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Effect Of Leaf Surface Chemical Properties On Efficacy Of Sanitizer For Rotavirus Inactivation

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1 Effect of Leaf Surface Chemical Properties on the Efficacy of Sanitizer for Rotavirus
2 Inactivation
3

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25 **ABSTRACT**

26 The use of sanitizers is essential for produce safety. However, little is known about how
27 the sanitizer efficacy varies with respect to the chemical surface properties of produce. To
28 answer this question, the disinfection efficacy of an oxidant-based sanitizer and a new surfactant-
29 based sanitizer for porcine rotavirus strain OSU (PRV) was examined. PRV was attached to the
30 leaf surfaces of two kale cultivars with high epicuticular wax content, and one cultivar of endive
31 with low epicuticular wax content and then treated with each sanitizer. The efficacy of the
32 oxidant-based sanitizer correlated with leaf wax content as evidenced in 1-$\log_{10}$ PRV disinfection
33 on endive surfaces (low wax content) and 3-$\log_{10}$ disinfection with the cultivars with higher wax
34 content. In contrast, the surfactant-based sanitizer showed similar PRV disinfection efficacies
35 (up to 3-$\log_{10}$) independent of the leaf's wax content. A statistical difference was observed with
36 disinfection efficacies of the oxidant-based sanitizer for suspended and attached PRV, while the
37 surfactant-based sanitizer showed similar PRV disinfection efficacies. A significant reduction of
38 entry and replication of the PRV was observed after treatment with either disinfectant. Moreover,
39 the oxidant-based sanitizer-treated PRV reduced sialic-acid specific binding to the host cells,
40 whereas the surfactant-based sanitizer increased non-specific binding of PRV to the host cells.
41 These findings suggest that the surface properties of fresh produce may affect the efficacy of
42 virus disinfection, implying that food sanitizers should be carefully selected for different surface
43 characteristics of fresh produce.

44 **IMPORTANCE**

45 Food sanitizer efficacies are affected by the surface properties of vegetables. This study
46 evaluated disinfection efficacies of two food sanitizers, oxidant-based sanitizer and surfactant
47 based-sanitizer, on porcine rotavirus strain OSU adhering to leaf epicuticular surfaces of high

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48 wax and low wax content cultivars. The disinfection efficacy of the oxidant-based sanitizer was
49 affected by the surface properties of vegetables, while the surfactant-based sanitizer was
50 effective for both high and low wax leafy vegetable cultivars. This study suggests that the
51 surface properties of vegetables may be an important factor interacting with the disinfection of
52 rotaviruses adhering to fresh produce with food sanitizers.

53 **INTRODUCTION**

54 There were 48 million reported annual incidents of foodborne illness in the United States,
55 from 2000 to 2008 [1-3]. Of these incidents, 128,000 cases required hospitalization and 3,000
56 resulted in death [1-3]. The annual cost associated with foodborne illness, including human
57 morbidity and mortality, is estimated to range between $14.1- 152 billion [4-6].

58 Viruses including norovirus and rotavirus cause foodborne outbreaks, especially due to
59 their persistence in the environment and their low infectious doses (10 – 100 particles) [7, 8].
60 Murine norovirus, Tulane virus, and sapovirus have been found to attach to the surface of or
61 within the tissues of romaine lettuce and strawberries [9-13], suggesting that pre-harvest viral
62 contamination is a public health concern. To reduce the incidence of viral foodborne illness
63 caused by contaminated produce, it is essential to understand environmental and agricultural
64 factors that control the stability and therefore infectivity of foodborne viruses.

65 There are several conditions where fresh produce can become contaminated with viral
66 pathogens. This may occur during the pre-harvest period, when plants come into contact with
67 contaminated irrigation water or runoff [14-16]. Typically, fresh produce is stored at around
68 4 °C to maintain post-harvest shelf life and prevent bacterial growth. However, this condition is
69 favorable for stability of various types of viruses [7, 17, 18]. In addition, the chemical
70 composition, surface roughness, and hydrophobicity of fresh produce also play an important role

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71 in virus adhesion to produce surfaces [19-22]. For example, the presence of wax crystals on the
72 cuticular surface of 24 vegetable cultivars was found to reduce rotavirus adhesion [20].
73 Moreover, rotavirus particles attached to the surfaces of these 24 cultivars persisted even after
74 washing with phosphate buffer saline [20]. A similar trend was observed with hepatitis A virus
75 adsorption to lettuce, fennel, and carrots washed with potable water [23]. These findings
76 emphasize the importance of the disinfection practice for fresh produce.

77 The current sanitation treatments employed in the food industry may not effectively
78 inactivate viruses that have adsorbed to fresh vegetables [24-27]. Chlorine-based sanitizers are
79 the most commonly used sanitizer in the food industry. However, chlorine is consumed by
80 organic matter present on produce, which may result in unstable disinfection efficacy [28]. These
81 shortcomings motivate the development of alternative sanitation methods, using non-chlorine
82 based sanitizers [29-31], ozone [24, 32], ultrasound [33, 34], heat [35, 36], cold atmospheric
83 gaseous plasma [26], and electron beam [37]. However, it remains unclear how the non-chlorine
84 chemical sanitizers inactivate viruses on fresh produce.

85 To fill this research gap, this study aimed (i) to determine the efficacy of a surfactant-
86 based and an oxidant-based food sanitizer on rotaviruses adhering to the surfaces of three fresh
87 vegetables with different epicuticular wax composition; and (ii) to identify which stage of the
88 rotavirus replication cycle was inhibited by the sanitizers. Two cultivars with high wax crystals
89 ('Red Russian' and 'Stabor' kales) and a cultivar with low wax content ('Totem' Belgian endive),
90 as characterized by our previous study [20], were selected. Tsunami®100, an oxidant acid-based
91 food sanitizer authorized by EPA and potentially stronger disinfectant than chlorine [38, 39], and
92 another sanitizer, a mixture of malic acid with TDS were chosen because their disinfection
93 efficacy on viruses remains unclear. We chose rotavirus as our model virus because it is a major

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94 cause of gastroenteritis worldwide, especially in children under five years old [40]. Although
95 rotavirus vaccines have been used worldwide, rotavirus has been frequently detected in treated
96 wastewater, river water, and fresh produce [8, 41-43]. A better understanding of the survival of
97 foodborne viral pathogens adhering to fresh produce will improve disinfection strategies for
98 fresh produce to prevent foodborne illness.

99 **MATERIALS AND METHODS**

100 **Sanitizers**

101 An oxidant-based sanitizer (Tsunami 100®) was purchased from Ecolab (Saint Paul, MN).
102 Ingredients of a new surfactant-based sanitizer, malic acid and thiamine dilauryl sulfate (TDS)
103 were purchased from Sigma Aldrich (St. Louis, MO) and Sanigen Co. Ltd. (Juam dong, Korea),
104 respectively. *Vibrio cholerae* neuraminidase was purchased from Sigma Aldrich (St. Louis, MO).

105 **Greenhouse production of leafy vegetables**

106 In this study, 'Red Russian' kale (*Brassica napus*; total leaf wax concentration: 81.3 ±
107 3.7 $\mu g/cm^2$) and 'Starbor' kale (*Brassica oleracea*; total leaf wax concentration: 78.4 ± 1.4
108 $\mu g/cm^2$) were chosen as cultivars with high epicuticular wax concentrations. 'Totem' Belgian
109 endive (*Cichorium intybus*; total leaf wax concentration: 6.3 ± 0.2 $\mu g/cm^2$) was chosen as a
110 cultivar with low epicuticular wax content [20]. All three plants were grown in the greenhouse as
111 previously described [20]. Greenhouse conditions were consistently maintained throughout the
112 study so that replicated samples of produce could be obtained over a period of months. All seeds
113 were purchased from Johnny's Selected Seeds (Winslow, ME). Seeds of each cultivar were
114 germinated in 32-cell plant plug trays filled with Sunshine LC1 (Sun Gro Horticulture,
115 Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a
116 university greenhouse under a 25 °C/17 °C and 14 h/10 h: day/night temperature regimen with

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117 supplemental lighting. The greenhouse is disinfected regularly, affording vegetable growth
118 without substantial microbial contamination that may be detrimental to the plants. Twenty days
119 after germination, seedlings were transferred to 4 L pots. Leaf tissues from each type of plant
120 were harvested 50-65 days after sowing seeds. Leaves from median internodes from each leafy
121 vegetable were harvested at market maturity for analysis.

