PORCINE ROTAVIRUS ADSORPTION TO 24 SALAD VEGETABLES AND SANITIZATION BY FREE CHLORINE

BY

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THESIS

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ABSTRACT[1]

Foodborne diseases are a persistent problem in the United States and worldwide. Fresh produce, especially those used as raw foods like salad vegetables, can be contaminated, causing illness. In this study, we determined the number of rotaviruses adsorbed on produce surfaces using group A porcine rotaviruses and 24 cultivars of leafy vegetables and tomato fruits. We also characterized the physicochemical properties of each produce's outermost surface layer, known as the epicuticle. The number of rotaviruses found on produce surfaces varied among cultivars. Three-dimensional crystalline wax structures on the epicuticular surfaces were found to significantly contribute to the inhibition of viral adsorption to the produce surfaces ($p=0.01$). We found significant negative correlations between the number of rotaviruses adsorbed on the epicuticular surfaces and the concentrations of alkanes, fatty acids, and total waxes on the epicuticular surfaces. Partial least square model fitting results suggest that alkanes, ketones, fatty acids, alcohols, contact angle and surface roughness together can explain 60% of the variation in viral adsorption. The results suggest that various fresh produce surface properties need to be collectively considered for efficient sanitation treatments. Up to 10.8 % of the originally applied rotaviruses were found on the produce surfaces after three washing treatments, suggesting a potential public health concern regarding rotavirus contamination.

[1] This abstract was published on *PLoS ONE*. Lu L, Ku K-M, Palma-Salgado SP, Storm AP, Feng H, Juvik JA, et al. (2015) Influence of Epicuticular Physicochemical Properties on Porcine Rotavirus Adsorption to 24 Leafy Green Vegetables and Tomatoes. PLoS ONE 10(7): e0132841. doi:10.1371/journal.pone.0132841

To Family and Friends

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CHAPTER 1
INTRODUCTION[2]

As a persistent problem in the United States, every year, foodborne pathogens cause illness for approximately 48 million Americans, and among these cases, 3,000 are fatal (1). According to the U.S. Food and Drug Administration (2), foodborne illnesses are estimated to cost the U.S. economy $10-83 billion each year, from medical expenses, reduced productivity, and other costs. Of all foodborne illnesses in the U.S., human enteric viruses are a leading cause (3). In a number of foodborne outbreaks, epidemiological evidence suggests that contaminated produce surfaces during pre- and post-harvest are critical in viral transmission (4). Produce contamination before harvest, such as being irrigated or spayed with contaminated water, is an important safety concern, especially for raw and fresh produce (5-10). Although certain inactivation strategies such as thermal inactivation and high hydrostatic pressure are effective in viral inactivation, they are mostly not applicable to fresh vegetable produce used for raw consumption because they either cook the produce or cause significant tissue damage and changes in produce appearance and taste (11, 12). In the U.S., reports of foodborne illnesses associated with contaminated raw produce have increased, with salad greens and fruits found as the vehicles for pathogen transmission (13). Despite the role of fresh produce in viral transmission, only limited information is available concerning the factors controlling viral adsorption to produce surfaces.

Several factors, including surface charge, roughness, and hydrophobicity, were found to contribute to viral adsorption to produce (14-17). Ionic strength and pH of

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solutions containing viruses and temperature also influenced viral adsorption and survival (15, 17, 18). The presence of stomata and exposed carbohydrates of plant cell walls were found to enhance adsorption of viruses to romaine lettuce (19-21). Produce surfaces or cuticles are composed of various layers that serve separate functions in plant development, protection, and adaptation to the growing environment (22). The outermost layer of produce surfaces is composed of epicuticular waxes and is the most likely to interact with microorganisms attaching to produce surfaces. The epicuticular waxes were proposed to act as a physical barrier to prevent infection by plant viruses or fungi, in addition to other critical functions, including drought tolerance and tissue protection from ultraviolet light (23-25). While previous studies show an important role of the physicochemical characteristics of produce surfaces for several species influencing viral adsorption, a comprehensive study of the major leafy green salad species commonly consumed by humans as fresh or minimally processed produce has not been conducted.

To fill this knowledge gap, we conducted a study to determine the correlations between viral adsorption and physicochemical characteristics of produce surfaces using 24 cultivars of leafy green vegetables and tomato fruits. Group A rotavirus, the most common cause of severe diarrhea in children under the age of five and of gastroenteritis among all ages (4, 10, 26), was selected as a model enteric viral pathogen. Although a rotavirus vaccine was developed and applied in both developed and developing countries, in such areas as Africa and Asia, rotavirus is still a major cause of pediatric gastroenteritis. Rotavirus vaccine efficacy is only 48% in Asia and 30% in Africa, with the lowest efficacy found in Mali (17%) (27-30). Group A rotaviruses were detected in wastewater and surface water in Kenya (31), irrigation water and even receiving

vegetables in South Africa (10). Group A porcine rotavirus (OSU strain), a surrogate for human rotavirus Wa, was used in our study due to its stability and similar structural proteins to the human rotavirus strain Wa (32). Twenty-four different cultivars of leafy greens and tomatoes were grown in the greenhouse and used as model produce. Compared to previous work (14-20, 33), the greater number of cultivars from various species in this study provides more comprehensive information on viral adsorption to produce and generates a larger database for future investigations. Undamaged vegetable leaves and tomato fruit skins were characterized and tested in our viral assays to generate correlations between viral adsorption and physicochemical characteristics of the outermost layers of produce surfaces. The produce surface hydrophobicity, roughness, stoma number and length, the presence or absence of 3-D epicuticular wax crystals, and the chemical compositions of the epicuticular wax layers were determined based on the measurements of contact angle, laser confocal microscopy, scanning electron microscopy (SEM), and chemical identification using gas chromatography.

CHAPTER 2
MATERIALS AND METHODS[3]

2.1 Cell culture and OSU rotavirus propagation and purification

Group A porcine rotaviruses OSU strain (ATCC # VR-892) were propagated by infecting embryonic African green monkey kidney cells (MA-104 cells) grown in minimum essential medium (MEM, Gibco) containing 5% fetal bovine serum (FBS) (34, 35). Porcine rotaviruses OSU strain and MA-104 cells were purchased from ATCC (Manassas, VA). Before virus infection, the MA-104 cells were grown in roller bottles with an inner surface area of 850 cm^2 (Thermo Fisher Scientific Inc., Waltham, MA) and incubated at 37˚C under 5% CO_2 for 5-6 days. The medium was replaced by fresh medium on the third day of incubation. After a confluent cell monolayer was visible on the bottle wall, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) solution. After the PBS buffer was removed, rotaviruses were activated with 10 μg/ml of trypsin for 30 min. The viruses were added to maintain around 2-5 focus-forming units per cell (FFU/cell). After 90 min of incubation at 37˚C under 5% CO_2, the cells were washed twice with PBS and then incubated in MEM without the serum for 16 to 18 h. Once the infected cells were fully detached from the bottle wall, the cells and viral solution were collected and stored at 4˚C until further purification. The rotavirus solution underwent three sequential freezing and thawing treatments at -80˚C and 37˚C. Then, the viruses were separated from the cell debris by centrifugation at 1000 × g for 10 min at 20e cell debris by centrifugation at 1000 e wall, incubation. After a confluent cell monolayer was visiblThermo Scientific, Nalgene,

[3] This chapter was included in the same plication on *PLoS ONE*.

