A COMPARISON STUDY OF EFFICACY OF FOOD SANITIZERS WITH FRESH LEAFY VEGETABLES WITH DIFFERENT SURFACE PROPERTIES AND ANALYSIS OF THE ROTAVIRUS REPLICATION CYCLE AFTER INACTIVATION BY FOOD SANITIZERS

BY

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THESIS

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ABSTRACT

Foodborne illness is a serious concern in the United States. Fresh produce is recognized as a vehicle of food borne illness pathogens including viruses. To prevent causes of foodborne illness, the use of sanitizers is essential for fresh produce. However, it is considered that the current sanitizers used in food industry may not be effective on virus inactivation on fresh produce, and little is known how the performance of sanitizers varies on virus inactivation on different fresh produce surface properties. In this study, we evaluated the efficacy of two kinds of food sanitizers Tsunami® 100 and malic acid + TDS on rotaviruses attached onto three cultivars' surfaces with different surface properties. Moreover, we investigated the inhibitory effects of the sanitizers on the rotavirus replication cycle at a molecular level. As a result, while differences of sanitation efficacy on rotaviruses on the three cultivars were observed by Tsunami® 100, malic acid + TDS had similar disinfection efficacies on rotaviruses attached onto the produce. Moreover, the entry and replication step of the rotavirus replication cycle was significantly inhibited by the sanitizer treatments with rotaviruses, while the sanitizers did not inhibit the binding of rotaviruses onto cells. These observations suggest that the surface properties of fresh produce may affect the efficacy of sanitation on viruses, implying that food sanitizers should be carefully selected for different surface characteristics of fresh produce, and to identify how the food sanitizers function on rotaviruses, further studies on identification of virus damage caused by the sanitizers' effect are needed.

ACKNOWLEDGEMENTS

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CHAPTER 1
INTRODUCTION

In the United States, foodborne illness is a serious concern. It is estimated based on data from 2000-2008 that there were 48 million annual incidents of foodborne illness, including 128,000 hospitalized patients and 3,000 fatalities [1-3]. The annual cost associated with foodborne illness is estimated from $14.1 billion to $152 billion [4-6]. Fresh produce such as fruits and vegetables contaminated with pathogens is considered as a vehicle of foodborne illness pathogens and is a public health concern [7]. It is estimated that fresh produce annually accounts for approximately 50 % of the total foodborne outbreaks [8]. One of the most contamination routes of fresh produce is pre-harvest period, when crops are exposed to contaminants in the environment such as waterborne pathogens from irrigation water [7, 9, 10]. Viruses such as norovirus and rotavirus have been shown to cause foodborne outbreaks, especially because of their resistance in the environment and their low infectious doses (10 – 100 particles) [11, 12]. It is also shown that pathogen viruses can not only attach to fresh produce, but also become internalized in produce from contaminated soil [13-17]. Based on these facts, pre-harvest viral contamination is considered as a threat to human public health. In order to address viral foodborne illness caused by pre-harvest contamination, it is essential to understand what factors are contributing to the transmission of viral foodborne illnesses with fresh produce.

Several factors were found to be involved with viral survival and adsorption on fresh produce. For example, pH and ionic strength were found to correlate with viral adsorption with lettuces [18]. Temperature and humidity were also shown to be contributing to the viral survival on cantaloupe, lettuce, and bell peppers [19], tomato, cabbage, carrot, lettuce, parsley, pepper, and strawberry [20]. Typically, fresh produce is stored at temperature around 4^{o} C to prevent the

bacterial growth, however, this condition was favorable for the survival of various types of viruses [11, 20]. In addition, chemical composition, surface roughness, and hydrophobicity were found to play an important role on virus attachment [21-24]. A recent study characterized physicochemical properties of 24 kinds of vegetables and conducted viral adsorption study with the vegetables with different surface chemistry [22]. Based on their characterization, the chemical composition of vegetables' surface was found to be partially responsible for the viral adsorption, contributing to causes of foodborne illness [22]. Moreover, viral particles attached to the surfaces of these 24 kinds of vegetables and persistently remained on the produce surface even after the washing treatment [22], as a similar trend observed previously with lettuce, fennel, and carrots [25]. These findings emphasize the importance of the sanitation practice of fresh produce. However, it is estimated that the current sanitation treatment methods employed in the food industry may not effectively remove viruses attached onto fresh vegetables [26-29]. Chlorine-based sanitizers are the most commonly used sanitizer in food industry. However, chlorine forms harmful disinfection by products (DBPs) and is consumed by organic matter present on produce, which may result in instability of the disinfection effect [30]. Therefore, alternative sanitizer methods are needed to be developed. As modified sanitation methods, various types of decontamination technologies are being developed and studied, such as cold atmospheric gaseous plasma [28], thermal inactivation [31, 32], electron beam inactivation [33], and ozone inactivation [34] as well as different types of sanitizers [35-37] . Regardless of these novel food sanitation methods, washing is still required for fresh produce to remove unwanted contaminants and improve produce appearance [38-40]. Antimicrobial substances are usually added to the washing water because this method is more convenient and requires less initial investment than physical sanitation such as irradiation [34, 41]. However, it remains unclear the sanitation efficacy of chemical food sanitizers on viruses on fresh