122 **Cell culture and rotavirus**

123 Porcine rotavirus strain OSU (PRV) was used in this study due to the structure of its outer
124 protein being similar to human rotavirus strain Wa, and its stability in the environment [44].
125 PRV was propagated in the monkey MA104 cell line was purchased from ATCC and maintained
126 at 37 °C in a 5 % CO_2 incubator with minimal essential medium (MEM) with 10 % fetal bovine
127 serum (FBS). PRV was propagated using confluent cells in a 150 cm^2 flask and cell were washed
128 by pre-warmed serum-free MEM three times as recommended [45]. PRV was activated with
129 trypsin at a final concentration of trypsin 10 µg/mL for 30 min at 37 °C. Following dilution by
130 serum-free MEM, trypsin-activated rotavirus solution was added to these confluent cells. After
131 the incubation at 37 °C for 60 min in a 5 % CO_2 incubator, the viral solution was aspirated and
132 washed by serum-free MEM twice. Then, serum-free MEM was added into the flask and
133 incubated for 4-5 days at 37 °C without the presence of trypsin until most of the cells were
134 detached. After this propagation step, rotavirus solution was sequentially frozen at -80 °C and
135 thawed three times. Rotavirus solution was centrifuged at 1,000 × g for 10 min at room
136 temperature and filtered through a 0.45 µM pore size filter to remove the cell debris. After this
137 step, MEM was removed from the virus stock by filtering the virus suspension through a 100
138 kDa ultrafiltration membrane as described previously [44]. Rotavirus was re-suspended in 1 mM

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139 NaCl + 0.1 mM $CaCl_2$ and stored at 4 °C for up to 4 weeks. Virus titers were quantified by using
140 the FFU assay, as previously described [46].

141 **Focus forming unit (FFU) assay**

142 Trypsin-activated PRV stock was serially diluted with serum-free MEM. Next, PRV
143 aliquots were applied to MA104 cellular monolayers in a 96-well plate and incubated at 37 °C
144 for 30 min in a 5 % CO_2 incubator. Virus was aspirated and each well was washed twice using
145 serum-free MEM. A final 50 µL of serum-free MEM was added to each well and the plates were
146 incubated at 37 °C for 18 h in a 5 % CO_2 incubator to allow viruses to replicate.

147 Next, the media were removed from each well and cells were fixed by adding 100 µL of
148 9:1 methanol: glacial acetic acid per well and incubated for 2 min. 100 µL 70 % ethanol was
149 added into each well and incubated for 5 min to rehydrate cells, before adding 100 µL 50 %
150 ethanol and incubating for another 5 min. Endogenous peroxidase activity was quenched by
151 adding 50 µL 3 % H_2O_2 in wash buffer (96 mM TRIS-HCl, 350 mM NaCl, 29 mM TRIS-Base,
152 and 0.25 % Triton X-100) per well and incubated for 10 min at room temperature. After washing
153 fixed cells with wash buffer, 50 µL of wash buffer containing 5 % normal goat serum was added
154 to each well and incubated for 20 min at room temperature. Then, 50 µL of primarily antibody
155 (rabbit anti rotavirus group A, AbD serotec, Raleigh, NC; 1:100) in wash buffer was added to
156 each well and incubated at 37 °C for 1 h. Wells were rinsed twice with wash buffer. Then, 50 µL
157 of wash buffer containing secondary antibody (biotinylated goat anti-rabbit lgG, Vector
158 Laboratories, CA; 1:200) and 1.5 % normal goat serum was added to each well and incubated for
159 20 min at room temperature. Wells were rinsed twice with wash buffer. Then, 50 µL of
160 Vectastain ABC reagent containing 2 % of reagent A and 2 % reagent B (VECTASTAIN ABC
161 Kit, Vector Laboratories, CA) first incubated in wash buffer for 30 min was added to each well

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162 and incubated for 20 min. After washing wells twice with wash buffer, 50 μL of DAB solution
163 diluted in distilled water following the manufacturer's instruction (DAB Substrate Kit, Vector
164 Laboratories, CA) was added to each well and incubated for 2 min. DAB was solution aspirated
165 and Mili-Q water was replaced into each well. Focus forming units were enumerated using a
166 microscope.

167 **Disinfection experiments for PRV adhering to leaves**

168 Each set of leaves in this study, consisting of three biological replicates for each cultivar,
169 was gently washed with Milli-Q water and the water on the leaves was then lightly wiped off
170 with a Kim wipe (Kimberly-Clark, Irving, TX). Two disks were excised from each leaf with a
171 15.6 mm diameter cork borer. One disk was sampled on the adaxial surface, and the other on the
172 abaxial surface. Two drops of 20 μL of viral solution (PRV) were applied onto each disk surface
173 and incubated for 1 h to allow virus adsorption. Next, each disk was washed with 4 mL of each
174 kind of pre-chilled sanitizer solution at 4 °C in a well of a 12-well plate as a function of time.
175 Each sanitizer concentration was as follows; the oxidant-based sanitizer (Tsunami 100®) at 50
176 ppm at pH 3.7; the surfactant based-sanitizer (0.25 % malic acid with 0.025 % TDS) at pH 2.7.
177 Next, 10 μL of 1M NaOH was immediately added into the sanitizer-containing solution to raise
178 the pH to 7. The leaf was removed from the well and the remaining PRV on the leaf was eluted
179 with 500 μL of serum-free MEM in a 1.5 mL centrifuge tube by vortexing for 30 s. 400 μL from
180 the tube was transferred into a new 1.5 mL centrifuge tube for trypsin activation of both
181 sanitizer-treated samples and rotavirus stock with known-FFU. Integrated cell culture-qPCR
182 (ICC-qPCR) was then conducted.

183 **Rotavirus decay experiment**

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184 Following the PRV adsorption to leaves, decay experiments were conducted to clarify the
185 effect of each sanitizer on PRV suspensions without vegetable leaf tissue. PRV suspensions were
186 treated with the same concentration of each sanitizer described above as a function of time at
187 4 °C. As a control, PRV suspensions were treated with distilled water at 4 °C. As above, the
188 solutions were adjusted to pH 7. Following the trypsin activation, the PRV solution was
189 immediately diluted with serum-free MEM. ICC-qPCR was then conducted.

190 **ICC-qPCR**

191 ICC-qPCR was used to quantify infectious viruses remaining after the disinfection
192 experiments for PRV adhering to leaves and rotavirus decay experiment. This method was
193 employed instead of the focus forming unit (FFU) assay because this methodology allows for the
194 detection of infectious viruses more rapidly and sensitively than the FFU assay [47]. In addition,
195 this method allows for the quantification of the RNA genomes of the viruses replicated inside the
196 host cells. The quantitative principle of this method is based on a calibration curve for the
197 number of copies of NSP3 genes from the replicated viruses inside MA104 cells infected by
198 either the virus solutions with known FFU or the infectious viruses, which remained after the
199 exposure to the sanitizers. The X axis of this calibration curve is $\log_{10}$ copy numbers of NSP3
200 genes from infectious rotaviruses. The Y axis of this calibration curve is $\log_{10}$ FFU obtained
201 from the virus solution with known FFU. A monolayer of confluent cells on a 24-well plate was
202 washed by pre-warmed serum-free MEM twice, and then trypsin-treated rotavirus from
203 disinfection experiments for PRV adhering to leaves, rotavirus decay experiment, or serially
204 diluted rotavirus stock with known FFU was added onto the cells and incubated for 37 °C for 30
205 min in a 5 % CO_2 incubator. After the infection step, cells were washed by serum-free MEM
206 twice and incubated with 500 μL of serum-free MEM at 37 °C for 18 h in a 5 % CO_2 incubator.