Rochester, NY) to further remove cell debris. Then, the filtrate was subjected to membrane dialysis using a 100 kDa ultrafiltration membrane (Koch Membrane, polymer polyvinylidene fluoride; Koch, Wilmington, MA) in an Amicon stirred cell (Millipore) to remove the medium and to concentrate the viruses, as described in previous work (36). In this dialysis membrane system, the rotaviruses were retained on the membrane surface and subsequently washed with a solution containing 0.1 mM of $CaCl_2$ and 1 mM $NaHCO_3$. This concentration of Ca^{2+} was used to keep the rotavirus capsid stable (37).

The infectivity assay (FFU assay) was adopted from previous publications [38, 39], and the protocol is briefly described here. The rotavirus stock was treated with 10 μg/ml of trypsin for 30 min and made into a series of dilutions in serum-free MEM. After confluent MA104 monolayers in a 96-well plate had been rinsed twice with PBS, 50 μl of each diluted virus solution from the rotavirus stock was applied to the monolayers and incubated at 37˚C under 5% CO_2 for 30 min. Afterward, the virus solutions were removed from the plate and the cell monolayers were washed twice with serum-free MEM. The cells were then incubated with 100 μl of serum-free MEM in each well at 37˚C under 5% CO_2 for 18 h prior to immunocytochemical quantification of infected cells by rotaviruses.

After 18-h incubation, the cell monolayers were rinsed twice with PBS, and fixed with 9:1 methanol (Sigma-Aldrich, St. Louis, MO) – glacial acidic acid (Fisher Scientific, Waltham, MA) for 2 min. The monolayers were then hydrated with 70% and 50% ethanol subsequently for 5min, and then subjected for 10-min treatment with 3% H_2O_2 (30%; Fisher Scientific, Waltham, MA) diluted in wash buffer (125 mM Tris (Fisher Scientific, Waltham, MA), 350 mM NaCl (Fisher Scientific, Waltham, MA), and 0.25%

Triton X-100 (Sigma-Aldrich, St. Louis, MO); pH = 7.6). Afterward, the cells were rinsed with wash buffer for 10 min and incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min to block nonspecific bindings of primary antibodies. After this step, the primary antibodies (Dako, Carpinteria, CA; catalog no. B218) diluted 1:100 in wash buffer were applied to the monolayers and incubated at 37 ℃ for 1 h. After being rinsed twice with wash buffer, the cells were incubated with the secondary antibodies (biotinylated goat anti-rabbit immuno-globulin G; Vector Laboratories, Burlingame, CA) diluted 1:200 in wash buffer for 20 min. Two washings with wash buffer were applied to the monolayers after this step. After washing, the ABC reagent (Vector Laboratories, Burlingame, CA), made 30 min prior to use and diluted as 1 (reagent A):1 (reagent B):50 (wash buffer), was applied to the monolayers for 20 min. Afterward, the cells were rinsed twice with wash buffer and then incubated with the stain peroxidase chromogen (KPL, Gaithersburg, Maryland) for less than 9 min to avoid nonspecific cell staining. Deionized (DI) water was then added to each well, and the brown-stained cells, which were the infected cells by rotaviruses, were quantified using an inverted microscope. This assay using a 96 well plate has a detection limit of 1200 FFU (50 μl of 2.4×10^4 FFU ml^{-1} viral solution).

2.2 Rotavirus adsorption assay to leafy vegetables and tomato fruits

Harvested vegetable heads, leaves, or tomato fruits were rinsed with DI water to remove soil particles attached to their surfaces and dried by gently laying a Kimwipe (Kimberly-Clark, Irving, TX) on the adaxial surface. When no water was visible on the produce surfaces, two 15.6 mm diameter disks were excised from each leaf and fruit sample. For each cultivar, two leaves, heads, or fruits from three different plants were

harvested for 6 replicate measurements. Each piece was gently transferred, with a tweezer, onto the top of a droplet of 300 µl diluted porcine rotavirus solution in PBS on a 35-mm-glass-bottom-dish (MatTek Corporation, Ashland, MA). See Figure 1 for the experimental schema. Rotavirus concentration in this droplet was determined to be 7.17 ± 0.05 $\log_{10}$ genome copies/ml (N=4) by RT-qPCR (see below). The petri dish containing the produce piece and the viral droplet was loosely capped and incubated for 2 h at room temperature in a biological cabinet. After this incubation period, the produce pieces were transferred with a tweezer, in the same way as described above, to a 24-well plate, which had 700 µl PBS in each well. The plate was gently shaken for 15 s, and the PBS solution was then carefully removed. This washing step was repeated three times before the produce pieces were removed from the well plate and the disks were excised with another corker borer (with a diameter of 11.1 mm) into smaller diameter pieces to remove the cut edges. Since viruses might be attached to the edges during the incubation period or washing steps, this treatment was important to avoid potentially confounding results. Each piece was transferred with a tweezer into a 1.7 ml labeled tube for RNA extraction and RT-qPCR. The adaxial surfaces were kept facing up throughout the whole experiment, except during incubation and washing.

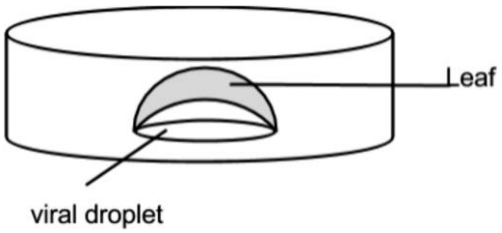

Figure 1. Experimental schema for the OSU rotavirus adsorption assay to leafy vegetables and tomato fruits.[4]

[4] This figure was included in the same plication on *PLoS ONE*.

The negative controls for these assays underwent the same procedure except that they were incubated on PBS droplets without porcine rotaviruses for 2 h. Based on the results obtained from the negative controls, we concluded that the leaves were not previously contaminated with rotaviruses. Due to the pool of 24 cultivars whose mature tissues were available at different times, the viral assays were conducted over an 8-week period from March to May, 2014. All viral adsorption experiments were conducted using the same OSU rotavirus stock (see below for concentration determination). Infectivity assays showed that the OSU rotavirus stock had approximately 5×10^7 FFU/ml. Infectivity assays were also conducted for the viral adsorption experiments, however, the concentration of infective rotaviruses on the produce samples was generally below the detection limit of the infectivity assay using a 96 well plate (2.4×10^4 FFU ml^{-1} viral solution). While the infectivity assay may be sensitive to aggregation of viruses, the qPCR method is not because it is based on the extracted genomes of all viruses.

