the Food Safety Modernization Act’s Produce Safety Rule(FDA- CFR- Title 21 n.d.). Small scale and medium scale producers have increasingly begun retrofitting washing machines as it is a cost-efficient process when compared to the use of traditional spin-drying machines. However, available trainings and other support only offers basic postharvest practices that help to reduce risk as there is a dearth of research conducted to support the
implementation of Sanitation Standard Operating Procedure’s (SSOPs) for standardizing the cleaning and sanitation procedures for many applications. Writing SOPs will help outline what areas need to be cleaned, how often, which detergents and sanitizers to use, how to clean and/or sanitize the surface, and who is responsible for completing the task. Furthermore, such research to support cleaning and sanitation SOPs for practices more uniquely utilized by small and medium-sized processors can especially be lacking. Hence, the main goal of this research was to conduct comparative studies between two spin drying machines that are drastically different from one another based on various factors such as cost, ease of use, potential for spread of microbial contamination, ease and efficiency of cleaning and sanitation applications. We believe the research findings will help to better inform the producers which machine is best suited to cater towards their specific requirements and on farm conditions. From the results, it was seen there was a loss of $3.23\pm 0.56$ log CFU/ml of *Listeria innocua* on initially inoculated leaves into the wash water prior to loading into both the machines irrespective of the initial microbial load. As the removal of microbial load into the water was in very high numbers, it can be said that this can be one of the major contributing factors towards further spread within the different areas of the machines as the water acts as a carrier. The primary different between the RWM and CGM is the number of layers in each machine. The RWM is a three-layered machine including the external loading basket and the CGM is a two layered machine where the first layer is an in-built loading basket. The layers of both the machines and the contact points are depicted in Figures 3A and 3B. In both the machines the highest degree of microbial recovery was seen in the contact points B1, B2 and the contact points that came in direct contact with the inoculated spinach such as L1 and R1.
Interestingly, when comparative study was conducted between the RWM and CGM it can be inferred that the degree of microbial recovery was higher from the former machine that the latter. Additionally, when comparing the individual high recovery points in both the machines (B1, B2), it was seen that in CGM there was significantly higher recovery from B2 when compared to B1, however, the recovery from both the points were statistically similar when enumerated from the RWM. This is mainly due to the stagnation of the wash water in the contact layer B2 and the difficulty in draining it into water collecting chamber located at the bottom of the CGM. The researchers believe a remodification of the water draining hole at the bottom of the machine is strictly required to ensure this does not lead to further issues. This need was further supported by the research findings after the application of cleaning and sanitation procedures. It was seen from the results that it was not possible to obtain a complete removal of the residual microorganisms in the contact point B2 in CGM even after following proper FDA recommended practices in EPA concentrations. This is of particular importance for *Listeria*, as it has been well known to be able to environmentally persist in food processing plants and equipment, often in the form of difficult to remove biofilms. In addition, there was also rust formation in the contact layer B2 in CGM and that is a major concern as it can lead to quality concerns in the fresh produce introduced into the machine.

Various other factors of both the machines were also taken into consideration when conducting comparative studies. The differences in both the machines are listed in Table 1. The major concern in RWM is the difficulty in accessibility of the 3rd layer as the 2nd layer must be unscrewed by using a power drill. The results suggest that there is a
significant microbial recovery from the 3rd layer specifically in the contact point B3, hence being able to efficiently clean and sanitize this layer is highly important for risk reduction. Another concern in using the RWM is improper water draining system. It has been known that wash water is one of the major contributing factors towards microbial spread in machines. Hence, in this machine the water is collected externally by attaching a tube and it is important to ensure that this wash water is treated and discarded accordingly. In CGM the water collecting chamber in built in and is present in a separated compartment at the bottom of the machine which aids in easy removal. However, this advantage cannot be used to its full potential as the water draining hole and B2 layer of the machine is not designed efficiently which is a major concern. In addition, it is to be noted that our research is mainly catered towards small scale and medium scale farmers. Hence cost of the individual machines is major factor, the CGM costs twice as much as RWM. This is the main reason many local producers have growingly started retrofitting washing machines. It was also seen that CGM’s centrifugal force had an impact on the physical quality of the leafy greens. The individual baby spinach leaves had bruises on its surface post spin drying. However, currently further research is being conducted by the Kinchla research team at University of Massachusetts Amherst to support this claim. In addition, further research can be done to determine the measures required to ensure that a microbial removal is obtained specifically in contact point B2 in CGM. This can be done by further investigating variables such as sanitizer contact time and sanitizer concentrations. ATP Lumitester machines are commonly used as on-site sanitation indicators. Hence, these machines can be used to further confirm the efficiency of the cleaning and sanitation practices. In addition, there are different types of
retrofitted washing machines available in the market based on the cost of the machines.

Hence. Further investigation can be done to conduct comparative studies with these machines. The results of this work will help inform areas of concern for producers to stay in compliance with the recently implemented Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) that states specific sanitary regulatory conditions and there are specific regulations that discuss postharvest handling and sanitation. By looking at the research findings it is up to the readers to decide which machine best suits their individual needs as there are pros and cons in using both the types.

<table>
<thead>
<tr>
<th>RETROFITTED WASHING MACHINE</th>
<th>COMMERICAL SPIN DRYING MACHINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>High microbial recovery</td>
<td>Low microbial recovery</td>
</tr>
<tr>
<td>Inefficient wash water removal</td>
<td>Efficient wash water removal= water collecting chamber</td>
</tr>
<tr>
<td>Absence of lid = aerosolized microorganisms spread.</td>
<td>Presence of lid</td>
</tr>
<tr>
<td>Efficient sanitation</td>
<td>Inefficient sanitation = contact point B2 due to water stagnation</td>
</tr>
<tr>
<td>No visible physical damage to the leafy greens.</td>
<td>Higher physical damage to the leafy greens and rust formation in the contact point B2.</td>
</tr>
</tbody>
</table>

Table 3: Comparative study differences between Retrofitted washing machine and Commercial Green machine based on various factors.
CHAPTER 6:

6. INACTIVATION OF FOODBORNE VIRUSES BY CINNAMALDEHYDE NANOEMULSIONS REQUIRE A LIPID ENVELOPE

6.1 Introduction:

Human norovirus is the leading cause for foodborne illness globally accounting for 200,000 deaths annually. Noroviruses also account for the majority of foodborne illnesses, as well as the fourth leading cause of foodborne death. It is a single stranded positive sense RNA, non-enveloped virus of the Caliciviridae family. One considerable challenge to their control is their general resistance to many commonly used inactivation agents such as UV radiation, ethanol, and chlorine-based treatments. Norovirus consists of a negatively charged, stable protein capsid concealing an RNA genome. From previous research it can be seen that there are a few efficacious disinfectants for noroviruses, many of which inactivate virus by oxidizing the non-enveloped protein capsid, however, the challenge faced is that none of them are food grade (P. Kamarasu, Hsu, and Moore 2018).