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207 During this 18 h incubation, only infectious viruses can enter cells and replicate. This method
208 enables us to quantify infectivity of viruses remaining after the sanitizer treatment, using known
209 infectivity of a viral stock. After the incubation, 350 µL of lysis buffer from an RNA extraction
210 kit (E.Z.N.A.® Total RNA Kit I, Omega Bio-Tek) was added to each well and incubated for 30
211 min at room temperature. RNA extraction was conducted according to the manufacturer's
212 instructions. After the extraction, reverse-transcription quantitative PCR (RT-qPCR) was
213 performed using the rotavirus gene specific primers and cellular gene specific primers.

214 **Reverse-transcription quantitative PCR**

215 Reverse transcription quantitative PCR (RT-qPCR) was conducted to quantify the PRV
216 NSP3 and cellular GAPDH transcripts in infected MA104 cells using an iTaq™ Universal
217 SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA) in MicroAmp® optical 384-
218 well reaction plates with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster,
219 CA). Plasmids containing rotavirus NSP3 gene (Integrated DNA technologies, Coralville, IA)
220 were used to develop a standard curve for the amount of cDNA present (X axis, expressed as
221 $\log_{10}$ genome copies/ µL) versus Ct values obtained from qPCR on the Y axis. For the
222 quantification of cells which were exposed to PRV, genomic RNA extracted from cells were
223 used as a standard. A total reaction mix 10 µL for quantification of rotavirus consisted of 3 µL of
224 RNA sample, 5 µL of 2× iTaq universal SYBR® Green reaction mix, 0.125 µL of iScript reverse
225 transcriptase, 0.3 µL of 10 µM JVK forward primer (5'-CAGTGGTTGATGCTGAAGAT-3'),
226 0.3 µL of 10 µM JVK reverse primer (5'-TCATTGTAATCATATTGAATACCCA-3') [48, 49],
227 and 1.275 µL of nuclease free water. For quantification of cells, a total reaction mix 10 µL for
228 quantification of rotavirus consisted of 3 µL of RNA sample, 5 µL of 2× iTaq universal SYBR®
229 Green reaction mix, 0.125 µL of iScript reverse transcriptase, 0.6 µL of 10 µM GAPDH forward

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230 primer(5'-AATCCCATCACCATCTTCCAG-3'), 0.6 µL of 10 µM GAPDH reverse primer (5'-
231 AAATGAGCCCCAGCCTTC-3') [50], and 0.675 µL of nuclease free water. The thermal
232 cycling condition for both the NSP3 gene and GAPDH gene quantification was as follows;
233 reverse transcription reaction at 50 °C for 10 min, polymerase activation and DNA denaturation
234 at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing/extension
235 at 60 °C for 1 min. After these steps, a dissociation stage for dissociation curve analysis was
236 conducted at 95 °C for 15 s, at 60 °C for 15 s, and 95 °C for 15 s. Data were obtained from the
237 software package SDS 2.4.2 (Applied Biosystems, Foster, CA) after RT-qPCRs. In this study,
238 the amount of rotavirus RNA was normalized by the number of housekeeping gene copies of
239 GAPDH in cellular RNA.
240 The qPCR specificity was checked by a gel electrophoresis using RT-qPCR products,
241 with 2 % agarose containing SYBR® Safe DNA Gel stain (Thermo Fisher Scientific, Waltham,
242 MA). Only one band between 100 and 200 bp was observed for both products amplified with
243 JVK primers and GAPDH primers.
244 **Virus entry and replication assay**
245 PRV were treated with each sanitizer at 4 °C as a function of time. After this sanitation
246 step, the viral solution was adjusted to pH 7 and activated with trypsin. This solution of activated
247 rotaviruses was diluted by serum-free MEM, then 300 µL of which was added to the monolayer
248 of confluent MA104 cells and incubated for 30 min at 37 °C in a 5 % CO_2 incubator. Following
249 this incubation, cells were washed by serum-free MEM twice and incubated for 18 h at 37 °C in
250 a 5 % CO_2 incubator. RNA extraction and RT-qPCR were conducted as described above.
251 **Virus binding assay**

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252 The assays for detection of PRV binding to MA104 cells were conducted similarly to the
253 virus entry and replication assay. The only difference from the entry and replication assay was
254 that after the sanitizer treatment of PRV at 4 °C for 5 min, the sanitizer-treated PRV was
255 incubated with MA104 cells at 4 °C for 1 h, followed by aspiration of the viral solution and wash
256 of MA104 cells by serum-free MEM twice. RNA extraction and RT-qPCR were conducted as
257 described above. For the control experiment to check adhesion of intact PRV to MA104 cells,
258 viruses were treated with distilled water without sanitizer at 4 °C for 5 min. To check for non-
259 specific binding of rotavirus to cells, A549 cells without the receptors for PRV (GM3 receptors)
260 [51, 52] were used for the binding control experiment.

261 **Binding assay with neuraminidase**

262 Confluent monolayers of MA104 cells in a 6-well plate were washed by pre-warned
263 serum-free MEM twice. 1 mL of serum-free MEM containing neuraminidase *V. cholerae* at a
264 final concentration of 40.3 mU/mL was added to cells in each well and incubated for 1 h at 37 °C
265 in a 5 % CO_2 incubator. As a positive control for each sample, 1 mL of pre-warmed serum-free
266 MEM without neuraminidase was added to cells and incubated similarly as written above. Intact
267 PRV as a control experiment and sanitizer-treated PRV were adjusted to pH 7 and activated with
268 trypsin before being incubated with cells and washed in the same manner as the virus binding
269 assay, followed by RNA extraction and RT-qPCR.

270 **Plaque assay with neuraminidase**

271 The plaque assay for detection of PRV-infected MA104 cells were conducted similarly to
272 the binding assay with neuraminidase. The only difference from the binding assay with
273 neuraminidase was that the incubation of sanitizer-treated PRV with MA104 cells was conducted
274 at 37 °C for 1 h in a 5 % CO_2 incubator, followed by washing cells by serum-free MEM twice.

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275 The cells were overlaid with MEM containing 1 % agarose, 2.2 μg/mL trypsin at a final
276 concentration, 7.5 % sodium bicarbonate, 15 mM HEPES, 0.1 mg of kanamycin/ml, 0.05 mg of
277 gentamicin/ml and incubated at 37 °C for 72 h, in a 5 % CO_2 incubator. Following the incubation,
278 the cells were fixed by 10 % formaldehyde in 1× PBS and stained by 0.05 % crystal violet in
279 10 % ethanol. PRV-infected MA104 cells were visualized and counted.

280 **Statistical analyses**

281 Statistical analyses were conducted with OriginPro 2016 (OriginLab Corporation, MA,
282 USA). For the disinfection experiments for PRV adhering to leaves, significant differences in
283 disinfection efficacies between cultivars and sanitizer treatments were determined by a three-way
284 analysis of variance (ANOVA). Also for the rotavirus decay experiment, disinfection efficacies
285 were analyzed for sanitizer treatments by a two-way ANOVA. For other assays, t-tests were
286 conducted. Relationships were considered significant when $P < 0.05$.