2.3 Viral RNA extraction from inoculated produce surfaces

The RNA extraction was conducted with E.Z.N.A Total RNA Kit I (Omega, Norcross, GA) following the manufacturer's protocol in a sterilized RNA extraction cabinet to avoid RNA contamination and degradation. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC) water and stored at -80˚C before quantification by real-time quantitative PCR (RT-qPCR).

2.4 Detection of OSU rotavirus adsorption by RT-qPCR

We first determined the concentration of the OSU rotavirus stock (8 $\log_{10}$ genome copies/ml) by conducting one-step RT-qPCR in parallel with a standard calibration curve

for known concentrations of a plasmid cDNA standard (2207 bp) containing the inserted rotavirus NSP3 gene (212 bp). The 'JVK' Primers (Forward: CAGTGGTTGATGCTCAAGAT and Reverse: TCATTGTAATCATATTGAATACCCA) were used as described in previous studies (38, 39) to specifically amplify the NSP3 gene of the OSU rotaviruses. The primers and the plasmid cDNA standards were purchased from Integrated DNA Technologies (Coralville, IA). The concentration of dissolved plasmid DNA in DI water was measured by Qubit dsDNA HS assay kit (Life Technologies, Grand Island, NY), according to the manufacturer's manual. The measured cDNA standard concentration (1.88 µg/ml) was then converted into copy numbers (11.9 $\log_{10}$ genome copies/ml). For experiments with plant tissues, the extracted RNA from the OSU stock with known concentration (8 $\log_{10}$ genome copies/ml) was used to determine a detection limit of 3.9 $\log_{10}$ genome copies/ml with the corresponding Ct value at 34.3 $\pm$ 0.1 (N=3).

The number of adsorbed rotaviruses on each produce sample surface was determined by one-step RT-qPCR using an iTaq One-Step Universal SYBER RT-qPCR kit (Bio-Rad Laboratories, Hercules, CA). The overall volume of each qPCR reaction was 10 µl, composed of 2 × iTaq Mix, 0.3 mM of each primer, 125 × iScript reverse transcriptase, 3 µl RNA template, and DNase/RNase-free distilled water. The prepared PCR reactions were conducted with a Bio-Rad Unicon qPCR machine (Hercules, CA). The qPCR program was 48˚C, 10 min (reverse transcription), and 95d 0 min (reverse transcription), PCR program was 48was 48TechnologiiTaq polymerase), with cycles of 95˚C, 15 s (melting DNA double strands), 54˚C, 20 s (primers annealing), 60˚C, 30 s (elongation), 60-95˚C, and 0.05 s increments. The PCR specificity was checked on a gel

after qPCR, and only one band at around 200bp was observed under a Bio-Rad Universal Hood II Imager (Hercules, CA). The number of RNA genome copies from OSU rotaviruses adsorbed to each sample disk was calculated via equations obtained from standard curves conducted for every set of qPCR. For example, $Y = -3.497X + 47.536$ (R^2 = 0.99, efficiency = 93%). Y is the RNA amount (log_{10} genome copies/ml), and X is the Ct value. The number of adsorbed OSU rotaviruses was expressed as log_{10} genome copies normalized by the produce sample area in cm^2.

Leaves from two cultivars with epicuticular wax crystals ('Top Bunch' collards and 'Red Russian' kale) and two cultivars without epicuticular wax crystals ('Two Star' lettuce, and 'Totem' Belgian endive) were selected as additional controls for RT-qPCR inhibitors. RT-qPCR inhibitors were tested by spiking RNA extracted from the OSU virus stock into the extracted solutions from these four cultivars used as controls. The measured Ct values from these four controls were compared with those determined for the extracted RNA at the same concentration. The extracted RNA with a concentration of 5.3 log_{10} genome copies /mL showed an average Ct value of 28.72 ± 0.4 (N=6), while the controls with the same concentration had Ct values of 28.89 ± 0.2 (N=8). No significant difference was found between these two sets of Ct values (P=0.35), and therefore no inhibitors were present in this system. The negative controls for rotavirus adsorption assays showed their Ct values as "NA", indicating no rotaviruses present on the 24 vegetables prior to the viral adsorption assays. The same Ct readings ("NA") were obtained for qPCR negative controls, which used DNase/RNase-free distilled water as templates, suggesting no contamination in the qPCR reactions.

CHAPTER 3
RESULTS[5]

3.1 OSU rotavirus adsorption to epicuticular surfaces

As confirmed by RT-qPCR results, OSU rotaviruses were found on the adaxial surfaces of all 24 cultivars when the produce surfaces were incubated with the viral suspension for 2 h at room temperature. The number of adsorbed rotaviruses on the leaf or tomato fruit surfaces (an area with a diameter of 11 mm) ranged from approximately 3.7 to 5.6 $\log_{10}$ genome copies/cm^2 (Table 1). The three species with the highest levels of viral adsorption included 'Southern Giant Curled' mustard greens (5.6 ± 0.1 $\log_{10}$ genome copies/cm^2), Tatsoi (5.4 ± 0.1 $\log_{10}$ genome copies/cm^2), and 'Racoon' spinach (5.6 ± 0.2 $\log_{10}$ genome copies/cm^2). The three species with the lowest the number of rotaviruses adsorbed on epicuticular surfaces were 'Top Bunch' collard (3.7 ± 0.1 $\log_{10}$ genome copies/cm^2), 'Sun Gold' tomato (3.9 ± 0.4 $\log_{10}$ genome copies/cm^2) and 'Outredgeous' romaine lettuce (4.1 ± 0.5 $\log_{10}$ genome copies/cm^2). Within the *Solanum* genus, the cultivar 'Rose' had the highest number of adsorbed rotaviruses (4.4 ± 0.3 $\log_{10}$ genome copies/cm^2), followed by 'Indigo Rose' (4.2 ± 0.4 $\log_{10}$ genome copies/cm^2), and 'Sun Gold' tomatoes (3.9 ± 0.4 $\log_{10}$ genome copies/cm^2). The percentage of rotaviruses that adhered to each cultivar was calculated using the numbers of rotaviruses adsorbed on the produce surfaces divided by the rotavirus genome copies in the initial virus suspension. From 0.1% to 10.8% of the initial viruses were found on produce surfaces, suggesting that the surface physicochemical properties of the produce may play an important role on viral adsorption. Control experiments with 'Stabor' kale and 'Red

[5] This chapter was included in the same plication on *PLoS ONE*.