Alternative plant-based sanitation methods such as bioactive phytocompounds present in essential oils (EO) are being explored. EO’s are generally recognized as safe (GRAS) and is approved for food use (21 CFR 182.60) by the Food and Drug Administration (FDA) to impart flavor in numerous foods. Hence, they could be considered an alternative way to impede the proliferation of foodborne pathogens, especially on food contact surfaces. From research conducted by Calo et al (Calo et al. 2015) it was seen that EOs comprise numerous compounds such as aromatic
hydrocarbons, terpene (monoterpenes and sesquiterpenes), terpenoids, esters, alcohols, acids, aldehydes and ketones, and their antibacterial activity is not solely contributed by any one compound. A challenge faced by the researchers has been evaluating the most potent antimicrobial compound due to their chemical complexity. To date, most studies have focused on studying the antimicrobial activity of EOs with little discussion on activity of individual components in the EO or their mechanism of action. It was also noted that antimicrobial activity of EOs is not reliant on one specific mode of action; instead, EOs can attack several targets in a cell to inactivate it (Hyldgaard et al. 2015). One such efficient essential oil is acquired from the bark of cinnamon and the primary compound is cinnamaldehyde, which makes up 90% of the essential oil. Cinnamon essential oils have displayed antimicrobial, antitumor, anti-inflammatory and antioxidant activities (Angane et al. 2022). Further, they have the potential to oxidize proteins, making them an ideal candidate for noroviral inactivation. Researchers have demonstrated that cinnamaldehyde has strong antibacterial effect against S. epidermidis (Albano et al. 2019), S. aureus (Zhang et al. 2015) and Aeromonas hydrophila (Liu et al. 2020) by causing cell membrane distortion, leakage and in addition to polarization of cytoplasmic content. Cinnamaldehyde has demonstrated promising results against bacteria and fungi, however, their efficiency against viruses has not been explored to an extent. M fabra et al conducted research to evaluate the efficiency of cinnamaldehyde against human norovirus surrogates such as murine norovirus, feline calicivirus and hepatitis A virus and results demonstrated very low inactivation efficiency (Fabra et al. 2016). This inability to successfully inactivate these viruses can be attributed to their high levels of stability compared to bacteria and more specifically in
the case of human norovirus as it is a non-enveloped virus, a feature that can make them more stable and more resistant than enveloped viruses to thermal, chemical and environmental inactivation (Li and Chen 2015).

Nanoemulsions have been used increasingly in pharmaceutical, food and cosmetic industries due to their advantage over traditional emulsions. They have the ability to improve the appearance, stability, release properties and bioavailability of the active compounds. One major drawback of incorporating cinnamaldehyde in food systems are their low water solubility and strong odor which can be overcome by incorporating the compound in a nanoemulsion (Ozogul et al. 2022). Further, some work has demonstrated that incorporation of EOs or their active ingredients in nanoemulsions can enhance their efficacy against bacteria and fungi. Specifically, research conducted by Paudel SK et al resulted in enhanced inactivation compared to similar concentration of cinnamon essential oil in more than 7.7 and 5.5 log reduction of Listeria monocytogenes and Salmonella enterica Typhimurium respectively (Paudel, Bhargava, and Kotturi 2019). This increase in inactivation efficiency can be attributed towards the nanomolecular size of the emulsion particles which enhances surface area relative to volume of active ingredient used and makes them easier to reach the target and in turn inactivate target cells, as well as the introduction of charge in the emulsions that can enhance their delivery to the pathogen target. Although the targets mentioned above have a lipid envelope, allowing for a strong attraction of the lipophilic nanoemulsion to the target, the norovirus capsid has hydrophobic protein patches in the capsid protein that are involved in folding and maintaining icosahedral contacts that keep the viral capsid intact.
The potential of these nanoemulsions to better deliver inactivation agent to these hydrophobic patches and disrupt capsid stability has not been tested.

There are various different methods of preparation of nanoemulsions, which can be more broadly divided into high energy and low energy techniques. Low energy techniques, such as spontaneous emulsification, are based on the spontaneous formation of small droplets when the composition of the surfactant-oil-water is changed in a controlled manner at a constant temperature (Matthews et al. 2012). This method does not require any additional external energy, thereby it is cost efficient, quick and user friendly. The rapid movement of components from one phase to another leads to the generation of supersaturated regions that promotes nucleation and droplet growth phenomena (Quintanar-Guerrero et al. 1997). This method has been used to form several kinds of nanoemulsion based delivery systems.

Addition of cationic surfactant such as CTAB to essential oil nanoemulsions has been found to further enhance the antimicrobial ability and can enhance the delivery of the disinfectant to the negatively charged pathogen targets, like norovirus particles. Hence, the purpose of this work was to evaluate the potential of cationic cinnamaldehyde nanoemulsions (CNME) made by the spontaneous method to enhance the efficacy of cinnamaldehyde against a norovirus surrogate. Further, the stability and shelf-life of these nanoemulsions will be evaluated.

Due to the recent pandemic caused by SARS-CoV2 there have been many concerns raised about the spread of the virus through food or food packaging materials. The absence of standard therapeutic options for the pandemic COVID-19, such as vaccines and antibiotics, has led to supportive therapies that involve the use of natural agents.
Alternative plant-based therapies such as bioactive Phyto compounds are being explored as they have demonstrated antimicrobial and anti-inflammatory activities against respiratory viruses such as SARS-CoV-2 (Kulkarni et al. 2020). SARS-CoV2 viruses are a group of enveloped viruses with single-stranded RNA genomes with size ranging from 26 kb to 32 kb. Mpro is a key enzyme of coronaviruses and has a pivotal role in mediating viral replication and transcription, making it an attractive drug target for SARS-CoV-2. According to a trial by Chojnacka et al., approximately seventeen prospective SARS-CoV-2 Mpro inhibitors phytochemical compounds were recognized (Chojnacka et al. 2020). These Mpro inhibitors were assigned to the compounds belonging to pseudo-peptides, floronates, and flavonoid. Additionally, lipophilic monoterpenic compound such as 1,8-Cineole (Eucalyptol, C10H18O) which is the major component of Eucalyptus oil, has shown efficient antiviral, immunomodulatory and broncho dilatory effect against many enveloped respiratory viruses (Sadlon and Lamson 2010) (Usachev et al. 2013). However, there has not been any previous research conducted on evaluating the efficiency of cinnamaldehyde essential oil as a possible inhibitor for enveloped viruses such as coronavirus. Hence, the aim of this project is to evaluate the efficiency of cationic cinnamaldehyde nanoemulsions (CNME) and cinnamaldehyde oil (CNMO) as a disinfectant against enveloped virus human coronavirus-229E (HCoV-229E), a surrogate for SARS-CoV-2 and non-enveloped virus MS2 bacteriophage (surrogate for human norovirus).
6.2 Materials and methods:

6.2.1 Materials:

Pure cinnamaldehyde essential oil (W228613-100G-K) was purchased from Sigma-Aldrich, Springfield, MA. Tween-80 and Centrinium Bromide CTAB obtained from BioBasic, Ontario, Canada. Nature’s way Medium Chain Triglyceride (MCT oil) was purchased from Amazon, USA. coronavirus-229E (ATCC-VR740), MS2 bacteriophage (ATCC 15597-B1) were obtained from ATCC, Manassas, Virginia. The HUH7 cell line was obtained from Dr Brett Lindenbach’s laboratory at Yale University, New Haven, Connecticut.