287 **RESULTS**

288 **Disinfection of PRV adhering to leaf surfaces**

289 The ratio of the number of infectious PRV remaining post-sanitizer treatment (FFU) over
290 the initial number of infectious PRV (FFU_0) were determined to identify the effectiveness of
291 each sanitizer in inactivating PRV when PRV was adsorbed to the plant surfaces tested. The
292 disinfection of PRV was expressed as disinfection ratios (FFU/FFU_0) obtained by the ICC-RT-
293 qPCR method. Figure 1 and Figure 2 show the comparisons of $\log_{10}$ reduction of PRV treated
294 with oxidant-based sanitizer at 50 ppm and surfactant-based sanitizer (0.25 % malic acid with
295 0.025 % TDS) on leaves of the three vegetable cultivars, respectively.

296 As shown in Figure 1, the oxidant-based sanitizer caused statistically similar disinfection
297 rates of PRV that was adsorbed to the 'Starbor' kale and 'Red Russian' kale surfaces ($P > 0.05$).

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298 In contrast, only approximately 1-$\log_{10}$ PRV disinfection was observed when PRV was adsorbed
299 to the 'Totem' Belgian endive surfaces. In contrast as shown in Figure 2, the surfactant-based
300 sanitizer exhibited similar disinfection of PRV on all the three cultivars ($P > 0.05$). The
301 disinfection of PRV adhering to the 'Totem' Belgian endive surfaces achieved approximately 3-
302 $\log_{10}$ inactivation when the surfactant-based sanitizer was used, while the oxidant-based sanitizer
303 showed only 1-$\log_{10}$ inactivation. These results suggest that the surfactant-based sanitizer is more
304 effective than the oxidant-based sanitizer in inactivating PRV adhering to the leaf surface of
305 'Totem' Belgian endive. Taken together, these results imply that the different sanitizer efficacies
306 for leafy vegetables vary with different epicuticular chemical properties.

307 **Disinfection of suspended PRV without vegetables**

308 To determine how each sanitizer interacted with PRV to prevent virus adhering to
309 vegetable surfaces, we explored how PRV in suspension, and in the absence of plants, was
310 disinfected. Figure 3 shows the comparisons of $\log_{10}$ disinfection of suspended PRV treated with
311 distilled water (control), the oxidant-based sanitizer, or surfactant-based sanitizer. Similar to
312 Figure 1 and Figure 2, the disinfection of PRV was expressed as disinfection ratios FFU/FFU_0
313 obtained by the ICC-RT-qPCR method. Compared to the control experiment, both sanitizers had
314 statistically different disinfection ratios ($P < 0.05$). When comparing the disinfection efficacies of
315 the surfactant-based sanitizer, it was observed that the efficacies were statistically similar when
316 comparing disinfection of PRV in solution versus PRV adsorbed to plant leaves (Figure 3 as
317 compared to Figure 2). However, the oxidant-based sanitizer disinfected suspended PRV to a
318 statistically significant greater degree than it did when PRV was associated with the plant leaves
319 ($P < 0.05$). Thus, the efficacy of the oxidant-based sanitizer was different between when PRV
320 was in suspension versus attached to vegetable surfaces, whereas the surfactant-based sanitizer

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321 showed a similar disinfection efficacy on PRV with or without vegetable leaf tissue. These data
322 implied that PRV interaction with the leaf surface could reduce the efficacy of the oxidant-based
323 sanitizer.

324 **Influence of sanitizers on the PRV replication cycle**

325 It is unknown how sanitizers inactivate PRV on a molecular level. As a means to
326 understand this process, we examined how each sanitizer impacted virus entry and replication,
327 and binding steps. For these assays, the suspension of PRV that was exposed to the sanitizers (as
328 opposed to PRV adhering to vegetable surfaces) was examined. Figure 4 shows the comparison
329 of the number of PRV RNA copies (NSP3 gene) replicated in MA104 cells after the sanitizer
330 treatments of PRV following 18 h post-infection. PRV treated with the oxidant-based sanitizer
331 and the surfactant-based sanitizer had significant reductions in the number of PRV RNA copies
332 replicated in MA104 cells 18 h post-infection, compared to the control where PRV was exposed
333 to distilled water instead of a sanitizer. Treatment with the oxidant-based sanitizer or the
334 surfactant-based sanitizer lead to significantly less PRV RNA copy numbers compared to the
335 control ($P < 0.05$). Thus, each sanitizer altered PRV particles in a manner that prevented PRV
336 entry and replication in MA104 cells.

337 One possibility is that the inhibition of PRV entry and replication was due to transformed
338 PRV particles that could no longer bind to the host cells. This question was answered by
339 conducting a virus-binding assay shown in Figure 5. In this experiment, sanitizer-treated PRV
340 was incubated with MA104 cells, monolayers were washed, and PRV particles that remained
341 attached to cells were quantified by RT-qPCR. As shown in Figure 5, PRV genes were more
342 abundant when PRV particles were treated with either disinfectant compared to water. This
343 finding indicated that sanitizers increased binding of PRV to the MA104 cells. This same assay

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344 was performed in parallel using A549 cells, cells that lack GM3 receptors [52], which allows
345 PRV to initialize penetration into the host cells through an entry receptor integrin. When using
346 the A549 cell line, the highest number of bound PRV was observed with PRV treated with the
347 surfactant-based sanitizer, followed by intact PRV and PRV treated with the oxidant-based
348 sanitizer. The increased binding of PRV after treatment with sanitizers suggests that sanitizers
349 themselves may alter the capsid such that there is an increase in non-specific binding of PRV to
350 both A549 cells and MA104 cells.

351 Although A549 cells do not express the GM3 receptor [52], both A549 and MA104 cells
352 express sialic acid on the cellular surface [53], a molecule that can serve as an attachment
353 receptor for PRV. Therefore, the effect of the sanitizers on disrupting PRV-sialic acid
354 interactions cannot be evaluated using this assay. To determine whether sanitizer treatment
355 influences PRV binding to sialic acid on MA104 cellular surface, binding assays were conducted
356 in which MA104 cells were incubated with *V. cholerae* neuraminidase to digest sialic acid
357 moieties on the cellular surface (Figure 6). The removal of sialic acid from the cellular surface
358 significantly reduced the number of untreated and oxidant-based sanitizer treated PRV that
359 bound to MA104 cells, as determined by RT-qPCR. This observation suggests that sialic acid
360 plays an important role in PRV attachment to MA104 cells, where receptor-specific binding of
361 the control sample (intact PRV) and oxidant-based sanitizer-treated PRV were reduced by the
362 removal of sialic acid ($P < 0.05$). However, no statistical difference was observed when using
363 surfactant-based sanitizer-treated PRV ($P > 0.05$), indicating that non-specific binding of PRV to
364 the cellular surface occurred regardless the presence of sialic acid. These data imply that each
365 sanitizer may alter PRV in distinct manners.

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366 To identify if the sialic acid digestion by neuraminidase affects PRV infection, a plaque
367 assay was conducted. As shown in Figure 7, a significant difference in plaque formation (PRV
368 infection) was observed with the control sample (intact PRV) between non-treated infected cells
369 and neuraminidase-treated infected cells ($P < 0.05$). A similar trend was observed with PRV
370 treated with the oxidant-based sanitizer and surfactant-based sanitizer ($P < 0.05$). The removal of
371 sialic acid from the ganglioside on the cellular surface reduced PRV infection, as well as the
372 attachment of intact PRV and PRV treated with oxidant-based sanitizer to MA104 cells. This
373 indicates that non-specifically bound PRV to MA104 cells could not replicate effectively in the
374 cells.