Russian' kale showed that viral adsorption was statistically similar for adaxial and abaxial surfaces (P = 0.89 for 'Stabor' kale; P = 0.18 for 'Red Russian' kale).

Table 1. Chemical composition of epicuticular waxes from 24 vegetable leaves and tomato fruits and the genome copies from adsorbed rotaviruses on these produce surfaces. [6]

	Epicuticular wax concentration (μg/cm²)					Viral copy numbers	
Sample name	Alkanes	Fatty acids	Alcohols	Ketones	Total wax content	Log $_{10}$ copies/cm²	%[a]
Tokyo bekana	2.0 ± 0.6	1.6 ± 0.5	1.5 ± 0.3	0.2 ± 0.3	5.2 ± 2.7	4.5 ± 0.2	0.7 ± 0.3
'Perseo' radicchio	1.0 ± 0.3	1.8 ± 1.5	3.1 ± 0.4	0.0 ± 0.0	5.9 ± 3.9	4.9 ± 0.4	2.0 ± 1.1
'Rhodos' endive	0.6 ± 0.6	0.9 ± 0.3	2.1 ± 0.2	0.0 ± 0.0	3.5 ± 0.2	4.5 ± 0.2	0.8 ± 0.4
'Southern Giant Curled' mustard	3.8 ± 1.7	4.1 ± 1.9	1.6 ± 0.4	0.4 ± 0.3	9.8 ± 2.9	5.6 ± 0.1	9.3 ± 1.1
Mizuna	0.9 ± 0.6	2.5 ± 0.1	0.8 ± 0.3	0.0 ± 0.0	4.4 ± 1.2	5.2 ± 0.1	3.4 ± 0.9
'Tyee' spinach	5.8 ± 0.2	1.0 ± 0.1	2.9 ± 0.6	0.0 ± 0.0	9.7 ± 0.8	5.3 ± 0.2	4.2 ± 1.4
'Racoon' spinach	6.9 ± 0.4	1.2 ± 0.6	3.1 ± 0.3	0.0 ± 0.0	11.5 ± 0.5	5.6 ± 0.2	10.8 ± 7.4
'Carmel' spinach	6.1 ± 0.1	1.2 ± 0.4	2.8 ± 0.3	0.0 ± 0.0	10.0 ± 0.7	4.9 ± 0.1	1.6 ± 0.2
Tatsoi	2.7 ± 3.3	0.8 ± 0.2	2.0 ± 0.7	0.0 ± 0.0	5.7 ± 3.8	5.4 ± 0.1	6.1 ± 1.8
'Top Bunch' collard	44.2 ± 4.8	8.1 ± 1.1	3.3 ± 0.3	24.1 ± 3.3	79.9 ± 9.2	3.7 ± 0.1	0.1 ± 0.0
'Starbor' kale	35.0 ± 2.6	5.9 ± 4.3	1.9 ± 0.2	35.5 ± 2.1	78.4 ± 1.4	4.9 ± 0.2	2.1 ± 1.0
'Red Russian' kale	44.0 ± 2.2	6.3 ± 1.2	3.3 ± 0.1	27.7 ± 0.9	81.3 ± 3.7	4.7 ± 0.1	1.3 ± 0.4
Arugula	2.2 ± 0.9	6.4 ± 2.4	1.3 ± 0.5	0.5 ± 0.5	10.4 ± 3.2	5.0 ± 0.03	2.2 ± 0.2
'Totem' Belgian Endive	1.5 ± 0.2	1.7 ± 0.2	3.1 ± 0.2	0.0 ± 0.0	6.3 ± 0.2	5.2 ± 0.1	3.9 ± 1.2
'Two Star' lettuce	0.5 ± 0.3	0.7 ± 0.6	4.4 ± 1.2	0.0 ± 0.0	5.7 ± 2.0	4.6 ± 0.2	0.9 ± 0.4
'Tropicana' lettuce	1.3 ± 1.9	2.6 ± 2.1	6.1 ± 3.6	0.0 ± 0.0	10.1 ± 7.6	4.9 ± 0.1	1.7 ± 0.6
'Outredgeous' romaine lettuce	1.6 ± 0.8	2.9 ± 0.8	15.3 ± 9.2	0.0 ± 0.0	19.9 ± 8.2	4.1 ± 0.5	0.4 ± 0.4
'Super Red' cabbage	6.7 ± 2.4	2.2 ± 1.2	1.2 ± 0.6	2.6 ± 3.2	14.2 ± 5.6	4.9 ± 0.2	1.8 ± 0.8
'Gonzales' cabbage	15.7 ± 7.7	5.9 ± 3.3	0.6 ± 0.2	12.3 ± 6.4	34.5 ± 17.5	4.5 ± 0.1	0.8 ± 0.1
'Ruby Perfection' cabbage	5.3 ± 3.5	1.7 ± 1.1	0.9 ± 0.5	3.3 ± 2.3	11.5 ± 6.7	4.9 ± 0.3	2.1 ± 0.9
'Alcosa' cabbage	40.8 ± 4.3	6.6 ± 1.3	2.3 ± 0.4	26.2 ± 2.3	76.2 ± 7.5	4.4 ± 0.1	0.6 ± 0.2
'Sun Gold' cherry tomato	26.4 ± 0.9	5.6 ± 0.9	1.9 ± 0.6	0.0 ± 0.0	33.9 ± 1.0	3.9 ± 0.4	0.2 ± 0.2

[a] The percentage was calculated using number of adsorbed rotaviruses divided by OSU rotavirus genome copies in the initial viral solution (7.17 ± 0.05 $\log_{10}$ genome copies/ml). LSD value was calculated by Student's T-test at $P = 0.05$.

[6] This table was included in the same plication on *PLoS ONE* and created by Dr. Kang-mo Ku. Data of epicuticular wax content were provided by Dr. Kang-mo Ku.

3.2 Correlations within and between physicochemical properties of epicuticle and viral adsorption

Based on the statistical analysis, 16 significant correlations were found among and between physicochemical properties of epicuticular layer and the number of adsorbed rotaviruses (Table 2). Correlations within physicochemical properties of the produce were conducted from data generated from produce collected at the same time. Contact angle showed significant positive correlations with alkane ($r = 0.659$, $P < 0.001$), fatty acid ($r = 0.442$, $P = 0.031$), ketone ($r = 0.637$, $P < 0.001$), and total wax concentrations ($r = 0.647$, $P < 0.001$). Different wax concentrations were positively and significantly correlated with each other. Previous research that used leeks as a model found that epicuticular wax biosynthesis is initiated by the conversion of fatty acids to aldehydes, then alkanes, alcohols, and ketones (40). This shared biosynthetic pathway would explain the co-correlation of the concentrations of various waxes.