6.2.2 Preparation of cinnamaldehyde essential oil nanoemulsions:

Cinnamaldehyde essential oil-in-water nanoemulsion was prepared by spontaneous method as shown in Figure 1. This was done by homogenizing 10 % wt oil phase with 90%wt aqueous phase. The components of the oil phase such as the essential oil (27.7 μg/ml), MCT oil (stabilizer) were initially mixed with constant agitation of speed 700 rpm using a magnetic stirrer at room temperature for 5 minutes. Followed by that the non-ionic surfactant (Tween 80) was added to the oil phase and the mixture was stirred again for about 3 minutes. Simultaneously pure distilled water was used as a base for the aqueous phase and the ionic surfactant (CTAB) was dissolved in it. The spontaneous emulsification process as shown in Figure 2 starts when 10% of the oil phase is slowly added into 90% aqueous phase while it is stirring at a constant speed of 700 rpm for 30 minutes on the magnetic stirrer. The rapid movement of components from one phase to
another leads to the generation of supersaturated regions that promotes nucleation and droplet growth phenomena. This mechanism has been described as a “diffusion and stranding” mechanism as mentioned by Quintanar et al (Quintanar-Guerrero et al. 1997).

The fabrication parameters affecting the characteristics of the nanoemulsion were analyzed by varying the different components such as the essential oil, surfactants, and the stabilizer oil in a range of concentrations. The composition that resulted in ideal particle diameter (around 100 nm), zeta potential (25 mv) and polydispersity index of above 98% was chosen to conduct the antiviral studies. In addition to the nanoemulsion, antiviral analysis was also conducted with plain cinnamaldehyde essential oil to conduct comparative studies.

Figure 6.1: The low energy emulsification, spontaneous emulsion method is depicted showcasing the spontaneous droplet formation process.

Figure 6.2: The nanoemulsion formation takes place in two stages. Initial discontinuous microemulsion phase and followed by from one phase to another leads to the generation of supersaturated regions that promotes nucleation and droplet growth phenomena.
6.2.3 Shelf-life study of the cinnamaldehyde nanoemulsion:

After fabrication, the visual stability and the characteristic properties of the nanoemulsion were analyzed. The nanoemulsion was stored at a range of temperatures (4°C, 20°C and 37°C) for a period ranging from 0 to 28 days and the characteristic properties were analyzed on 0,1,3,7,14,21 and 28th day. Zetasizer nano machine based on dynamic light scattering mechanism from Dr Julian McClements laboratory was used to analyze characteristic properties of the nano particles such as mean diameter, polydispersity index and the zeta potential. Before use, each sample was diluted with 1:100 ratios of deionized water. The disintegration of the components in the emulsion were analyzed visually. Stability measurements were carried out in triplicates.

6.2.4 Antimicrobial activity:

6.2.4.1 MS2 Bacteriophage:

The antiviral activity of CNMO and CNME was tested against MS2 Bacteriophage which is a BSL-1 safe surrogate for human norovirus. *Escherichia coli* phage was used as a host to propagate MS2 bacteriophage. The number of bacteriophages in plaque forming units per milliliter (PFU/ml) were determined by the overlay agar plating method. To do that, Tryptic soy broth (TSB), 0.5% Tryptic soy agar soft media and 1% Tryptic soy agar (TSA) plates were prepared. The propagation and purification of bacteriophage were as follows: Overnight culture of Ecoli was prepared by inoculating a loop of freeze thawed
bacterial culture into 5 ml of TSB media. The following day, 300 μL of the culture was further inoculated into 29.7 ml of TSB and placed in an incubator on a shaker at 100 rpm for 2 hours 15 minutes until it reaches an Optical Density of 0.6 measured in an UV-Vis Spectrophotometer. Followed by that, suspension assay was conducted for different variables of reaction time periods by using a neutralizing buffer (3% Beef extract) and varying concentrations of the disinfectant against the phage. This was followed by serially diluting the MS2 bacteriophage: disinfectant samples and adding 700 μL of the sample, 300 μL of Ecoli and 36 μL of the supplements to 9 ml of 0.5% overlay agar and vortex it before carefully pouring it on the TSA plates. Neutralization control and negative control were also plated, and they were incubated overnight at 37°C incubators.

All bacteriophage tests were performed in triplicates.

6.2.4.2 HUH7 tissue culture for 229E propagation and antiviral studies:

HUH7 cells were cultured in 37°C at 5% CO2 with DMEM medium supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, F7524-500ML, Burlington, MA, USA) and 1% (v/v) Penicillin-Streptomycin. HUH7 cells were seeded with a density of 500,000 cells/well in 6-well plates. The suspension inactivation assay of coronavirus-229E was conducted with CNMO and CNME. Freeze thawed 100 μL of viral culture was mixed in the flipper with 100 μL of the disinfectants for varying reaction time periods (5 minutes, 15 minutes, and 30 minutes). In addition to the disinfection samples, negative control (100 μL virus + 100 μL PBS) and neutralization control (100 μL disinfectant+ 1080 μL of FBS) were also analyzed. All the samples were neutralized with 10% v/v
FBS. All the sample analysis were conducted in triplicates. Followed by that the individual controls were serially diluted up to 6 dilutions and 300 μL of sample was added to each well in the 6 well plates. The samples were sealed on top of the wells by adding 2 ml of 1:1 dilution of 2.4% Avicel solution and 2xDMEM and the plates were placed in the 33°C 5% Co2 incubator for 4 days to allow plaque formation. On the 4th day, 3.7% formaldehyde solution was prepared by diluting it in regular media. The plates were removed from the incubator and the avicel solution was slowly removed and replaced by adding 2 ml of the formaldehyde solution. The formaldehyde helps to interlink the cells and makes sure it does not get affected during the following staining process. After an incubation period of 1 hour the plates are again removed from the incubator and washed with cell grade water to remove any excess formaldehyde solution followed by staining with 200 μL of crystal violet per well. This is allowed to sit for 30-60 minutes with gentle shaking to stain the plaques. Water is used to remove the crystal violet and discarded in labelled waste containers. The plaques were counted, and the PFU/ml was calculated.

6.2.4.3: Cytotoxicity assay:

Different concentrations of CNMO and CNME were added to 96-well cell culture plates containing a monolayer of HUH 7.5 cells and incubated 33°C 5% Co2 incubator for 4 days to allow plaque formation. Cytotoxicity effects were determined by visual inspection under the optical microscope.
6.2.5 Statistical analysis:

The experimental results from triplicate measurements were expressed as mean values and standard deviation (mean ± SD). Graphpad prism was used to conduct Turkey-test and One way- ANOVA and multiple comparison Turkey-test was performed using GraphPad Prism version 9.3.1 (350) for Mac OS X, GraphPad Software LLC, San Diego, California, USA, (www.graphpad.com). A P value less than 0.05 was considered significant.