375 **DISCUSSION**

376 The oxidant-based sanitizer, containing 15.2 % peroxyacetic acid combined with 11.2 %
377 hydrogen peroxide, has been used in the food industry because of its low reactivity with organics
378 [38] and is less pH dependent compared to chlorine [54]. In the food industry, the concentration
379 range allowed for food sanitation is from 30 to 80 ppm, with a 1.5 minute mixing time [55]. In
380 this study, we employed the concentration at 50 ppm with the exposure time from 30 sec to 8
381 min. Oxidants such as hydrogen peroxide has been shown to inactivate viruses [44, 56]. For
382 example, 0.32-$\log_{10}$ disinfection of human rotavirus strain Wa was observed with 0.6 mM
383 hydrogen peroxide after 8 min of exposure at 25 °C [44]. Furthermore, 0.2 mM hydrogen
384 peroxide achieved 2-$\log_{10}$ inactivation on MS2 coliphage after a 30 min incubation [56]. The
385 hydrogen peroxide concentration in the studied sanitizer is 2 mM, which is much higher than the
386 concentration used in the previous studies. The high concentration of hydrogen peroxide and also
387 another oxidant, peroxyacetic acid, used in our study allowed 3-$\log_{10}$ inactivation of PRV
388 adhering to vegetables' leaf surfaces with high epicuticular wax concentrations ('Starbor' kale

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389 and 'Red Russian' kale) and 1-$\log_{10}$ inactivation of PRV adhering to a vegetable cultivar with
390 low wax concentrations ('Totem' Belgian endive). This 100-fold difference in PRV disinfection
391 efficacy by this sanitizer suggests that a strong interaction between PRV and the more
392 hydrophilic surfaces of endive protected the PRV adhering to the leaf surface from disinfection.
393 Rotaviruses including PRV are negatively charged and hydrophilic [57, 58]. A weak interaction
394 between hydrophilic PRV and hydrophobic kale surfaces may allow the oxidants to inactivate
395 the adsorbed PRV. However, for PRV attached strongly to hydrophilic leaf surfaces, a sanitizer
396 that can denature or oxidize the attached capsid is more desirable.

397 The surfactant-based sanitizer consists of 0.25 % malic acid, an organic acid, which have
398 been used as antimicrobials [29, 54], and 0.025 % thiamine dilauryl sulfate (TDS), a vitamin B1
399 derivative and also a negatively-charged surfactant. This combination of malic acid and TDS has
400 been recently proposed and not yet in use in the food industry [29]. The disinfection efficacies of
401 10 % malic acid and 1 % TDS on *E. coli* O157:H7 on alfalfa seeds was as effective as 20,000
402 ppm chlorine [29]. TDS was found to have synergistic effects for the inactivation of total
403 mesophilic bacteria and coliforms, when TDS was combined with a commercially available
404 sanitizer, such as ethanol, chlorine, and hydrogen peroxide [59]. Similarly, chlorine at 200 ppm
405 in combination with one of the following surfactants sodium dodecyl sulfate (SDS), Triton X-
406 100, or NP40 had higher sanitation efficacies than chlorine alone for murine norovirus adhering
407 to the surface of strawberries, raspberries, cabbage, and romaine lettuce [25]. Moreover, each of
408 these surfactants had virucidal effects against murine norovirus when the viruses were incubated
409 with one of the surfactants at 37 °C for 72 h, disrupting the outer protein of murine norovirus
410 [25]. Charged surfactants have been found to have the ability to bind to and denature protein [60].
411 In our study, despite the strong interaction between PRV and hydrophilic surface of 'Totem'

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412 Belgian endive, the surfactant-based sanitizer was still effective, suggesting that TDS could
413 denature the protein capsid of PRV adhering to the leaf surface. Moreover, the surfactant-based
414 sanitizer also had similar inactivation efficacies on PRV adhering to leaves of the two kale
415 cultivars (‘Starbor’ kale and ‘Red Russian’ kale). One possible reason behind these similar
416 inactivation efficacies could be the ability of TDS and malic acid with both hydrophilic and
417 hydrophobic groups to reach PRV adhering to both kale and endive leaf surfaces. Taken together,
418 these findings presented in this study demonstrate that the surfactant-based sanitizer effectively
419 inactivated PRV adhering to both leafy surfaces with high and low wax concentrations.

420 The observed effectiveness of the oxidant-based sanitizer for PRV adhering to the leaf
421 surfaces suggests that the surfaces of PRV were oxidized by the sanitizer. The PRV capsid may
422 also be denatured by surfactant-based sanitizers. We hypothesize that the oxidized or denatured
423 capsids could non-specifically bind to MA104 cells; however, the non-specifically bound PRV
424 cannot replicate in MA104 cells effectively. This hypothesis was tested with the binding assays
425 to determine the effects of the sanitizers on the PRV attachment to MA104 cells and replication
426 inside the cells. We found that the sanitizer treatment of PRV led to an increase of PRV attached
427 to MA104 cells, indicating that non-specific binding of PRV particles to MA104 cells, or
428 specific binding to the cellular receptors followed by non-effective penetration or replication in
429 the cells, given that PRV treated with the sanitizers could not effectively replicate in MA104
430 cells as observed in the virus entry and replication assay. Furthermore, we conducted binding
431 experiments with neuraminidase to digest sialic acid on the cellular surface, which is an
432 important factor to initialize the PRV penetration step into the host cell. Rotaviruses attach to
433 terminal sialic acid on their receptor (GM3 receptor for PRV strain OSU) and utilize sialic acid
434 binding to penetrate into the cell via integrin, which is an entry receptor for rotaviruses [61, 62].

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435 Therefore, by digesting sialic acid moieties on the cellular surface, specific-binding of PRV to
436 sialic acid moieties was reduced as found with intact PRV and PRV treated with the oxidant-
437 based sanitizer. However, the surfactant-based sanitizer treatment did not cause a reduction in
438 PRV bound to the cellular surface with or without sialic acid digestion. This observation can be
439 attributed to non-specific binding caused by the surfactant-based sanitizer treatment to PRV
440 surface. In the plaque assay with neuraminidase, PRV infection (plaque formation) was reduced
441 by digesting sialic acid from MA104 cells by neuraminidase in all the samples, whereas the
442 surfactant-based sanitizer treated PRV binding to MA104 cells was not affected by the removal
443 of sialic acid in the binding assay with neuraminidase, indicating that the plaque assay to analyze
444 PRV binding was needed to evaluate non-specific binding, as well as the binding assay
445 quantified by RT-qPCR.

446 In summary, our mechanistic studies show that the effectiveness of the oxidant-based
447 sanitizer depends on the sanitizer properties and epicuticular leaf surface properties. We found
448 that the oxidant-based sanitizer was less effective for the inactivation of PRV adhering to
449 hydrophilic leaf surfaces, due to the stronger interactions with PRV. This knowledge will
450 facilitate the selection of effective food sanitizers for virus disinfection. Based on our results,
451 non-specific binding of PRV was increased by the surfactant-based sanitizer treatment. Future
452 studies will identify what factors provided by the sanitizers are contributing to the non-specific
453 binding of PRV to MA104 cells. A potential factor could be capsid damage on rotavirus particles
454 after the exposure to sanitizers. To identify capsid damage caused by oxidative stress, carbonyl
455 groups on oxidatively-damaged viral particles can be marked using biotin [63]. Alternative
456 methods could be applied in this study and the sanitizer effect on PRV particles can be further
457 analyzed.