Table 2. Correlation coefficients (r) between epicuticular physiochemical properties and the number of rotaviruses adsorbed on the produce surfaces.[7]

	1	2	3	4	5	6	7	8	9
1. Contact angle (°)	1.000								
2. Surface roughness (μm)	-0.169								
3. No. of stoma	0.393	0.174							
4. Stoma length (μm)	-0.225	**0.516**	0.101						
5. Alkanes (μg/cm^2)	**0.659**	-0.247	0.370	-0.065					
6. Fatty acids (μg/cm^2)	**0.442**	0.221	**0.637**	**0.535**	**0.701**				
7. Alcohols (μg/cm^2)	-0.418	-0.202	-0.292	-0.019	-0.179	-0.237			
8. Ketones (μg/cm^2)	**0.637**	**-0.420**	0.074	-0.398	**0.809**	0.317	-0.071		
9. Total wax (μg/cm^2)	**0.647**	-0.303	0.300	-0.123	**0.974**	**0.642**	-0.055	**0.899**	
10. Log_{10} viral adsorption (genome copies/mL)	-0.019	0.360	-0.356	0.112	**-0.498**	**-0.466**	-0.226	-0.246	**-0.473**

Pearson's correlation coefficients (r) were calculated by mean values of each variables from each cultivar, and the r values in bold are significantly correlated at $P < 0.05$.

[7] This table was included in the same plication on *PLoS ONE* and created by Dr. Kang-mo Ku. Data were provided by Dr. Kang-mo Ku.

Three significant correlations were found between the number of adsorbed rotaviruses and physicochemical properties of the epicuticle. The numbers of OSU rotaviruses adsorbed on the produce surfaces showed significant negative correlations with alkane ($r = -0.498$, $P = 0.013$), fatty acid ($r = -0.466$, $P = 0.022$), and total wax concentrations ($r = -0.473$, $P = 0.020$). Contact angle ($r = -0.019$, $P = 0.930$), surface roughness ($r = 0.360$, $P = 0.084$), stoma numbers ($r = -0.356$, $P = 0.089$), stoma lengths ($r = 0.112$, $P = 0.518$), alcohols ($r = -0.226$, $P = 0.195$), and ketones ($r = -0.246$, $P = 0.174$) were not correlated with the number of adsorbed rotaviruses. The six major epicuticular variables, alkane, fatty acid, alcohol, and ketone concentrations, contact angle, and surface roughness, were used to generate a PLS model (Figure 2) to predict the number of rotaviruses adsorbed on the epicuticular surfaces. Total wax was excluded because this is a redundant indicator for individual wax components. Stomata lengths and numbers were also excluded because viral adsorption was found on stomata-free tomato fruits. The performance of the final PLS model is evaluated according to the coefficient of determination (R^2) and the root mean square error of prediction (RMSEP) in the prediction set. Generally, R^2, which describes how well the data of the training set is mathematically reproduced, varies between 0 and 1 (with 1 indicating a perfectly fitted model). In PLS model six factors were extracted to get a maximized prediction value as the van der voet T^2 statistic tests did not differ significantly from the optimized model (3 factors extraction, $R^2=0.60$) with the minimum predicted residual sum of squares (PRESS) value. As RMSEP is a good measure of how accurately the model predicts the response, lower values of RMSEP indicate a better fit. A VIP score indicates how important this factor contributes to describing the variation in viral adsorption to

vegetable surfaces, compared to other variables. A VIP ≥ 0.8 is considered the cut-off value for a variable making a significant contribution to dimensionality reduction (41). We found that the RMSEP was 0.25 when 6 PLS factors were extracted in the prediction model and the PLS model explained 60% (adjusted R^2=0.60) of the experiment-wide variation in the number of adsorbed rotaviruses using the physicochemical data. The alkane concentration showed the highest variable importance for projection value (VIP = 1.15), followed by fatty acids (1.12), contact angle (0.97), ketones (0.95), alcohols (0.92), and surface roughness (0.85).

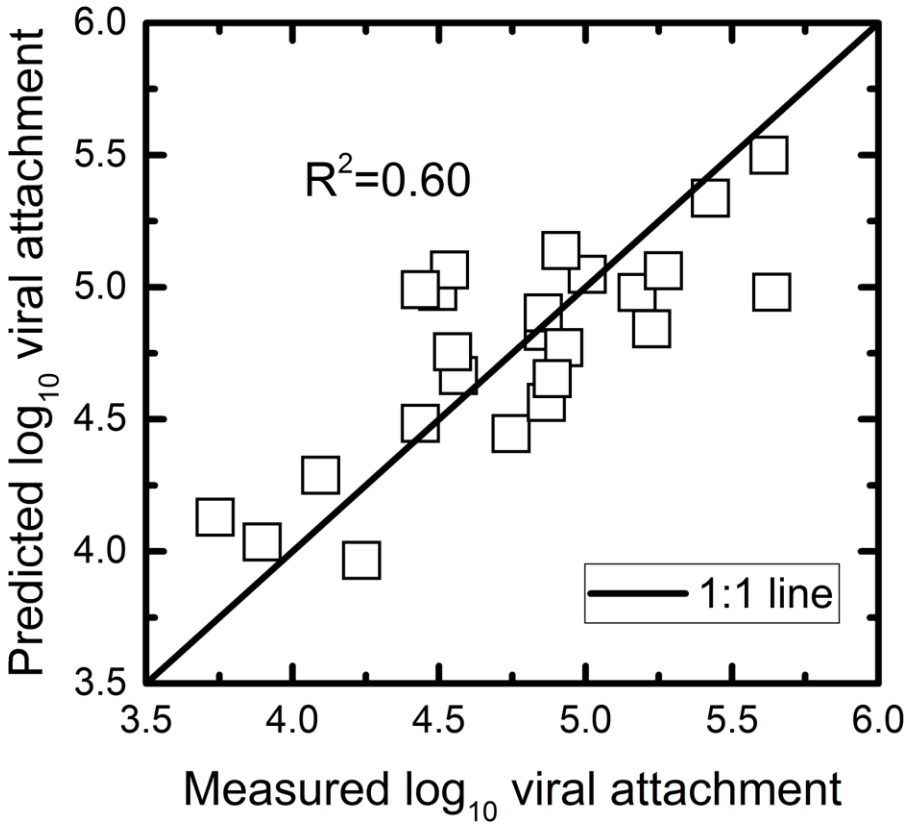

Figure 2. Partial least squares prediction model for the number of adsorbed viral particles on produce surfaces using six epicuticular physicochemical properties, including concentrations of alkanes, fatty acids, alcohols, and ketones, contact angle, and surface roughness.[8]

[8] This figure was included in the same plication on *PLoS ONE*.