6.2.3 Results:

6.2.3.1 Characteristics of nanoemulsion:

The physical characteristic changes in the nanoemulsion were analyzed over a period when it was placed in incubators at three different temperatures: 4 °C, 20 °C and 37°C; to reflect refrigeration, room, and body temperature, respectively. Samples were analyzed on the 1st, 3rd, 7th, 14th, 21st and 28th day. From the results, it was noted that as the temperature kept increasing the color of the nanoemulsion became brighter and there was an increase in the milkiness appearance of the emulsion as seen in Figure 3. Additionally, there was visual disintegrated separation of the water phase and the oil phase of the nanoemulsion after the 4th day when it was stored at 37 °C.
To obtain an optimum composition, analysis was conducted with varying the concentration of the different components of the nanoemulsion. From the results, it was seen that as the concentration of the oil increased above 27.7 μg/ml the particle size diameter also increased. When the concentration of cinnamaldehyde was 38.8 μg/ml the particle size measured was 569.8-729.6 nm. Hence, it was imperative to maintain the concentration of cinnamaldehyde essential oil added at 27.7 μg/ml. There was also a drastic increase of the particle size diameter from 150.16 +/-16.5 nm on the 3rd day to around 962.88 +/-16.3 nm on the 28th day when the samples were stored at 37°C and measured in the zetasizer nano machine which is depicted in Figure 4. A drastic change in the particle size was only seen in the samples stored at 37°C and no significant change was noticed when the nanoemulsion was stored at 4°C and 20°C. Although, there was a steady slight increase in the trajectory of the size of the emulsion particles when stored at 20°C. The size of the emulsion particles remained constant when it was stored at 4°C. From the results, it can be inferred that the storage temperature conditions are important while handling nanoemulsion and storing it at lower temperatures such as refrigerator conditions was more favorable. It was also noted that the emulsion particle size was
consistent throughout the nanoemulsion and maintained at about 99% polydispersity index when stored at the ideal temperature conditions.

The ζ- Potential is a parameter used to measure the electrical charge on the nanoemulsion droplets. It is an indicator of the stability of nanoemulsion (Pilong et al. 2022). The zeta potential helps to measure the charge of the emulsion and in the case of the research at hand it was imperative to ensure the zeta potential was positively charged for all the samples analyzed. It was seen that the lowest concentration of the cationic surfactant Centrinium Bromide (CTAB) of about 0.02% was enough to obtain cationic nanoemulsion ranging between +10-12 mv. Additionally, when the concentration of CTAB was increased the particle size diameter of the emulsion also increased to about 520 +/-12.8 nm which is not ideal. Hence, it was imperative to maintain the quantity of CTAB added to minimal quantity. When the nanoemulsion was stored at different temperature conditions, regular ζ- Potential measurements were taken on 0, 1st, 3rd, 7th, 14th and 28th day. From the results as seen in Figure 5, there were no drastic fluctuations in the charge of the emulsion when stored at all the different temperatures. However, the

\[\text{Figure 6.4-6.5: Nanoemulsion particle size diameter (nm) recorded when stored at 4 °C, 20 °C and 37°C for a period ranging from 0-28 days; Nanoemulsion ζ- Potential recorded when stored at 4 °C, 20 °C and 37°C for a period ranging from 0-28 days}\]
emulsion potential was the most stable when stored at 4°C measuring at 11.11+/−0.09 mv and it measured at 11.34+/−0.24 mv and 12.15+/−0.13 when stored at 20°C and 37°C respectively.

6.2.3.2 Antiviral activity against MS2 Bacteriophage:

The antiviral activity of CNMO and CNME was measured against MS2 bacteriophage, an HuNoV surrogate. The inactivation studies were conducted for a range of concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%) of disinfectants as well as for a range of reaction time periods of 5, 15, 30 and 60 minutes for one specific concentration of cinnamaldehyde of 2.5% in nanoemulsion (27.7 ug/ml).

Cinnamaldehyde oil treatment: When treated with oil ranging from 0.5%-3.5% for 1 hour there was a 2.78 +/- 0.34 PFU/ml log reduction at the lowest concentration of 0.5%, 4.121 +/- 0.102 PFU/ml log reduction at 2%, 3.94+/−0.04 PFU/ml log10 reduction at 2.5% 4.84+/−0.7 PFU/ml at 3% and 4.04+/−0.05 PFU/ml log10 reduction at 3.5% as seen in Figure 6. At lower reaction time periods there was no significant reduction observed when treated for 5, 15, 30 mins.

Cinnamaldehyde nanoemulsion: When nanoemulsion was used as a disinfectant treatment ranging from 0.5%-3.5% for 1 hour, there was no reduction from the lowest concentration 0.5% and a maximum of 1.54 +/- 0.08 PFU/ml log reduction from the highest concentration of 3.5% as shown in Figure 7. However, this high concentration cannot be considered as at 3% and 3.5% of cinnamaldehyde concentration there was an increase of the particle size of the nanoemulsion to around 200 nm and around 600 nm at 3% and 3.5%
respectively. This particle size is too large in order for the disinfectant to be considered as a nanoemulsion.

6.2.3.3 Antiviral activity against human Coronavirus-229E:

The results from evaluating the efficiency of both CNMO and CNME against coronavirus 229E has shown high levels of reduction when compared to MS2 bacteriophage. When CNME was treated against the virus for 1 minute there was 1.35+/−0.23 PFU/ml, 2+/−0.43 PFU/ml, 4.13+/−0.21 PFU/ml, 5.56+/−0.3 PFU/ml and 5.98+/−0.12 PFU/ml log10 reduction for the concentrations 0.5%,1%,1.5%,2% and 2.5% respectively as seen in Figure 8. In addition, when CNMO was treated against coronavirus-229E for 1 minute there was 3.08+/−0.17 PFU/ml, 3.21+/−0.37 PFU/ml, 4.22+/−0.11 PFU/ml, 4.54+/−0.28 and 4.43+/−0.38 PFU/ml log10 reduction after treatment with concentrations 0.5%,1%,1.5%,2% and 2.5% respectively as seen in Figure 9. The results suggest that for lower concentrations there was higher efficiency when just CNME compared to CNMO.
However, overall highest efficiency of 5.98±0.12 PFU/ml log10 reduction was achieved when the concentration of cinnamaldehyde in nanoemulsion was increased to 2.5%.

![Coronavirus-229E inactivation by Cinnamaldehyde oil and nanoemulsion](image)

**Figure 6.8; Figure 6.9:** Coronavirus-229E microbial reduction post treatment with a range of cinnamaldehyde oil (0.5%-2.5%); Coronavirus-229E microbial reduction post treatment with a range of cinnamaldehyde nanoemulsion (0.5%-3.5%). 