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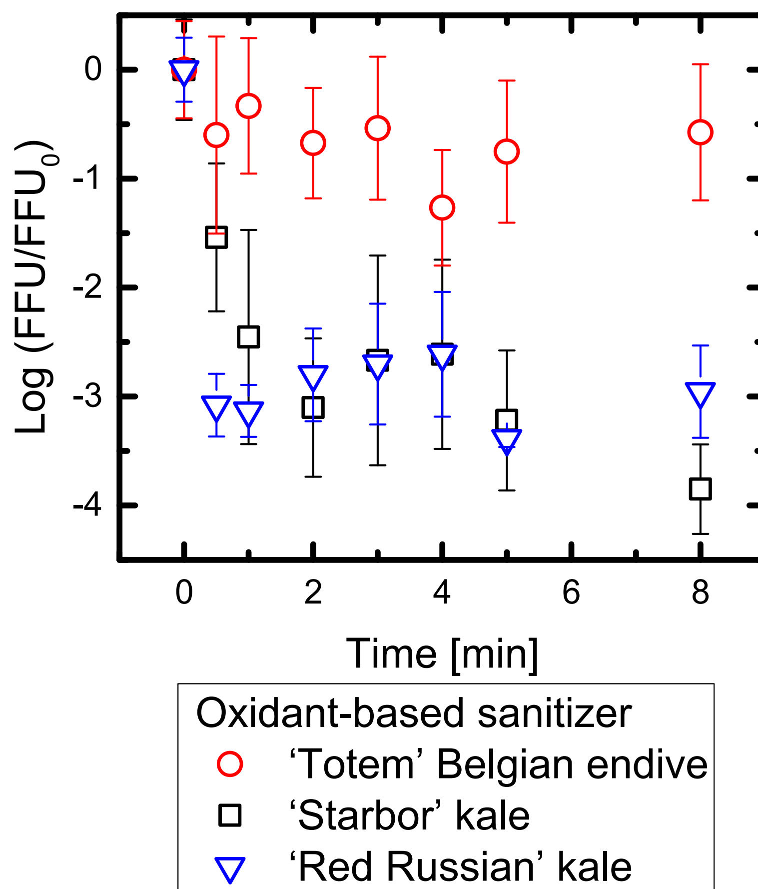
649 **Figure Legends**



650

651 **Figure 1.** Comparisons of $\log_{10}$ disinfection of PRV on 'Totem' Belgian endive, 'Starbor' kale,
652 and 'Red Russian' kale, by the oxidant-based sanitizer. Values in the figure are the averages of
653 results from biological replicates (n=6) at each contact time [min], with standard deviations
654 shown as vertical error bars. There was a significant difference on the disinfection ratios of PRV
655 between 'Totem' Belgian endive and 'Starbor' and 'Red Russian' kale ($P < 0.05$).

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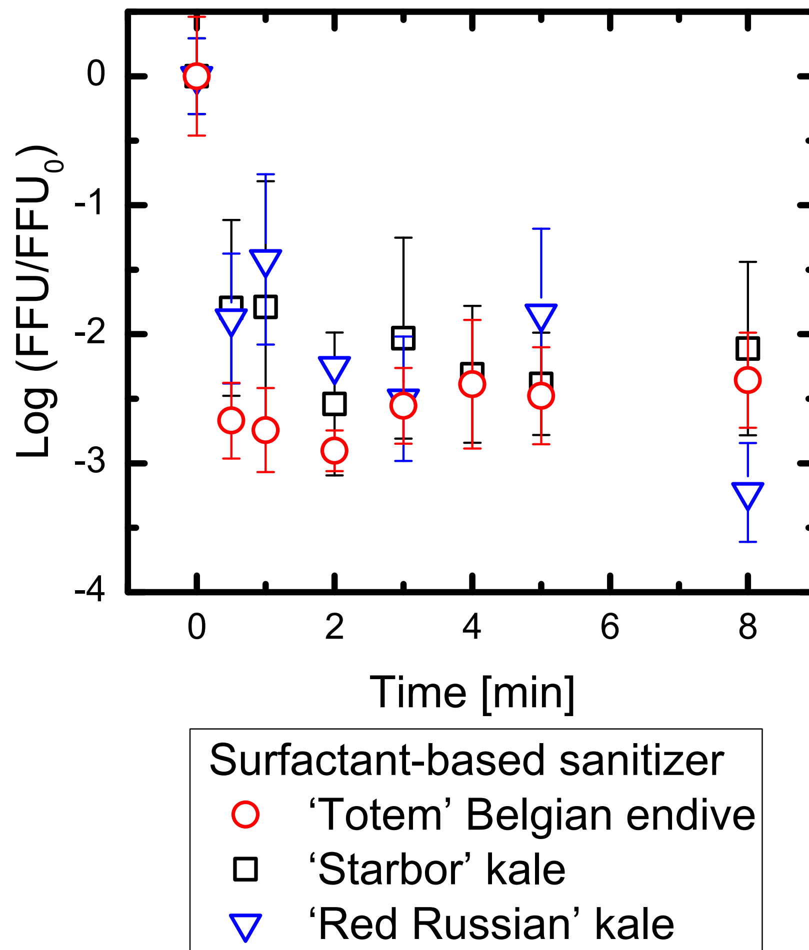

657 **Figure 2.** Comparisons of $\log_{10}$ disinfection of PRV on 'Totem' Belgian endive, 'Starbor' kale,
658 and 'Red Russian' kale, treated with the surfactant-based sanitizer. Values in the figure are the
659 averages of results from biological replicates (n=6) at each contact time [min], with standard
660 deviations shown as vertical error bars. There were no significant differences on the disinfection
661 ratios of PRV between all the cultivars ($P > 0.05$).

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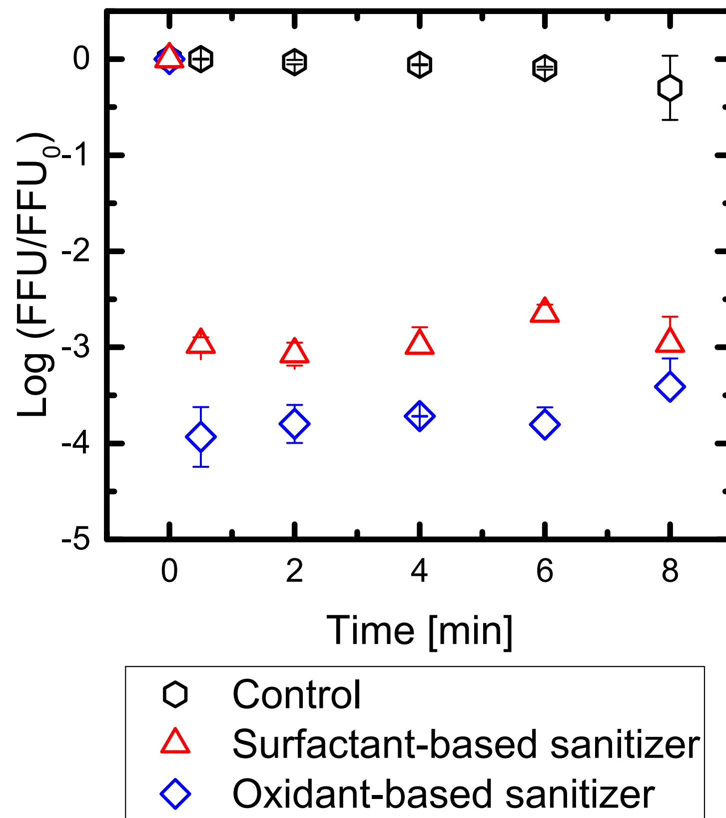

662

663 **Figure 3.** Comparisons of $\log_{10}$ disinfection of suspended PRV (in the absence of leafy vegetable

664 tissues), with distilled water (control), the oxidant-based sanitizer, or the surfactant-based

665 sanitizer. Values in the figure are the averages of results from biological replicates (n=4) at each

666 contact time [min], with standard deviations shown as vertical error bars. There was no

667 significant differences in the disinfection of PRV between the oxidant-based sanitizer and the

668 surfactant-based sanitizer ($P > 0.05$), while there was a significant difference in the disinfection

669 between each sanitizer treatment and the control ($P < 0.05$).