CHAPTER 4
DISCUSSION[9]

In this study, the influence of 3-D epicuticular wax crystals, the chemical components of epicuticular layers, hydrophobicity and roughness of the produce surfaces, and the presence of stomata were investigated to reveal major surface properties associated with the number of adsorbed rotaviruses. Significantly, negative correlations between viral adsorption and the concentrations of alkanes, fatty acids, and total wax on the epicuticular surface were observed (Table 2). Although concentrations of alkanes, fatty acids, and total wax were significantly correlated with contact angle, which is a measurement of surface hydrophobicity, this trait was not correlated with the number of adsorbed rotaviruses. The lack of correlation with contact angle implies that the inhibition effects of the epicuticular wax components on viral adsorption may not be directly associated with increased hydrophobicity of the surfaces, but rather by the presence of 3-D epicuticular wax crystals. Indeed, the presence of 3-D wax crystals on the epicuticular layers of the produce showed significantly lower rotavirus adsorption than those without 3-D crystalized wax structures ($P = 0.012$; Table 3). For example, we observed a significantly lower number of rotaviruses adsorbed on the epicuticular surfaces on 'Outredgeous' romaine lettuce than on the other two lettuces ('Two star' and 'Tropicana'). While these three lettuce cultivars had similar adaxial contact angles, only 'Outredgeous' romaine lettuce had 3-D epicuticular wax crystals. These results suggest that the presence of the 3-D epicuticular wax crystals may play a more important role in viral adsorption than surface hydrophobicity. Another explanation of the lack of correlation between OSU viral adsorption and hydrophobicity is that the measurement of

[9] This chapter was included in the same plication on *PLoS ONE*.

contact angle was likely influenced by both surface hydrophobicity and roughness (42, 43).

Table 3. Comparison of physiochemical epicuticular properties between cultivars with 2-D or 3-D wax crystals on leaf surfaces.

Variable	2-D wax	3-D wax	P-value
No. of cultivars	13	8	
Contact angle	77.3 ± 5.9	106.1 ± 8.5	**0.010**
Surface roughness	4.1 ± 0.5	2.0 ± 0.2	**0.003**
No. of stoma	10.3 ± 1.8	18.7 ± 2.5	0.425
Stoma length	13.6 ± 1.2	11.2 ± 1.1	0.180
Alkanes	2.7 ± 0.6	24.2 ± 6.6	**0.005**
Fatty acids	2.0 ± 0.4	5.0 ± 0.8	**0.003**
Alcohols	2.7 ± 0.4	3.6 ± 1.7	0.521
Ketones	0.10 ± 0.04	16.50 ± 4.80	**<0.001**
Total wax	7.6 ± 0.8	49.5 ± 11.4	**<0.001**
Log_{10} viral adsorption	6.43 ± 0.12	5.97 ± 0.14	**0.012**

Absence or presence of 3-D wax crystals was determined by SEM. Tomato cultivars were excluded because of different tissue type.[10]

Although previous work suggested that surface roughness favors microorganism adhesion and prevents detachment of *E. coli* from selected fruits and sprouting seeds when treated with a combination of organic acids and surfactants (44, 45), the smaller size of OSU rotaviruses compared to bacteria may allow for viral adsorption on the

[10] This table was included in the same plication on *PLoS ONE* and created by Dr. Kang-mo Ku. Table data were provided by Dr. Kang-mo Ku.

produce surfaces regardless of the roughness. Using the dynamic light scattering method described in previous studies (46, 47), we found that the rotavirus suspension showed one peak with an average diameter of 175 ± 1 nm, suggesting of a slight aggregation of every two viruses. This aggregation size is much lower than the height variations of adaxial surfaces (1.1 μm to 8.0 μm) within the 24 cultivars from various species. We suggest that the relatively smaller size of rotaviruses compared to the height variations of the leaf surfaces allow the adsorbed rotaviruses to be located in the "valleys" of produce surfaces and may not be removed by the washing treatments. Our results suggest that for nanometer-sized viruses, compared to micrometer scale bacteria like *E. coli*, surface roughness may not be a critical factor controlling viral adsorption to produce surfaces.

Previous work found the aggregation of *E. coli* O157 and norovirus virus-like-particles on or inside the stomata (13, 19), suggesting that the presence of stomata may significantly help viruses adsorb to the vegetable surfaces. Hence, the contribution of stomata to viral adsorption was also investigated in our study by correlating adaxial stoma numbers and lengths with the number of adsorbed rotaviruses. While no significant correlations were found between the numbers of adsorbed rotaviruses and adaxial stoma numbers ($P = 0.113$) and lengths ($P = 0.689$), we found that vegetable samples with crystalized wax present on their surfaces showed significantly higher contact angles, and concentrations of alkanes, fatty acids, ketones, and total wax, as well as significantly lower surface roughness and the number of adsorbed rotaviruses, than the samples without 3-D crystalized wax present on the epicuticular surface (Table 3). This observation is consistent with a previous study reporting a reduced adsorption of the plant fungal pathogen, *Agathis robusta*, when stomata were covered by wax (48). In our study,

eight of the 24 vegetables had 3-D wax crystals on their epicuticular layers, and seven out of eight had stomata covered by wax crystals, as shown in Figure 3. The wax crystals shielding the stomata could prevent OSU rotaviruses from residing on or inside the stomata. Notably, 'Outredgeous' romaine lettuce did not have stomata covered by wax crystals, suggesting the potential for deposition of rotaviruses on or inside the stomata as observed in a previous study showing norovirus-like-particle aggregation at stomata of romaine lettuce leaves (19). In addition, up to 4.4 $\pm$0.3 $\log_{10}$ genome copies/ cm^2 OSU rotaviruses were able to adsorb to tomato fruit surfaces, which do not have stomata (49). These results suggest that for this comprehensive set of 24 fresh produce cultivars the presence of stomata is not necessary for rotavirus adsorption to produce surfaces, and the presence of 3-D epicuticular wax crystals covering stomata, rather than the stoma lengths and numbers, may play a more important role in the number of adsorbed rotaviruses associated with leaf stomata.

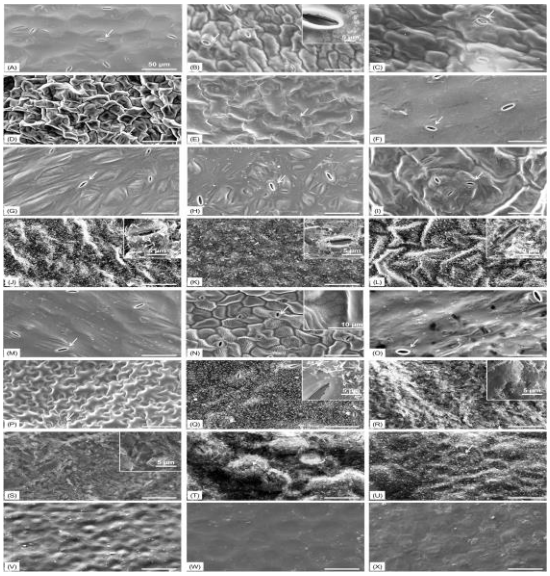

Figure 3. Epicuticular images from various vegetable leaves and tomato fruits.[11]

[11] This figure was included in the same plication on *PLoS ONE* and created by Dr. Kang-mo Ku.