produce with different chemical composition. Understanding of the disinfection mechanism about how food sanitizers inactivates at a molecular level would contribute to the improvement of food sanitation practice for viruses.

To fill this research gap, this study aimed (i) to determine the efficacy of a surfactant-based and a peroxide-based food sanitizers on rotaviruses attached onto three fresh vegetable surfaces with different wax contents, (ii) to identify which stage of the rotavirus replication cycle was inhibited by the sanitizer effects. A better understanding of the behavior of foodborne viral pathogens in pre-harvest environments will strongly enhance the perspective of developing effective sanitation strategies to prevent foodborne illness. To elucidate these unknowns, we conducted disinfection experiments with porcine rotaviruses OSU strain adsorbed onto fresh vegetables' surface with different chemical composition, using two kinds of food sanitizers, Tsunami®100 and malic acid with thiamine dilauryl sulfate (TDS). Tsunami®100 is a peroxyacetic acid-based food sanitizer authorized by EPA, known as a potentially stronger disinfectant than chlorine [42, 43]. Another kind of sanitizer, malic acid with TDS was chosen for this study because its disinfection efficacy on viruses remains unclear, even though it was found to be effective for disinfection of *E. coli* O157:H7 [35]. We chose rotavirus as our model virus which is a major cause of gastroenteritis worldwide, including the United States, especially in children under five years old [44]. Rotavirus is one of the major causes of foodborne illness associated with pre-harvest contamination [2, 12, 45], as well as the fact that rotavirus is one of the most detected viruses in water environments, such as treated wastewater and river water [12]. Porcine rotavirus OSU was used in this study due to the structure of its outer protein being similar to human rotavirus Wa, and stability in the environment [46].

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals

Tsunami 100® was purchased from Ecolab (Saint Paul, MN). Malic acid and thiamine dilauryl sulfate (TDS) were purchased from Sigma Aldrich (St. Louis, MO) and Sanigen Co. Ltd. (Juam dong, Korea), respectively.

2.2 Greenhouse production of leafy vegetables

'Red Russian' kale (*Brassica napus*), 'Starbor' kale (*Brassica oleracea*), and 'Totem' Belgian endive (*Cichorium intybus*) were grown in the greenhouse as previously described [22]. Greenhouse conditions were consistently maintained throughout the study so that produce replicates over many months can be obtained. All seeds were purchased from Johnny's Selected Seeds (Winslow, ME). Seeds of each cultivar were germinated in 32-cell plant plug trays filled with sunshine LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Urbana-Champaign under a 25 °C/17 °C and 14 h/10 h: day/night temperature regimen with supplemental lighting. Twenty days after germination, seedlings were transferred to 4 L pots. Leaf tissues of 'Red Russian' kale, 'Starbor' kale, and 'Totem' Belgian endive were harvested 50-65 days after sowing seeds. Leaves from median internodes from each leafy vegetable were harvested at market maturity for analysis. The greenhouse is disinfected regularly allowing us to grow vegetables without substantial contamination by bacteria and other microorganisms that may change the health of vegetables.

2.3 Cell and rotavirus

MA 104 cells were purchased from ATCC and maintained at 37 °C in a 5% CO_2 incubator with MEM with 10 % fetal bovine serum (FBS). Once cells were confluent, non-trypsinized porcine rotavirus strain OSU were propagated as it follows; confluent cells in a 150 cm^2 flask were washed by pre-warmed serum-free MEM three times as recommended [47]. Rotavirus stock was activated with trypsin in a final concentration of trypsin 10 ug/mL for 30 min at 37 °C and added to confluent cells. After the incubation at 37 °C for 90 min, the viral solution was aspirated and washed by serum-free MEM twice. Then, serum-free MEM was added into the flask and incubated for 4-5 days at 37 °C without the presence of trypsin until most of the cells were detached. After this propagation step, rotavirus solution was sequentially frozen at -80 °C and thawed three times. Rotavirus solution was centrifuged at 1,000 xg for 10 min at room temperature and filtered through a 0.45 μM pore size filter to remove the cell debris. After this step, membrane filtration was conducted to make pure rotavirus stock. Rotavirus Rotavirus suspension was placed onto an ultrafiltration membrane of 100 kDa, then MEM and trypsin from the propagation step was removed in this step. Rotavirus was re-suspended by 1 mM NaCl + 0.1 mM $CaCl_2$ and stored in a 4 °C refrigerator for short-term experimental use. The infectivity of this stock was quantified by FFU assay described below.