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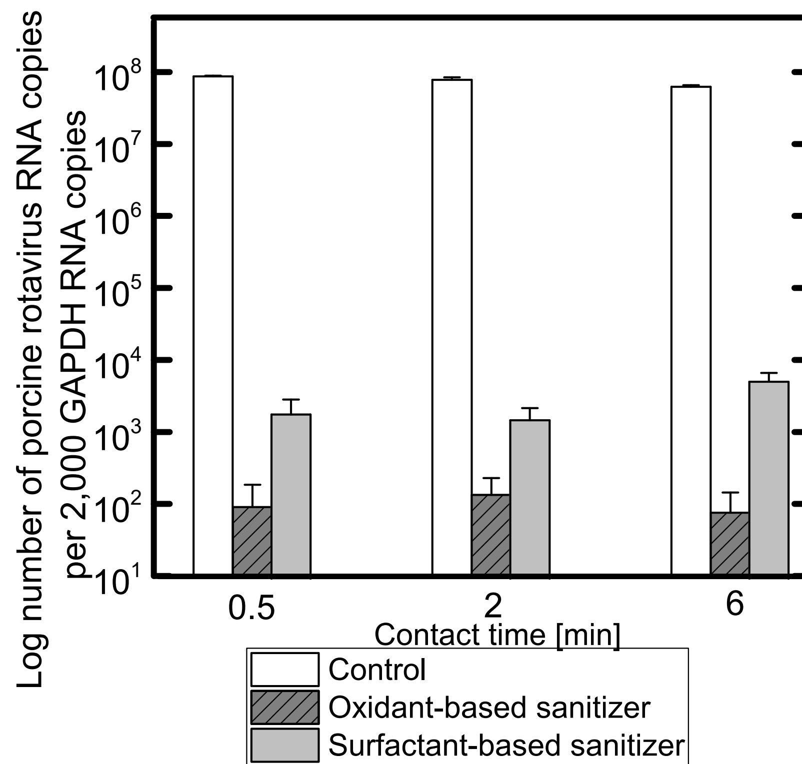

671 **Figure 4.** Comparisons of rotavirus NSP3 RNA transcript copy numbers replicated in MA104
672 cells after different sanitizer treatments. Values in the figure are the averages of results from
673 biological replicates (n=6), with standard deviations shown as vertical error bars. There was a
674 significant reduction in copy numbers in treatments with the oxidant-based sanitizer and the
675 surfactant-based sanitizer compared to the control ($P < 0.05$).

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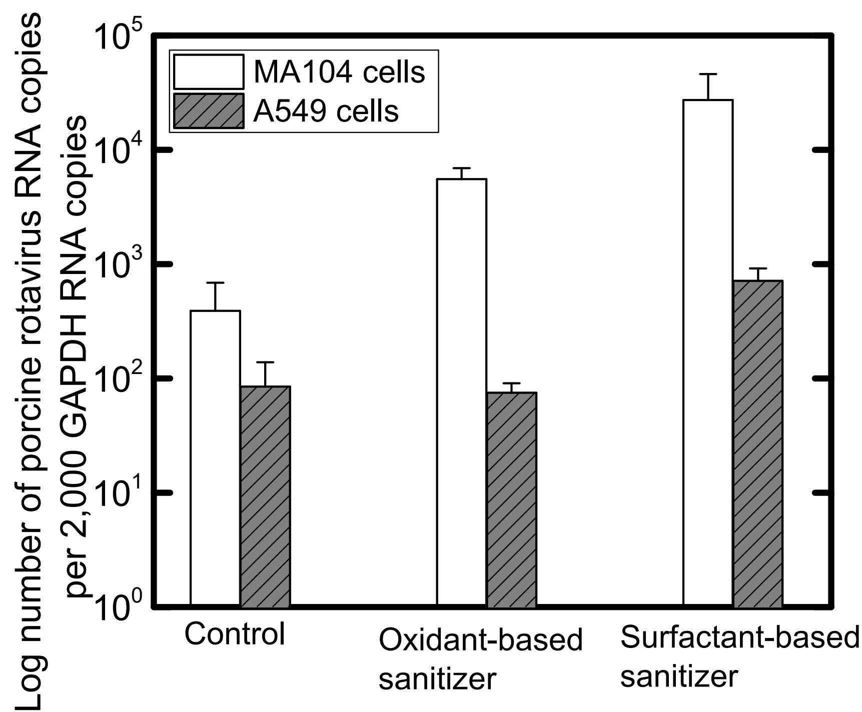

676

677 **Figure 5.** Comparisons NSP3 gene copy numbers bound to MA104 and A549 cells after
678 different sanitizer treatments. Values in the figure are the averages of results from biological
679 replicates (n=6), with standard deviations shown as vertical error bars. Viral binding to MA104
680 cells was significantly different between the control, the oxidant-based sanitizer, and the
681 surfactant-based sanitizer treatments ($P < 0.05$). Virus binding to A549 cells was significantly
682 different between the control and the treatment with the surfactant-based sanitizer ($P < 0.05$),
683 whereas there was no significant difference of viral binding to A549 cells between the control
684 and the treatment with the oxidant-based sanitizer ($P > 0.05$).

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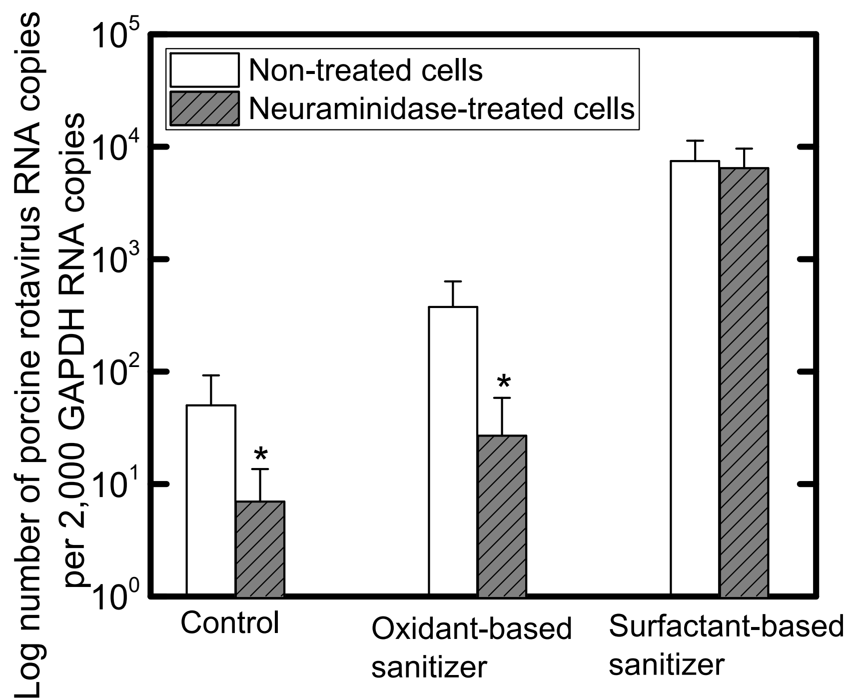

685

686 **Figure 6.** Comparisons of PRV attachment to MA104 cells pre-treated with 0 and 40.3 mU/ml
687 *Vivrio cholera* neuraminidase. PRV was treated with distilled water (control), oxidant acid-based
688 sanitizer, or surfactant-based sanitizer prior to the exposure to MA104 cells. Values in the figure
689 are the averages of results from biological replicates (n=6) at each contact time [min], with
690 standard deviations shown as vertical error bars. Statistical analyses were performed with *t* test. *,
691 $P < 0.05$.