*ns* - no significant difference

**6.2.4 DISCUSSION AND CONCLUSION:**

Essential oils (EOs) are aromatic, oil-like volatile substances present in plant materials such as fruits, bark, seeds, pulp, peel, root and whole plant. The use of synthetic antimicrobial compounds as food preservatives has raised consumers concerns, since they present numerous toxicological difficulties and may not be safe for human consumption. Hence, over the last two decades, natural antimicrobial agents such as EOs have received renewed interest from the scientific community, owing to their unique physicochemical properties and diverse biological activities (Asbahani et al. 2015). Researchers have demonstrated that cinnamaldehyde has strong antibacterial effect against *S. epidermidis*, *S. aureus* and *Aeromonas hydrophila* by causing cell membrane distortion, leakage and in addition to polarization of cytoplasmic content (Albano et al. 2019)(Zhang et al. 2015).
Several reports have documented that Cinnamon oil and its derivative, cinnamaldehyde, exhibit various biological activities in vitro, such as antibacterial activity (Chang, Chen, and Chang 2001), induction of apoptosis via reactive oxygen species (Ka et al. 2003), and inhibition of nitric oxide synthesis (H. S. Lee, Kim, and Kim 2002). However, little is known about their antiviral activity. This inability to successfully inactivate viruses can be attributed to their high levels of stability in comparison to bacteria and more specifically in the case of human norovirus as it is a non-enveloped virus, a feature that can make them more stable and more resistant than enveloped viruses to thermal, chemical and environmental inactivation (Li and Chen 2015) (Chen, Hoover, and Kingsley 2005). NoV has a protein capsid that is very resistant to lipophilic disinfectants (e.g. quaternary ammonium compounds) and solvents (e.g. alcohol) (Said, Perl, and Sears 2008). There has been previous research conducted where surfactant nanoemulsions were used for the prevention of murine influenza A virus pneumonitis (Hamouda et al. 2001). Antimicrobial nanoemulsions are novel water-in-oil formulations that are stabilized by the addition of small amounts of surfactants. The water-immiscible liquid phase is mixed into an aqueous phase by high stress mechanical extrusion, yielding a uniform population of droplets and these discrete droplets of oil selectively fuse with bacterial cell walls or viral envelopes destabilizing the organism’s lipid envelope and initiate disruption of pathogens (Hamouda et al. 2001). However, noroviruses are nonenveloped, but do have patches of hydrophobic amino acid residues in their major capsid protein that are critical to folding and maintaining the icosahedral contacts needed to form the norovirus capsid. In addition to cationic nanoemulsions being attracted to the capsid (negatively charged in neutral solution), the potential for the nanoemulsion to
enhance delivery to these contacts has not been tested. Hence, the main goal of this research was to evaluate the efficiency of cinnamaldehyde cationic nanoemulsions against negative protein capsid present nonenveloped virus human norovirus surrogate bacteriophage MS2. Contrary to this hypothesis, there was lower inactivation efficiency when the essential oil was incorporated into nanoemulsion when compared to disinfection with plain essential oil. There was a maximum of $1.54 \pm 0.08$ PFU/ml log reduction from the highest concentration of treatment with nanoemulsion and about $4.02 \pm 0.102$ PFU/ml log reduction when treated with plain cinnamaldehyde essential oil for 1 hour. One potential reason for this reduced efficacy could be the high proportion of carrier oil and nonionic surfactant relative to cinnamaldehyde required for spontaneous emulsification, which resulted in sequestering of the cinnamaldehyde. Further, it may simply be that the nanoemulsions are too lipophilic for nonenveloped viruses regardless of the presence of hydrophobic patches on the viral capsid protein, and thus only offer benefit with enveloped viruses. Additionally, it was seen that the nanoemulsion destabilized when stored at 37°C. Hien & Dao et al reported that nanoemulsion formulation methods like the low energy emulsion phase inversion method was more favorable for storing nanoemulsion at higher temperatures(Elaissi et al. 2012). It was also noted that preparation of nanoemulsion by following high energy methods such as the utilization of microfluidizer was more efficient at producing stable nanoemulsion, with a much lower proportion of carrier oil and surfactant required relative to active ingredient.

Hence, future research can be conducted by following these methods to prepare nanoemulsion to evaluate its efficiency. However, it can be noted that storing the cinnamaldehyde nanoemulsion at lower temperatures such as 4°C and 20°C have resulted
in constant maintenance in the stability, particle distribution and particle size diameter. In addition to looking at non enveloped virus human norovirus, it was also imperative to investigate disinfection of enveloped viruses such as SARS-CoV-2 surrogate human coronavirus 229E. The results show there was high levels of reduction with just 1 minute of treatment for both CNME and CNMO. When treated with the lowest concentration of 0.5%, there was $1.35 \pm 0.23 \text{ PFU/ml log10}$ reduction for CNME and there was $3.08 \pm 0.17 \text{ PFU/ml}$ when treated with CNMO. However, when the concentration was increased to 2.5%, the highest level of microbial reduction of $5.98 \pm 0.12 \text{ PFU/ml}$ was observed when treated with CNME when compared to CNMO which resulted in a reduction of $4.43 \pm 0.38 \text{ PFU/ml}$. This result suggests that the efficiency of the essential oil as a disinfectant against coronavirus-229E can be increased when it is incorporated in a nanoemulsion at the appropriate concentration. Additional studies can be conducted in the future to analyze the same experimental conditions for surface study to determine the efficiency of cinnamaldehyde nanoemulsion as a plant derived phytochemical based food contact surface sanitizer.
BIBLIOGRAPHY:

1. 2020, USFDA. “No Title.”
on Fresh Raspberries by Gaseous Ozone Treatment.” Food Microbiology 70: 1–6.
20. CDC- Bidart Prepackaged caramel apples. “Bidart Prepackaged Caramel Apples.”
24. CDC 2022- Fresh Express Packaged Salads. “No Title.”


100. Sapers. “No Title.”


117. USDA, FTS-328-01. “No Title.”


120. Warnes, Sarah L., Emma N. Summersgill, and C. William Keevil. 2015. “Inactivation of Murine Norovirus on a Range of Copper Alloy Surfaces Is