2.4 Focus forming unit (FFU) assay

Rotavirus stock was activated with trypsin in a final trypsin concentration 10 μg/mL at 37 °C for 30 min. Then, trypsin-treated rotavirus stock was serially diluted with serum-free MEM. These serially diluted trypsin-treated rotavirus stock was applied to a monolayer of MA 104 cells in a 96 well plate and incubated at 37 °C for 30 min in a 5% CO_2 incubator. After this incubation, the viral solution was removed from each well and washed by serum-free MEM twice. 50 μL of

serum-free MEM was added to each well and incubated at 37 °C for 18 h in a 5% CO_2 incubator to allow viruses to replicate.

After the 18 h incubation at 37 °C, serum-free MEM was aspirated from cells and cells were fixed by adding 100 µL of 9:1 methanol : glacial acetic acid per well and incubated for 2 minutes. 100 µL 70 % ethanol was added into each well for incubated for 5 minutes to rehydrate cells, before adding 100 µL 50 % ethanol and incubating for 5 minutes. Endogenous peroxidase activity was quenched by adding 50 µl of 3% H_2O_2 in wash buffer containing final concentrations of 96 mM TRIS-HCl, 350 mM NaCl, 29 mM TRIS-Base, and 0.25% Triton X-100 per well and incubated for 10 minutes at room temperature. After washing fixed cells by wash buffer, 50 µL of 5 % normal goat serum diluted by wash buffer was added to each well and incubated for 20 minutes at room temperature. This step is needed to avoid non-specific binding of primarily antibodies in the next step. Then, 50 µL of primarily antibody (rabbit anti rotavirus group A, AbD serotec, Raleigh, NC) diluted 1:100 by wash buffer was added to each well and incubated at 37 °C for 1 hour. After repeating wash step by wash buffer twice, 50 µL of second antibody (biotinylated goat anti-rabbit lgG, Vector Laboratories, CA) diluted 1:200 by wash buffer containing 1.5 % normal goat serum was added to each well and incubated for 20 minutes at room temperature. After repeating wash step by wash buffer twice, 50 µL of Vectastain ABC reagent containing 2 % of reagent A and 2 % reagent B (VECTASTAIN ABC Kit, Vector Laboratories, CA) in wash buffer incubated for 30 minutes prior was added to each well and incubated for 20 min. After wash step by wash buffer twice, 50 µL of DAB solution diluted in distilled water following the manufacturer's instruction (DAB Substrate Kit, Vector Laboratories, CA) was added to each well and incubated for 2 minutes. DAB solution was aspirated and Mili-Q water was replaced into each well. Focus forming unit was enumerated using microscope.

2.5 Sanitizer experiment

Each set of leaves in this study, consisting of three biological replicates for each cultivar, was gently washed with Milli-Q water and the water on the leaves was then wiped off with a Kimwipe (Kimberly-Clark, Irving, TX). Two disks were excised from each leaf with a 15.6 mm diameter cork borer: one disk to be sampled on the adaxial surface and the other on the abaxial surface. Spot inoculation of rotaviruses onto disks was employed before the sanitizer was applied; two drops of 20 µL of viral solution (porcine rotavirus stain OSU) were applied onto each disk surface and incubated for 1 h to allow these viruses to attach to the leaf. After the incubation, each disk was washed with 4 ml of each kind of cold sanitizer solution at 4 °C in a well of a 12 well plate for a certain time period. Each sanitizer concentration was as it follows; Tsunami 100® 50 ppm pH 3.7; 0.25 % malic acid with 0.025 % pH 2.7. After the sanitizer step, the leaf was taken out immediately from the well and the remaining rotaviruses on the leaf were eluted with 500 µl of serum-free MEM in a 1.5 ml centrifuge tube by vortexing for 30 seconds. After the elution step, 400 µl from the tube was taken into a new 1.5 ml centrifuge tube, then this rotavirus solution was activated by 4 µl of 1 mg/ml trypsin and incubated at 37 °C for 30 minutes. In this step, not only sanitizer-treated samples but also FFU-known rotavirus stock were also treated with trypsin.