Electrostatic forces, the presence of stomata, and exposed carbohydrates on epicuticular surfaces of plants have been suggested as important contributors to the number of rotaviruses adsorbed on the produce surfaces (15, 18, 19). Here we established a PLS prediction model to quantitatively explain the number of rotaviruses adsorbed on the epicuticular surfaces based on the physicochemical properties of the epicuticular surfaces. The PLS model was selected instead of multiple linear regression model because PLS allows for the inclusion of X variables that co-correlate (50). As described above, significant correlations between contact angles and concentrations of alkane, ketones, and fatty acids were observed. Based on the PLS model results, the major epicuticular properties which included the concentration of alkanes, fatty acids, alcohols, and ketones, contact angle, and surface roughness, together could explain 60% of the variation in viral adsorption among the cultivars. While we found moderate correlations between each individual variable and the number of adsorbed rotaviruses, none of these factors can individually explain more than 25% of the viral adsorption results. The highest coefficient of determination was observed between viral adsorption and alkane concentrations ($R^2 = 0.238$). The PLS model results suggest that these major epicuticular properties together impact the number of adsorbed rotaviruses. In addition, these major epicuticular properties are interdependent. For example, increasing wax contents may generate physical barriers that can increase contact angle. To the best of our knowledge, statistical modeling for prediction of viral adsorption has not been conducted, and this study for the first time calculated how these produce surface variables could quantitatively describe viral adsorption.

In summary, OSU rotaviruses were found to attach to a wide range of salad vegetables, suggesting a potential public health concern regarding rotavirus contamination during fresh produce pre-harvest production. This is the first report of lower viral adsorption on fresh produce surfaces associated with the presence of 3-D epicuticular wax crystals. In addition, the results obtained with 24 cultivars of leafy vegetables and tomato fruits commonly used in salads suggest that physical and chemical surface properties of the fresh produce need to be collectively considered for efficient sanitizer development. Future studies will determine whether commonly used sanitation strategies effectively remove adsorbed viruses and how these strategies influence the concentrations of alkanes, fatty acids, alcohols, and ketones on the produce surfaces that may allow for recontamination after sanitation.

CHAPTER 5
SANITIZATION

Introduction:

As the major pathogen that causes severe gastroenteritis among children, Group A rotaviruses can be a food safety concern through its pre-harvest contamination of produce (51). In South Africa, group A rotaviruses were detected in irrigation water and receiving vegetables, suggesting a potential human infection with rotavirus (10). Fresh produce, due to the raw consumption and little processing, puts consumers at a higher risk of being infected. Therefore, in this study, the sanitization effects of free chlorine, one of the most commonly used and cheap sanitizers in food industry, were tested on three species of salad vegetables at various contact-time lengths. Previous studies recommended to use free chlorine at above 50 mg/l, at a pH < 8.0, and with a contact time between 1-2 minutes for fresh vegetables (52). Hence, free chlorine was used at 50 ppm and pH = 7 in this study.

In order to detect only infectious rotaviruses at very low concentrations, a new quantification method was adopted from previous publication but modified in this experiment to greatly increase the sensitivity of viral infectivity assays (53). Instead of ELISA tests, FFU assays were integrated with RT-qPCR and eventually able to detect viruses at only 2 FFU. This experimental design was able to provide preliminary data on rotavirus removal by free chlorine from vegetable surfaces for future experiments and mechanism investigations. Meanwhile, the obtained results suggested a potential correlation of produce surface properties to rotavirus removal by free chlorine.

Materials and methods

In this study, 'Red Russian' kale, 'Starbor' kale and 'Totem' Belgian endive were selected as model produce and harvested at market maturity. OSU porcine rotaviruses (un-tripsinized) were propagated and harvested as described before (35). Free chlorine (sodium hypochlorite solution, pH = 7) was used in this study at 50 ppm.

Harvested leaves were gently rinsed with DI water to remove soil particles. Six disks (three for adaxial and three for abaxial inoculation) were exercised from each leaf of the three species. On the surface of each leaf disk, 2 drops of 20 µl OSU rotavirus (5.1 $\log_{10}$ FFU/ml) solution were carefully placed on either adaxial side or abaxial side. The inoculated disks were incubated for 1 h at room temperature in a biological cabinet for the droplets to dry. After the incubation, the leaf disks were treated with 4ml (1 g leaf : 150 ml sanitizer) sodium hypochlorite solution (50 ppm, pH =7) for different time lengths (0.5 min, 1min, 2min, 4min, 5min, 8min, and 10min) on ice. For each species, every time point had six replicates, three from adaxial and three from abaxial sides. The free chlorine solution was freshly made by diluting chlorine in DI water at a ratio of 1.84:1000, and the pH was adjusted to 7. The solution was stored at 4 ℃ before using. After the disks were sanitized by free chlorine for the expected lengths of time, 100 µl of 7 mM sodium thiosulfate solution was immediately added into the chlorine solution to stop disinfection reactions. Afterward, the leaf disks were carefully transferred with a tweezer into 1.5 ml centrifuge tubes, and 500 µl MEM without FBS was added into each tube for the elution of remaining rotaviruses on each leaf disk. Each sample was then vortexed for 30 s to completely remove rotaviruses from leaf surface into elution buffer.

The elution efficiency was checked by quantifying and comparing remaining rotaviruses on leaf disks and in elution buffer through RT-qPCR, and was found to achieve 100%.

The infectious rotaviruses in elution buffer were quantified through integrated cell culture and qPCR assay (ICC-qPCR) that was adopted and modified based on previous publication (53). The protocol is briefly described here. 400 out of 500 μl viral solution was activated with 10 μg/ml of trypsin for 30 min, and then added to confluent monolayers of MA-104 cells in 24-well plates for 30-min incubation at 37 ℃ under 5% CO_2. After 30 min, the cell monolayers were gently washed with serum-free MEM to remove unbound rotaviruses, and then incubated in 500 μl of fresh MEM without the serum for 18 h at 37 ℃ under 5% CO_2. 18 h later, the plates underwent RNA extraction for the collection of both cellular and viral RNA. The number of amplified rotaviruses and cells were quantified by running qPCR for the extracted RNA samples. See Chapter 2 for the detailed protocol of RNA extraction and qPCR quantification of OSU rotaviruses. The positive controls for each vegetable species underwent the same procedure except that they skipped chlorine sanitization treatment, and therefore right after the 1-h incubation with rotaviruses, the leaf disks were transferred into 1.5 ml centrifuge tubes for virus elution. As described in Chapter 2, the negative controls incubated with PBS instead of viral solution showed that the vegetables had not been previously contaminated with OSU rotaviruses.