121. Warriner. “No Title.”


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<thead>
<tr>
<th>VIRUS SURROGATES TESTED</th>
<th>INACTIVATION AGENT</th>
<th>RANGE OF CONCENTRATIONS</th>
<th>MEDIUM</th>
<th>QUANTIFICATION METHOD</th>
<th>RANGE OF REDUCTION(S)</th>
<th>REFERENCE</th>
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<tbody>
<tr>
<td>Murine norovirus-1 (MNV-1)</td>
<td>Gamma irradiation</td>
<td>3-10 kGy</td>
<td>Green algae-fulvescens (<em>Capsosiphon fulvescens</em>) and brown alage-fusiforme (<em>Hizikia fusiforme</em>)</td>
<td>Plaque assay</td>
<td>Fulvescens: 1.16-2.46 log reduction Fusiforme: 0.37-2.21 log reduction</td>
<td>(Park et al., 2016b)</td>
</tr>
<tr>
<td>Tulane virus (TV)</td>
<td>Gamma irradiation, electron beam (E-beam)</td>
<td>E-Beam: For PBS and DMEM-2-28 kGy For whole strawberries: 4-28 kGy Gamma: 3-23 kGy</td>
<td>PBS buffer, DMEM and whole strawberries</td>
<td>Plaque assay, RT-qPCR, porcine gastric mucin conjugated magnetic bead binding assay, transmission electron microscopy</td>
<td>NoV by E-Beam in media: RT-qPCR- 1.9- 4 log&lt;sub&gt;10&lt;/sub&gt; reduction PGM- 1.51- 4.21 No detection beyond 35.3 kGy.</td>
<td>(DiCaprio et al., 2016)</td>
</tr>
<tr>
<td>MNV-1</td>
<td>UV-A, UV-B and UV-C with and without titanium dioxide</td>
<td>0 to 10 mins</td>
<td>Solid agar surface</td>
<td>Plaque assay, SDS page, transmission electron microscopy, RT-qPCR and PMA pre-treatment RT-qPCR</td>
<td>UVA, UVA-TP- no obvious difference UVB, UVB-TP-2.3-2.8 after 10 mins UVC, UVC-TP- 4 log reduction, 5 log reduction Internalized treatment with UVC, UVC-TP- 2.7-3.2 log reductions</td>
<td>(Park et al., 2016a)</td>
</tr>
<tr>
<td>virus</td>
<td>UV treatment details</td>
<td>UV irradiance</td>
<td>Assay Methods</td>
<td>Log reductions after 24 hours</td>
<td>Ref.</td>
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<tr>
<td>MNV, recombinant human adenovirus</td>
<td>24, 48 and 72 hours Flocculated seawater sample in depuration tanks Plaque assay, flow cytometry MNV - greater than 6 log reductions after 24 hours Adenovirus - greater than 6 log reductions after 48 hours</td>
<td>0% - 1% Oxygen Phage suspension Plaque assay</td>
<td>0% - 4.98 log 0.25% - 5.69 log 0.5% - 5.93 log 0.75% - 7.06 log 1% - 5.73 log after 9 min</td>
<td>(Garcia et al., 2014).</td>
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<tr>
<td>MS2</td>
<td>Atmospheric pressure non-thermal plasma jet at varying oxygen feed gas concentrations in helium carrier gas</td>
<td>0% to 1% Oxygen Phage suspension Plaque assay</td>
<td>4 log reduction achieved with: MNV - 29 mJ cm$^{-2}$ FCV - 25 mJ cm$^{-2}$ Echovirus - 12 - 30 mJ cm$^{-2}$ MS2 - 70 mJ cm$^{-2}$</td>
<td>(Alshraiedeh et al., 2013)</td>
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<tr>
<td>MS2, echovirus 12, MNV-1, FCV</td>
<td>UV inactivation 0 to 100 mW cm$^{-2}$ PBS buffer Plaque assay</td>
<td>4 log reduction achieved with: MNV - 29 mJ cm$^{-2}$ FCV - 25 mJ cm$^{-2}$ Echovirus - 12 - 30 mJ cm$^{-2}$ MS2 - 70 mJ cm$^{-2}$</td>
<td>(Park et al., 2011)</td>
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<tr>
<td>MS2, MNV-1</td>
<td>UV-A and UV-B with and without titanium dioxide 0 to 1500 mJ cm$^{-2}$ PBS buffer Plaque assay and RT-qPCR</td>
<td>4 log reduction achieved with: MNV-UVA: 1400 mJ cm$^{-2}$, UVB: 367 mJ cm$^{-2}$, MS2 - UVA: 816 mJ cm$^{-2}$, UVB: 909 mJ cm$^{-2}$</td>
<td>(Lee and Ko, 2013).</td>
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<tr>
<td>Human norovirus</td>
<td>RT-qPCR- 0 to 900 mJ cm(^{-2})</td>
<td>Plaque assay</td>
<td>Human norovirus- 2.5 to 3 log reduction</td>
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<tr>
<td><strong>MNV-1, TV</strong></td>
<td>Pulsed light 1-16 pulses</td>
<td>PBS buffer, blueberry and strawberry surfaces</td>
<td>In PBS-MNV- greater than 5.8 log reduction TV- greater than 6 log reduction In berry surface-MNV- Strawberry- 0.7-0.9 Blueberry- 3.1-3.8</td>
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<tr>
<td><strong>TV, MNV-1</strong></td>
<td>Atmospheric cold plasma 0 to 120 seconds</td>
<td>Blueberry surface</td>
<td>TV – 3.5 after 120 seconds MNV- greater than 5 after 90 seconds</td>
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<tr>
<td><strong>MNV</strong></td>
<td>Continuous flow intense pulsed light system 12 to 40 l/min</td>
<td>Untreated ground water sample and PBS buffer</td>
<td>MNV in PBS- 6.69 log reductions at 3.43 J/cm(^2) for 30 sec. MNV in untreated ground water (pilot scale)- 3.35 log reduction at 4.30 J/cm(^2) for 89 sec</td>
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</tbody>
</table>

(Huang et al., 2017)

(Lacombe et al., 2017)

(Yi et al., 2016)
<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Time</th>
<th>Surface</th>
<th>Detection Method</th>
<th>Reduction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNV-1, bacteriophage B40-8 and bacteriophage X174</td>
<td>Hydrogen peroxide and UV light</td>
<td>1% to 20% hydrogen peroxide</td>
<td>Stainless steel surface, shredded iceberg lettuce</td>
<td>Plaque assay</td>
<td>2.1% Liquid hydrogen peroxide – 4 log reduction in MNV and X174 15% liquid hydrogen peroxide 4 log reduction</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>MS2, MNV-1, FCV</td>
<td>Steam ultrasound treatment</td>
<td>0 to 3 seconds</td>
<td>Plastic surfaces and fresh raspberries</td>
<td>Plaque assay, RT-qPCR</td>
<td>Plastic surface- FCV- 4.8 log MNV- 3.7 log MS2- &gt;6 log Raspberries- &lt;1 log reduction for all the viruses</td>
<td>(Schultz et al., 2012)</td>
</tr>
<tr>
<td>MNV-1, human norovirus virus like particles</td>
<td>Gamma irradiation</td>
<td>2.8 to 22.4 kGy</td>
<td>Strawberries, lettuce and spinach</td>
<td>Plaque assay, RT-qPCR, SDS page, transmission electron microscopy</td>
<td>MNV-1- greater than 6 log reduction in strawberries and lettuce and 5 log reduction in spinach after 22.4 kGy</td>
<td>(Feng et al., 2011)</td>
</tr>
<tr>
<td>FCV</td>
<td>Electron beam irradiation</td>
<td>81 kGy/min</td>
<td>Iceberg lettuce</td>
<td>Plaque assay and RT-qPCR</td>
<td>A maximum of 1-2 log reduction</td>
<td>(Zhou et al., 2011)</td>
</tr>
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</table>