2.6 ICC-qPCR (Integrated-Cell-Culture qPCR)

Integrated Cell Culture quantitative PCR (ICC qPCR) was used in this study to quantify infectious viruses remained after the sanitizer experiment described above. This method was employed instead of the focus forming unit (FFU) assay because of its ability to detect infectious viruses more rapidly and sensitively, compared to the FFU assay. The quantitative principle of this method is based on a calibration curve for the numbers of NSP3 genes from the replicated viruses inside MA 104 cells infected by either the virus solutions with known FFU or the infectious

viruses, which remained after the exposure to the sanitizers. The X axis of this calibration curve is $\log_{10}$ copy numbers of NSP3 genes from infectious rotaviruses. The Y axis of this calibration curve is $\log_{10}$ FFU obtained from the virus solution with known FFU. A monolayer of confluent cells on a 24 well plate were washed by pre-warmed serum-free MEM twice, and then trypsin-treated rotavirus from the sanitizer experiment or serially diluted rotavirus stock with FFU-known was added onto the cells and incubated for 37 °C for 30 minutes in a 5% CO_2 incubator. After the infection step, cells were washed by serum-free MEM twice and incubated with 500 µL of serum-free MEM at 37 °C for 18 hours in a 5% CO_2 incubator. During this 18 hours incubation, only infective viruses can infect cells and replicate. This method enables us to quantify infectivity of viruses remaining after the sanitizer treatment, using known infectivity of viruses. After the incubation, 350 µL of lysis buffer from an RNA extraction kit (E.Z.N.A.® Total RNA Kit I, Omega Bio-Tek) was added to each well and incubated for 30 min at room temperature. RNA extraction was conducted according to the manufacturer's instructions. After the extraction, reverse-transcription quantitative PCR (RT-qPCR) was performed using the rotavirus gene specific primers and cellular gene specific primers. Details of RT-qPCR were described in 2.7 reverse-transcription quantitative PCR.

2.7 Reverse-transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) was conducted to quantify the rotavirus NSP3 gene and GAPDH gene of MA104 cells using an iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA) in MicroAmp® optical 384-well reaction plates with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA). A cDNA plasmid containing rotavirus NSP3 gene obtained from Integrated DNA technologies (Coralville, IA) was used to develop a standard curve of the amount of cDNA present (X axis, expressed as log10

genome copies/ µL) versus Ct values obtained from qPCR on the Y axis. For the quantification of cells which were exposed to rotaviruses, genomic RNA extracted from cells were used as a standard. The primer sequences are reported in Table 2.1. A total reaction mix 10 µL for quantification of rotavirus consisted of 3 µL of RNA sample, 5 µL of 2× iTaq universal SYBR® Green reaction mix, 0.125 µL of iScript reverse transcriptase, 0.3 µL of 10 µM JVK forward primer, 0.3 µL of 10 µM JVK reverse primer 1.275 µL of nuclease free water. For quantification of cells, a total reaction mix 10 µL for quantification of rotavirus consisted of 3 µL of RNA sample, 5 µL of 2× iTaq universal SYBR® Green reaction mix, 0.125 µL of iScript reverse transcriptase, 0.6 µL of 10 µM GAPDH forward primer, 0.6 µL of 10 µM GAPDH reverse primer, 0.675 µL of nuclease free water. The thermal cycling condition for both NSP3 gene and GAPDH gene quantification was as follows; reverse transcription reaction at 50 °C for 10 min, polymerase activation and DNA denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing/extension at 60 °C for 1 min. After these steps, a dissociation stage for dissociation curve analysis was conducted at 95 °C for 15 sec, at 60 °C for 15 sec, and 95 °C for 15 sec. Data were obtained from software SDS 2.4.2 (Applied Biosystems, Foster, CA) after RT-qPCRs. In this study, the amount of rotavirus RNA was normalized by a number of housekeeping gene GAPDH of cellular RNA.

The qPCR specificity was checked by a gel electrophoresis using RT-qPCR products, with 2 % agarose containing SYBR® Safe DNA Gel stain (Thermo Fisher Scientific, Waltham, MA). Only one band between 100 and 200 bp was observed for both products amplified with JVK primers and GAPDH primers, under a Bio-Rad Universal Hood II Imager (Bio-Rad Laboratories, Hercules, CA).