The cell numbers in each well of a 24-well plate were determined by RT-qPCR using the same qPCR kit, program, reaction recipe as for rotaviruses quantification described in Chapter 2, except that different primers and standards for the generation of calibration curves were used. The primers (Forward: AATCCCATCACCATCTTCCAG

and Reverse: AAATGAGCCCCAGCCTTC) were used to specifically amplify cellular GADPH genes as described in previous study (54). The standard curve was generated by correlating cell concentrations of a serially diluted MA-104 cell stock, which had been quantified with hemocytometer, with the corresponding Ct values of dilutions from qPCR. The cell concentration of each sample was calculated from standard curves that were conducted for every set of RT-qPCR. For example, $Y = -3.15X + 42.515$ (R^2 = 0.99, efficiency = 107%). Y is the cell concentration (log_{10} cell numbers/ml), and X is the Ct value.

In order to quantify infectious viruses in elution buffer, the qPCR results from rotavirus samples after the 18-h incubation need to be correlated with the amount of infectious viruses before the ICC-qPCR step. To achieve this, a calibration curve was generated using the same OSU rotavirus solution as used in sanitizer assays. The OSU rotavirus solution (5.1 log_{10} FFU/ml) was activated with 10 μg/ml of trypsin for 30 min, and then serially diluted in serum-free MEM at 10, 100, and 1000 times to generate four standards. 400 μl of each standard was added to confluent cell monolayers in a 24-well plate, and quantified using ICC-qPCR as described above. Afterward, a standard curve was generated to correlate the number of infectious viruses (log_{10} FFU) before viral amplification in cells with the ratio of amplified viruses to cell concentrations (log_{10} amplified viruses per cell). Here the cell concentrations were taken into consideration in order to decrease the influence of different cell numbers on viral infectivity quantification. For example, $Y = 0.9238X + 2.4279$ (R^2 = 0.99). Y is the amount of infectious rotaviruses (log_{10} FFU), and X is the ratio of amplified viruses to cell

concentrations (log_{10} ratio). For every set of experiment, the standards went through the ICC-qPCR step the same time as the samples.

Data analysis was conducted with Microsoft excel.

Results and discussion

As shown in Figure 4, obtained data of remaining infectious rotaviruses on the vegetable leaf surfaces were plotted as log_{10} N/No versus lengths of contact time. N refers to the amount of remaining infectious viruses after sanitization treatment for certain time lengths, and No is the number of infectious rotaviruses in the initial OSU viral solution. Hence, the plots of log_{10} N/No versus contact time could be interpreted as rotavirus removal from the vegetable leaf surfaces by sodium hypochlorite solution with time. For positive controls, whose contact time was counted as zero, N equaled No and therefore log_{10} N/No equaled zero.

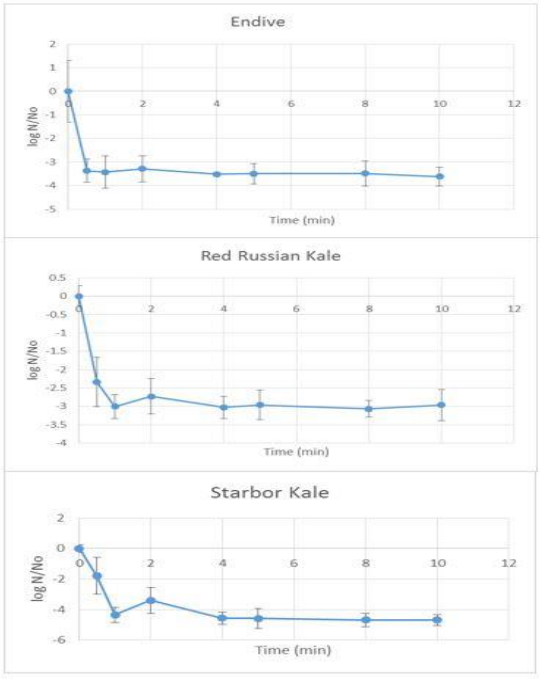

Figure 4. The plots of rotavirus removal from vegetable surfaces of three different species with time. Free chlorine was used as sanitizers at a concentration of 50 ppm.

In this experiment, 'Totem' Belgian endive ($\log_{10}$ N/No = -3.37 ± 0.5 at 0.5 min) showed the highest rate of rotavirus removal within the first 30 s, followed by 'Red Russian' kale ($\log_{10}$ N/No = -2.34 ± 0.67) and 'Starbor' kale ($\log_{10}$ N/No = -1.80 ± 1.2). These results seemed to imply that the rotavirus removal from leaf surfaces might be related to the wax contents of the epicuticular layers of each species. As in our published work, 'Totem' Belgian endive showed lowest wax concentration in the epicuticular layer while 'Red Russian' and 'Starbor' kale had high concentration of wax content (55). However, further experiments need to be conducted for confirmation.

The major virus reduction in this experiment was observed within 1 min. This result was similar to the previous report that no recoverable L. monocytogenes were found after treating the microbes with chlorine (≥ 50 ppm) for 20 seconds or longer (56). When exposed longer to free chlorine, the rotavirus removal did not go higher but stayed as constant. This was because very few infectious viruses were left after 1-min sanitization. The consumption of chlorine by the organic compounds on vegetable surfaces was not likely responsible for the "steady phase" of virus removal, since maximum 50 ppm of free chlorine was found to be consumed by organic compounds present on 'Red Russian' kale surfaces. For conservative evaluation of sanitization effects on rotavirus removal, the samples at longer contact time that had infectious rotaviruses under qPCR detection limit (3.9 $\log_{10}$ genome copies/ml) were also taken into consideration with the detection limit as their manually assigned results. In this way, the potentially stronger sanitization effects for longer contact time could be quantitatively and conservatively evaluated. However, this method might be influenced by background noise. Therefore, for the future experiment, a higher concentration of rotavirus solution is

highly recommended to use for the measurement of sanitization effects at longer contact time.

Compared to previously published work, our results showed higher virus reduction by free chlorine (57). The difference could come from different experimental materials, scale and procedure. For example, fewer viruses were used in this study. We only used 40 μl of rotavirus solution (5.1 log10 FFU/ml) for inoculation, while in the previous publication, the authors achieved a much higher inoculation level (1.5×10^8 PFU). In our experiment, small leaf disks (with a diameter of 15.6 mm) were manipulated instead of whole leaves or vegetables. The flat disk surfaces might be easier for the chlorine solution to wash off adsorbed viruses than the curved leaves with valleys on the surfaces.

CHAPTER 6
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