**GASEOUS AND VAPOR BASED TREATMENTS**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Concentration</th>
<th>Surface</th>
<th>Detection Method</th>
<th>TCID50 and plaque assay</th>
<th>Temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNV, FCV</td>
<td>Ozone inactivation</td>
<td>6.25 ppm for 0.5 to 10 mins.</td>
<td>Green onions, lettuce and water</td>
<td>TCID50 and plaque assay</td>
<td>FCV- Water- 6.79 log Lettuce-3.08 log Green onions- 2.02 log MNV- Water- 5.31 log Lettuce- 3.09 log Green onions- 3.78 log</td>
<td>(Hirneisen et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>MNV, TV</td>
<td>Ozone inactivation</td>
<td>6% ozone for 0 to 40 mins</td>
<td>Strawberries (surface and internalized) and romaine lettuce.</td>
<td>RT-qPCR, SDS PAGE, TEM</td>
<td>MNV after 10 mins-4.1 log reductions TV after 10 mins-0.5 log reductions Internalized in strawberry- 1.5 log reduction</td>
<td>(Predmore et al., 2015b)</td>
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<tr>
<td>MNV, TV</td>
<td>Atmospheric cold plasma</td>
<td>0 to 60 sec with 4 cfm</td>
<td>Blueberries</td>
<td>Plaque assay</td>
<td>TV- 3.5 log reduction after 120 sec</td>
<td>(Lacombe et al., 2017)</td>
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</tr>
<tr>
<td>MNV</td>
<td>Ozone inactivation</td>
<td>1-4 ppm</td>
<td>Raspberries</td>
<td>Plaque assay, RT-PCR</td>
<td>MNV- 3.3 log reduction in 1 min</td>
<td>(Brière et al., 2018)</td>
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<tr>
<td>TV</td>
<td>Gaseous chlorine dioxide</td>
<td>0.1 to 10</td>
<td>Blueberries</td>
<td>Plaque assay</td>
<td>&gt;2.5 log reduction after 30 min</td>
<td>(Park et al., 2016a)</td>
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<tr>
<td>MNV, TV</td>
<td>Ozone</td>
<td>6.25 ppm, 30 min</td>
<td>Alfalfa seeds</td>
<td>Plaque assay and RT-qPCR</td>
<td>MNV- &gt;6 log reduction TV- &gt;4 log reduction</td>
<td>(Wang et al., 2015)</td>
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</tr>
</tbody>
</table>

**INACTIVATION DURING NATURAL PROCESSES**

| MNV-1, FCV | Fermentation | 0 to 20 days post fermentation | Fermented Dongchimi | Plaque assay | FCV- 4.1 log reduction after 20 days MNV- 1.5 log reduction after 20 days | (Lee et al., 2012) |
| MNV-1, bacteriophage B40-8 | Mesophilic and thermophilic anaerobic digestion of pig slurry | 37 degrees and 52 degrees | Pig slurry | Plaque assay and RT-qPCR | Mesophilic- MNV- more than 4 log reduction  
Bacteriophage- 3 log reduction  
Thermophilic- more than 4 log reductions in both MNV and bacteriophage | (Baert et al., 2010) |
|--------------------------|-------------------------------------------------|--------------------------|-----------|--------------------------|-------------------------------------------------|--------------------------------|
| MNV-1, FCV               | Oyster fermentation                             | 5% and 10% salt added for native fermentations | Oysters   | Plaque assay             | FCV- 3 log reduction in 5%  
MNV- 1.56 log reduction in 5%  
FCV- 1.14 log reduction in 10%  
MNV- 0.9 log reduction in 10% | (Seo et al., 2014) |
<table>
<thead>
<tr>
<th>Solution Based Treatments</th>
<th>MNV-1, poliovirus (PV1)</th>
<th>Chlorine inactivation</th>
<th>0.1 mg l⁻¹ and 0.5 mg l⁻¹ free chlorine concentration</th>
<th>Drinking water</th>
<th>Plaque assay and RT-qPCR</th>
<th>PV1- 4.95 log at 0.5%</th>
<th>MNV- 4.04 log at 0.1%</th>
<th>(Kitajima et al., 2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNV-1, MS2</td>
<td>Chlorine and chlorine dioxide</td>
<td>0 to 0.3 mg/L, 0 to 5 mins</td>
<td>Drinking water</td>
<td>Plaque assay, short template RT-qPCR and long template RT-qPCR</td>
<td>Chlorine- MNV- 3 log MS2- 4 log Chlorine dioxide- MNV and MS2- 3.5 and 4 log, respectively</td>
<td>(Lim et al., 2010b)</td>
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<tr>
<td>MNV</td>
<td>Calcium hydroxide</td>
<td>Solution- 0.17% w/v Powder- 0.05 g and 0.1 g Suspension- 1%, 2.5% and 5% w/v</td>
<td>Rayon sheet and spiked feces</td>
<td>Plaque assay</td>
<td>Solution- greater than 4 log reduction in 30 sec Powder- 0.05g – 2.3 log reduction in 15 min 0.1g- 4.17 log reduction in 15 min suspension- 1.3,2.3 and 1.5 log reduction in 15 mins</td>
<td>(Sangsriratana kul et al., 2018)</td>
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<tr>
<td>FCV</td>
<td>Sodium hypochlorite, hydrochloric acid, citric acid, ethanol,Varies</td>
<td>Dried virus on polystyrene surface</td>
<td>Plaque assay</td>
<td>0.41 to &gt;5 log inactivation; generally sodium hypochlorite, acid, and monoethanolamine most</td>
<td>(Whitehead and McCue, 2010)</td>
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<tr>
<td>Virus</td>
<td>Preformulated disinfectants that were alcohol, acid, halogen, quaternary ammonium, hydrogen peroxide, and aldehyde-based</td>
<td>Concentration</td>
<td>Method</td>
<td>Effectiveness</td>
<td></td>
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<tr>
<td>MNV, FCV</td>
<td>Preformulated disinfectants that were alcohol, acid, halogen, quaternary ammonium, hydrogen peroxide, and aldehyde-based</td>
<td>Varies</td>
<td>Suspension, glove, and stainless steel surface</td>
<td>Generally 2-5 log reduction with different results being surface-dependent. Alcohol-containing formulations among most effective for MNV; halogen and acid-based formulations most effective for FCV (Zonta et al., 2016)</td>
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<tr>
<td>MNV</td>
<td>Chlorine solution, with sodium dodecyl sulfate (SDS), nondiet P-40, Triton-X100, Tween 20, Tween 50</td>
<td>200 ppm chlorine with 1-10,000 ppm detergent</td>
<td>Strawberry, raspberry, cabbage, and lettuce surfaces</td>
<td>About 2-3 log removal of virus when chlorine and detergent used together, 1 log for detergent alone (Predmore and Li, 2011)</td>
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<tr>
<td>MNV, FCV and SDS</td>
<td>Levulinic acid and SDS</td>
<td>Stainless steel surfaces</td>
<td>Plaque assay</td>
<td>Maximum log reductions: MNV- 4.21 FCV- 3.04 For 0.5% levulinic acid and 0.05% SDS</td>
<td>(Cannon et al., 2012)</td>
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<tr>
<td>SELF SANITIZING SURFACE</td>
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<tr>
<td>MNV</td>
<td>Copper alloys</td>
<td>70-80% copper nickel and 60-70% in brasses</td>
<td>Copper surfaces</td>
<td>Plaque assay and TEM</td>
<td>Rapid inactivation (greater than 4 log reduction) on alloys containing 79 to 89% copper, but intensity of reduction was lost at 70%, suggesting that a small difference in copper content, 70 to 79% can have a large effect on antiviral efficacy. Stainless steel and nickel did not have any antiviral activity.</td>
<td>(Warnes et al., 2015).</td>
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<tr>
<td>Virus</td>
<td>Inactivation Method</td>
<td>Concentration</td>
<td>Test Material</td>
<td>Method</td>
<td>Result</td>
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<tr>
<td>MNV</td>
<td>Sodium hypochlorite</td>
<td>1000 ppm to 2000 ppm</td>
<td>Lettuce, cabbage and ground pork on stainless steel surface</td>
<td>Plaque assay</td>
<td>At 1000 ppm there was complete inactivation (&gt;5 log reduction) on surface without food whereas on surface with food there was 1.4 log reduction on day 9 and it remained the same until the 30th day even with the highest concentration tested (2000 ppm) (Takahashi et al., 2011)</td>
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<tr>
<td>MNV, FCV</td>
<td>Grape seed oil</td>
<td>0.25-2 mg/mL for 2 hours</td>
<td>Lettuce surface</td>
<td>Plaque assay</td>
<td>Maximum reductions of MNV: 1.5 log FCV: 3 log (Ryu et al., 2015)</td>
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<tr>
<td>MNV, FCV</td>
<td>Curcumin</td>
<td>50 ug/mL, at 37°C and room temperature, 30 mins and 120 mins</td>
<td>DMEM suspension</td>
<td>Plaque assay</td>
<td>FCV: 37°C-4.6 log, room-3.2 log MNV: 37°C- 0.3 log, room- 0.23 log (Randazzo et al., 2016)</td>
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<tr>
<td>MNV</td>
<td>Heat denatured lysozyme</td>
<td>0 to 5 days at 4°C</td>
<td>4 different salads and 3 different dressings</td>
<td>Plaque assay</td>
<td>Coleslaw the infectivity decreased by 3 logs immediately; &gt;4 log reduction in vinaigrette and thousand island salads after 5 days. No significant decrease in egg salad. (Takahashi et al., 2016a)</td>
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</table>