Table 1. Information of primers used in this study

Target	Primer	Sequence (5'-3')	Reference
Rotavirus NSP3 gene	JVK forward primer	5'-CAGTGGTTGATGCTGAAGAT-3'	[48, 49]
	JVK reverse primer	5'-TCATTGTAATCATATTGAATACCCA-3'	
MA104/A549 cell GAPDH gene	GAPDH forward primer	5'-AATCCCATCACCATCTTCCAG-3'	[50]
	GAPDH reverse primer	5'-AAATGAGCCCCAGCCTTC-3'	

2.8 Virus binding assay

Rotaviruses were treated with each sanitizer (Tsunami 100® 50 ppm pH 3.7; 0.25 % malic acid with 0.025 % pH 2.7) at 4 °C for 5 minutes. After this sanitation step, 1M NaOH was added to the viral solution to adjust the pH to 7.0. Then, the rotavirus solution treated with sanitizer was treated with trypsin to activate rotaviruses in a final concentration of trypsin 10 μg/mL. This solution of activated rotaviruses was diluted by serum-free MEM, then 300 μl of which was added to monolayer of confluent MA 104 cells and being incubated for an hour at 4 °C. After this incubation time, the viral solution was aspirated and the cells were gently washed with serum-free MEM twice. 350 μL of lysis buffer from an RNA extraction kit (E.Z.N.A.® Total RNA Kit I, Omega Bio-Tek, GA) was added to each well and incubated for 30 min at room temperature. RNA extraction was conducted according to the manufacturer's instructions. After the extraction, reverse-transcription quantitative PCR was performed using the rotavirus gene specific primers and cellular gene specific primers. For the control experiment to check adhesion of intact virus to MA104 cells, viruses are treated with distilled water without sanitizer at 4 °C for 5 minutes. To check for non-specific binding of rotavirus to cells, A549 cells without the receptors for porcine rotavirus strain OSU (GM3 receptors) [51, 52] were used for the binding control experiment.

2.9 Virus entry and replication assay

The assays for detection of rotavirus OSU entering and replicating in MA104 cells were similarly conducted to the binding assay including the control experiment, except the incubation of sanitizer-treated rotaviruses with MA 104 cells was conducted at 37 °C for 30 min in a 5 % CO_2 incubator, followed by washing cells by serum-free MEM twice and a 18 hours incubation at at 37 °C for 18 h in a 5 % CO_2 incubator. RNA extraction and reverse-transcription quantitative PCR were also conducted in the same manner as the virus binding assay.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Sanitizer experiment

Figure 1 and Figure 2 show the comparison of log inactivation of rotavirus treated with two kinds of sanitizers, Tsunami® 100 at 50 ppm and 0.25 % malic acid + 0.025 % TDS from three kinds of vegetables, 'Totem' Belgian endive, 'Starbor' kale, and 'Red Russian' kale. The rotavirus inactivation was expressed as survival ratios FFU/FFU_0 obtained by the ICC-qPCR method.

As shown in Figure 1, Tsunami® 100 had similar rotavirus inactivation attached to the surfaces of 'Starbor' kale and 'Red Russian' kale. However, only approximately 1 log reduction of rotavirus inactivation was achieved on 'Totem' Belgian endive. On the other hand, as shown in Figure 2, malic acid + TDS exhibited similar inactivation of rotaviruses on the three cultivars 'Totem' Belgian endive, 'Starbor' kale, and 'Red Russian' kale. The rotavirus inactivation onto 'Totem' Belgian endive was significantly improved with malic acid + TDS up to approximately 3 log rotavirus inactivation, compared to Tsunami® 100. These results suggest that Tsunami® 100 might not be effectively inactivating rotaviruses on 'Totem' Belgian endive. Previously, the physical properties and chemical composition of 24 cultivars including these three cultivars' epicuticular layers were characterized [22]. Based on their characterization, 'Starbor' kale, and 'Red Russian' kale showed very similar properties, especially their high total wax content and high hydrophobicity, while 'Totem' Belgian endive had low total wax content and hydrophobicity. These results imply that the efficacy of rotavirus inactivation attached onto leaf surfaces may be affected by the surface properties of fresh produce.

Tsunami® 100, one of the food sanitizers used in this study, is a peroxyacetic acid-based food sanitizer combined with hydrogen peroxide. This sanitizer has a low reactivity with organic load, which could contribute to the stability of this sanitizer [42], and a low pH dependence

compared to chlorine [38]. Malic acid is a kind of organic acids which have been used as antimicrobials [35, 38]. TDS, thiamine dilauryl sulfate, which was added to malic acid in this study, is vitamin B1 derivative and also a surfactant. These days, the use of surfactants combined with sanitizers considered to be more effective compared to sanitizers themselves, based on the previous studies [27, 35]. Surfactants consist of both hydrophilic and hydrophobic groups, which results in lowering the surface tension between a solid and a liquid. This characteristic is considered to allow sanitizers to reach to pathogens on fresh produce more easily and provide higher inactivation rates.

Rotavirus is non-enveloped double-stranded RNA virus, which has hydrophilic capsid surrounding its genome. Due to this hydrophilicity, it is estimated that the attachment of rotaviruses onto hydrophilic fresh produce surfaces could stronger than the rotavirus attachment on hydrophobic fresh produce surfaces, especially based on the result that rotaviruses attached onto 'Starbor' kale and 'Red Russian' kale which have hydrophobic surfaces were effectively removed by Tsunami® 100, while 'Totem' Belgian endive which has hydrophilic surfaces had low inactivation rate of rotaviruses by the same sanitizer. Notably, when malic acid + TDS was applied to the leaves instead of Tsunami® 100, the inactivation of rotaviruses onto 'Totem' Belgian endive was improved and achieved similar log removal to 'Starbor' kale and 'Red Russian' kale. These observations imply that malic acid + TDS effectively inactivated rotaviruses attached to hydrophilic leaf surfaces, with the potential of the surfactant TDS allowing the malic acid to reach closer to rotavirus particles bound to the produce surfaces with less surface tension.

In order to analyze the relationship between viruses and sanitizers, especially how the rotavirus replication cycles were inhibited by these sanitizer effects, further molecular

experiments were conducted focusing on the binding step and entry + replication step of rotavirus.

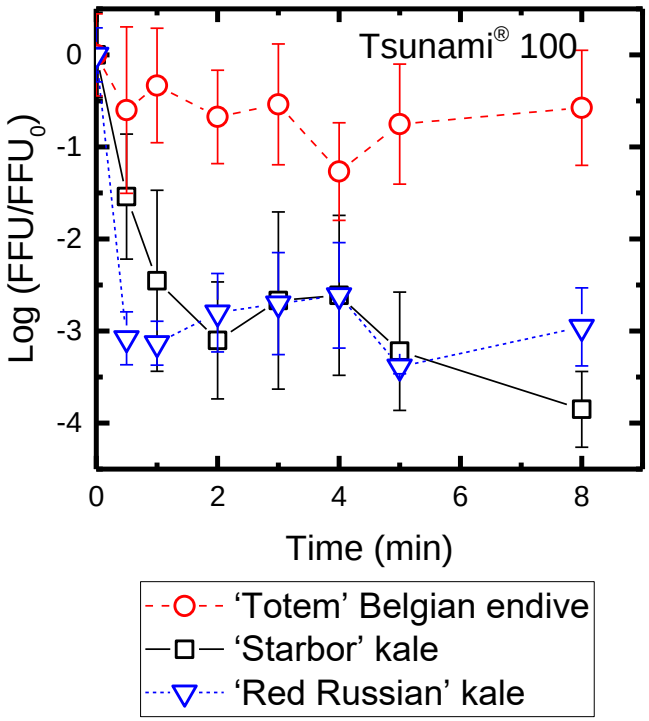

Figure 1. Comparison of log inactivation of rotavirus from 'Totem' Belgian endive, 'Starbor' kale, and 'Red Russian' kale, by Tsunami® 100. Values in the figure are the averages of results from biological replicates (n=6) at each contact time [min], with standard deviations shown as vertical error bars.