**OTHER INACTIVATION METHODS**

- **MNV, FCV**  
  Grape seed oil  
  0.25-2 mg/mL for 2 hours  
  Lettuce surface  
  Plaque assay  
  Maximum reductions of MNV: 1.5 log FCV: 3 log (Ryu et al., 2015)

- **MNV, FCV**  
  Curcumin  
  50 ug/mL, at 37°C and room temperature, 30 mins and 120 mins  
  DMEM suspension  
  Plaque assay  
  FCV: 37°C-4.6 log, room-3.2 log MNV: 37°C- 0.3 log, room- 0.23 log (Randazzo et al., 2016)

- **MNV**  
  Heat denatured lysozyme  
  0 to 5 days at 4°C  
  4 different salads and 3 different dressings  
  Plaque assay  
  Coleslaw the infectivity decreased by 3 logs immediately; >4 log reduction in vinaigrette and thousand island salads after 5 days. No significant decrease in egg salad. (Takahashi et al., 2016a)
<table>
<thead>
<tr>
<th>VIRUS SURROGATE TESTED</th>
<th>INACTIVATION AGENT</th>
<th>ORGANIC MATRIX</th>
<th>REDUCTION DIFFERENCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tulane virus (TV)</td>
<td>Gamma irradiation and electron beam</td>
<td>PBS buffer and whole strawberries</td>
<td>TV-2 log reduction difference between simple media and whole strawberries</td>
<td>(DiCaprio et al., 2016)</td>
</tr>
<tr>
<td>Murine norovirus-1 (MNV-1)</td>
<td>Pulsed light</td>
<td>PBS buffer, blueberry and strawberry surfaces</td>
<td>MNV in PBS- &gt;5.8 log reduction; strawberry: 4.9-5.1 log less reduction; blueberry: 2-2.7 log less reduction</td>
<td>(Huang et al., 2017)</td>
</tr>
<tr>
<td>MNV</td>
<td>Continuous flow intense pulsed light system</td>
<td>Untreated ground water sample and PBS</td>
<td>3.3 log less reduction in ground water than PBS</td>
<td>(Yi et al., 2016)</td>
</tr>
<tr>
<td>MNV, TV</td>
<td>High pressure treatment</td>
<td>Media, blueberries and oysters</td>
<td>Compared to reduction in media: Blueberry-TV- 2.8 log less reduction MNV- 1 log less reduction Oyster-TV-1.8 log less MNV- 1.5 log less reduction</td>
<td>(Li et al., 2013)</td>
</tr>
<tr>
<td>Feline calicivirus (FCV)</td>
<td>Chlorine exposure</td>
<td>In hard water and normal water</td>
<td>&gt;4 log reduction in both cases—no difference</td>
<td>(Nowak et al., 2011)</td>
</tr>
<tr>
<td>MNV</td>
<td>High hydrostatic pressure</td>
<td>Water and strawberry puree</td>
<td>300 MPa for 10 min-mineral water- 1.33 strawberry puree- 2.75 400 MPa-mineral water- &gt;5 log reduction (undetectable levels)</td>
<td>(Kovač et al., 2012)</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>Matrix Descriptions</td>
<td>Observation</td>
<td>Reference</td>
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<tr>
<td>MVN</td>
<td>Chlorine treatment</td>
<td>Clean artificial seawater and natural seawater</td>
<td>Natural seawater 1 log less reduction after 30 min</td>
<td>(de Abreu Corrêa et al., 2012)</td>
</tr>
<tr>
<td>MS2</td>
<td>Steam ultrasound</td>
<td>Plastic surface and surface of raspberries</td>
<td>~2 log less reduction on fresh and frozen raspberry surfaces compared to plastic surface</td>
<td>(Schultz et al., 2012)</td>
</tr>
<tr>
<td>MVN, FCV</td>
<td>Aqueous ozone</td>
<td>Green onions and lettuce and wash water</td>
<td>After 10 min treatment, compared to buffer: FCV: 3.7 log less (lettuce); 4.7 log less (onion) MVN: 2.2 log less (lettuce); 1.5 log less (onion)</td>
<td>(Hirneisen et al., 2011)</td>
</tr>
</tbody>
</table>

Table 2. Discrepancies between reduction observed with influence of different matrices and organic loads. Studies comparing inactivation of human norovirus surrogates in food or organic matrices side-by-side with buffer are presented. The degree of difference in efficacy of each agent between organic matrix and buffer is presented.