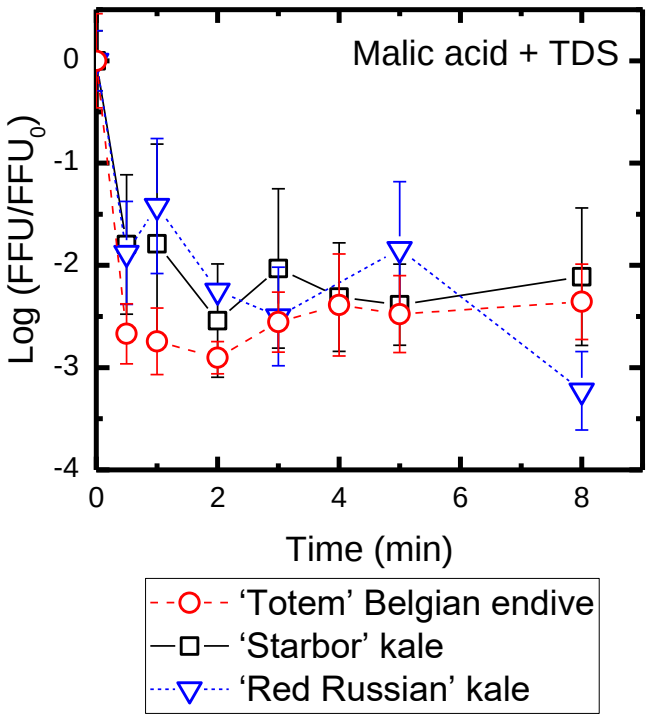

Figure 2. Comparison of log inactivation of rotavirus from 'Totem' Belgian endive, 'Starbor' kale, and 'Red Russian' kale, by Malic acid + TDS. Values in the figure are the averages of results from biological replicates (n=6) at each contact time [min], with standard deviations shown as vertical error bars.

3.2 Virus binding assay

In order to understand the mechanism of the rotavirus replication cycle inhibited by the sanitizer effects at a molecular level, virus binding assay was conducted with MA 104 cells.

As a binding control experiment, porcine rotavirus OSU was incubated with A549 cells which lack GM3 receptors, to identify the percentage of rotavirus particles nonspecifically that attach to cells. As shown in Figure 3, the highest binding of rotavirus to MA104 cells was observed in rotaviruses treated with malic acid + TDS, followed by Tsunami® 100 and the control experiment. Similarly, in the binding experiment with A549 cells, the highest binding of rotavirus was observed in rotaviruses treated with malic acid + TDS, followed by the control experiment and Tsunami® 100. Therefore, these findings imply that the binding of porcine rotavirus OSU strain treated with the sanitizers did not decrease after the sanitizer treatment. Considering that the rotaviruses treated with the sanitizers were inactivated in the sanitizer experiment, it is estimated that the entry and replication step of the rotavirus replication cycle may be inhibited due to the sanitizer effects, rather than the binding step. Based on this estimate, the virus entry + replication assay was further conducted to identify the inhibited replication step.

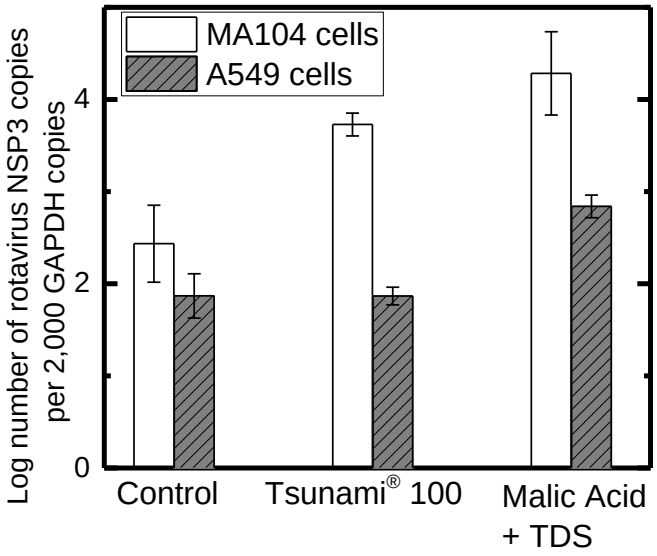

Figure 3. Comparison of numbers of rotavirus NSP3 gene bound to MA104 and A 549 cells after different sanitizer treatments. Values in the figure are the averages of results from biological replicates (n=6), with standard deviations shown as vertical error bars.

3.3 Virus entry and replication assay

Figure 4 shows the comparison of the amount of rotavirus particles replicated in MA 104 cells after the sanitizer treatments of rotaviruses. This assay was conducted to identify if the entry and replication step of rotavirus was inhibited by the sanitizer effect. As shown in Figure 4, rotaviruses treated with Tsunami® 100 and malic acid + TDS had a significant reduction of the amount rotavirus replicated in MA 104 cells after an 18 hours incubation, compared to the control experiment, which indicates that the two kinds of food sanitizers effectively inhibited the entry and replication step of the rotavirus replication cycle. Combined this finding with the results from the binding assay, it is estimated that the sanitizers did not inhibit the attachment of rotavirus to cells, but affected the entry and replication step significantly.

In the virus binding assay, the amount of rotaviruses attached to MA104 cells increased when rotaviruses are treated with the sanitizers. It is likely that the increased binding observed in the virus binding assay is nonspecific binding of rotavirus particles treated with the sanitizers to MA 104 cells, because the rotaviruses treated with the sanitizers could not effectively replicate in MA 104 cells which was observed in this entry + replication assay. This discrepancy between the results from virus binding assay and entry + replication assay could be explained by nonspecific binding of rotavirus particles to cells. Therefore, the identification of nonspecific binding of rotaviruses treated with the sanitizers should be further analyzed, especially to determine what factors provided by the sanitizers are contributing to the nonspecific binding of rotaviruses to cells. Moreover, a better understanding of the sanitizers' functions on rotaviruses is needed. It should be determined if capsid damage or genome damage of rotavirus particles after the exposure to sanitizers was caused or not, and naked RNA from sanitizer-treated rotavirus particles should be carefully inspected.

Identification methods on virus damage have been studied, especially to distinguish infectious and non-infectious viruses. Protease or RNAse could be applied to cells, because non-infectious virus particles tend to be susceptible to those enzymes and easily decomposed [53]. After this enzyme treatment, qPCR can be conducted. Also, to identify if the sanitizers damage rotavirus capsid or not, intercalation dye such as ethidium monoazide (EMA) could be applied to viruses followed by qPCR [54]. EMA can pass through damaged virus capsid and attach to its genome. Once EMA is exposed to light, the genome bound by EMA will be segmented. Moreover, to determine capsid damage caused by oxidative stress, carbonyl group on oxidatively-damaged virions can be marked using biotin [55]. To identify virus damage caused by the sanitizer effects, these alternative methods could be applied in this study and the sanitizer effect on rotavirus particles can be further analyzed at a molecular level. This knowledge will facilitate the functions of the food sanitizers and be useful to control foodborne rotavirus infections in food industry.

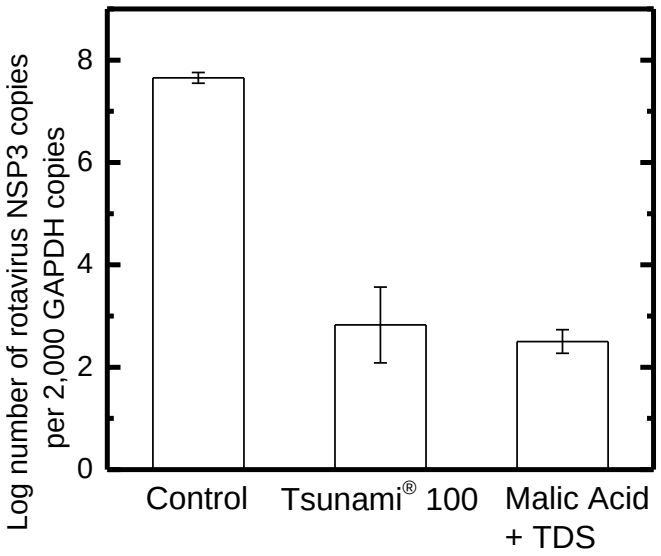

Figure 4. Comparison of numbers of rotavirus NSP3 gene replicated in MA104 cells after different sanitizer treatments. Values in the figure are the averages of results from biological replicates (n=6), with standard deviations shown as vertical error bars.

CHAPTER 4
CONCLUSIONS

1. In the sanitizer experiment, malic acid + TDS had similar rotavirus inactivation effects on all the three kinds of fresh produce ‘Totem’ Belgian endive, ‘Starbor’ kale, and ‘Red Russian’ kale, while Tsunami® 100 provided a higher rotavirus inactivation effect similarly on n ‘Starbor’ kale and ‘Red Russian’ kale and a lower rotavirus inactivation effect on ‘Totem’ Belgian endive. These observations could be explained by the interactions between rotavirus particles and different fresh produce surface properties.

2. In the virus binding assay, Tsunami® 100 and malic acid + TDS increased the amount of rotavirus particles attached to MA 104 cells, compared to the control experiment with intact rotavirus particles. In the virus binding control experiment, malic acid + TDS increased the amount of rotavirus particles attached to A549 cells and Tsunami® 100 had a similar amount of rotavirus particles bound to the cells to the control experiment.

3. In the entry + replication assay, both Tsunami® 100 and malic acid + TDS had significant reductions on the amount of rotavirus particles replicated in MA 104 cells compared to the control experiment with intact rotavirus particles.

4. The sanitizers’ functions on rotaviruses should be further analyzed, especially to determine if the sanitizers cause capsid damage or genome damage which both result in loss of infectivity. This could be examined by alternative identification methods to check virus damage, such as the use of EMA or PMA which bind to genome through damaged viral capsid.

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