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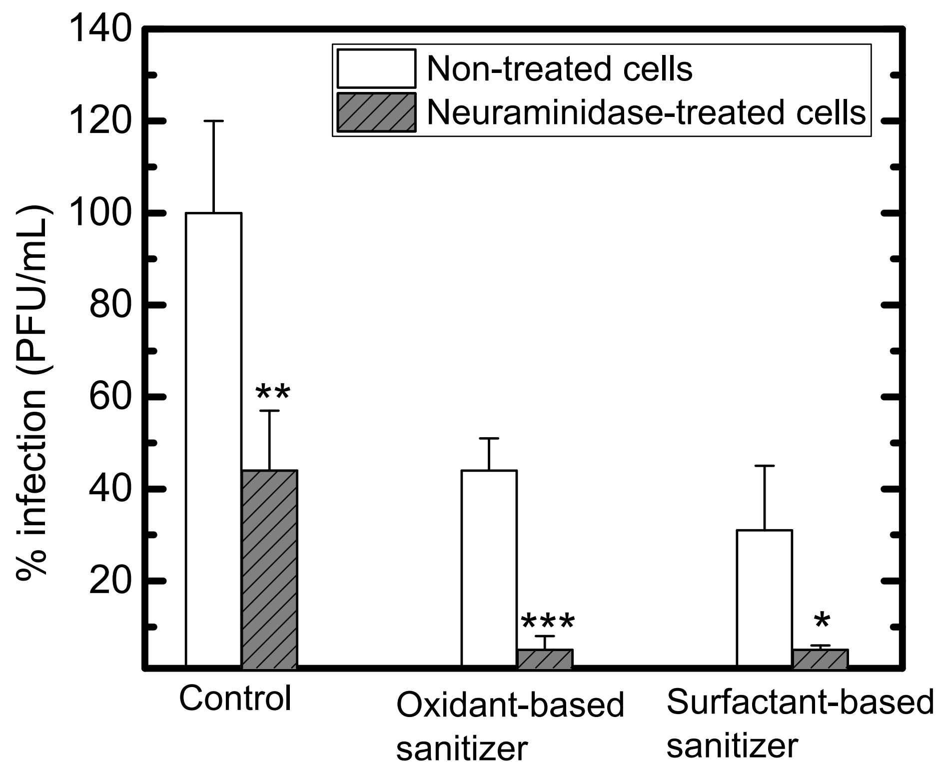

692

693 **Figure 7.** Comparisons of PRV infection to MA104 cells pre-treated with 0 and 40.3 mU/ml
694 *Vivrio cholera* neuraminidase. PRV was treated with distilled water (control), oxidant-based
695 sanitizer, or surfactant-based sanitizer prior to the exposure to MA104 cells. Values in the figure
696 are the averages of results from biological replicates (n=3) at each contact time [min], with
697 standard deviations shown as vertical error bars. Statistical analyses were performed with *t* test. *,
698 $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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701 **Table 1.** A table of the original titer, inactivated and recovered viruses for the PRV disinfection

702 experiments with sanitizers

		Virus inoculum [FFU/mL]	Inactivated viruses [FFU/mL]	Recovered viruses [FFU/mL]
Figure 1	'Totem' Belgian endive	1,000,000	755,014 ± 124,485	244,986 ± 124,485
	'Starbor' kale	1,000,000	994,500 ± 10,370	5,500 ± 10,370
	'Red Russian' kale	1,000,000	998,700 ± 733	1,300 ± 733
Figure 2	'Totem' Belgian endive	1,000,000	997,168 ± 1,180	2,832 ± 1,180
	'Starbor' kale	1,000,000	991,257 ± 5,493	8,743 ± 5,293
	'Red Russian' kale	1,000,000	987,500 ± 13,640	12,501 ± 13,640
Figure 3	Oxidant-based sanitizer	1,000,000	99,977 ± 14	23 ± 14
	Surfactant-based sanitizer	1,000,000	99,867 ± 55	132 ± 55

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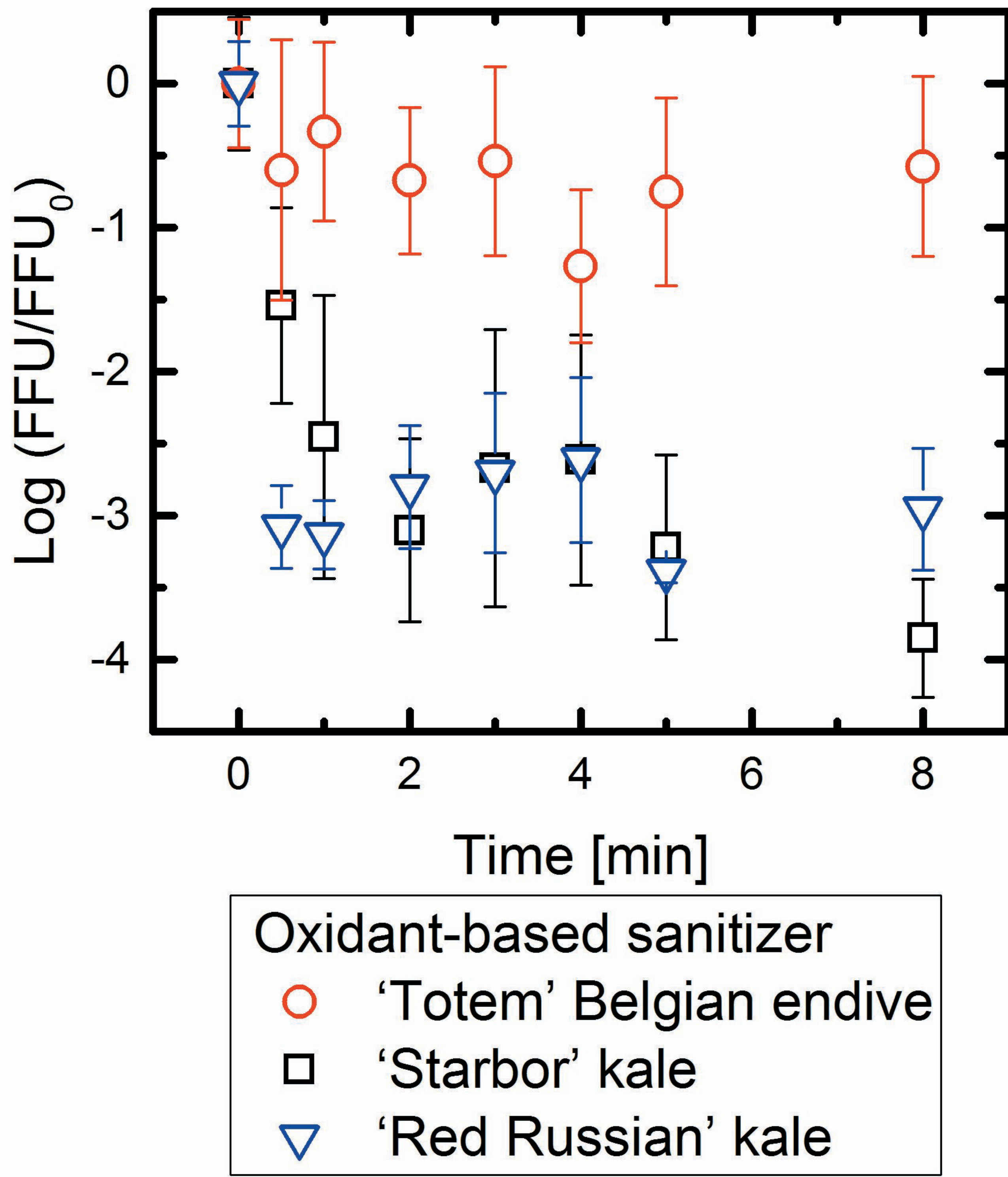

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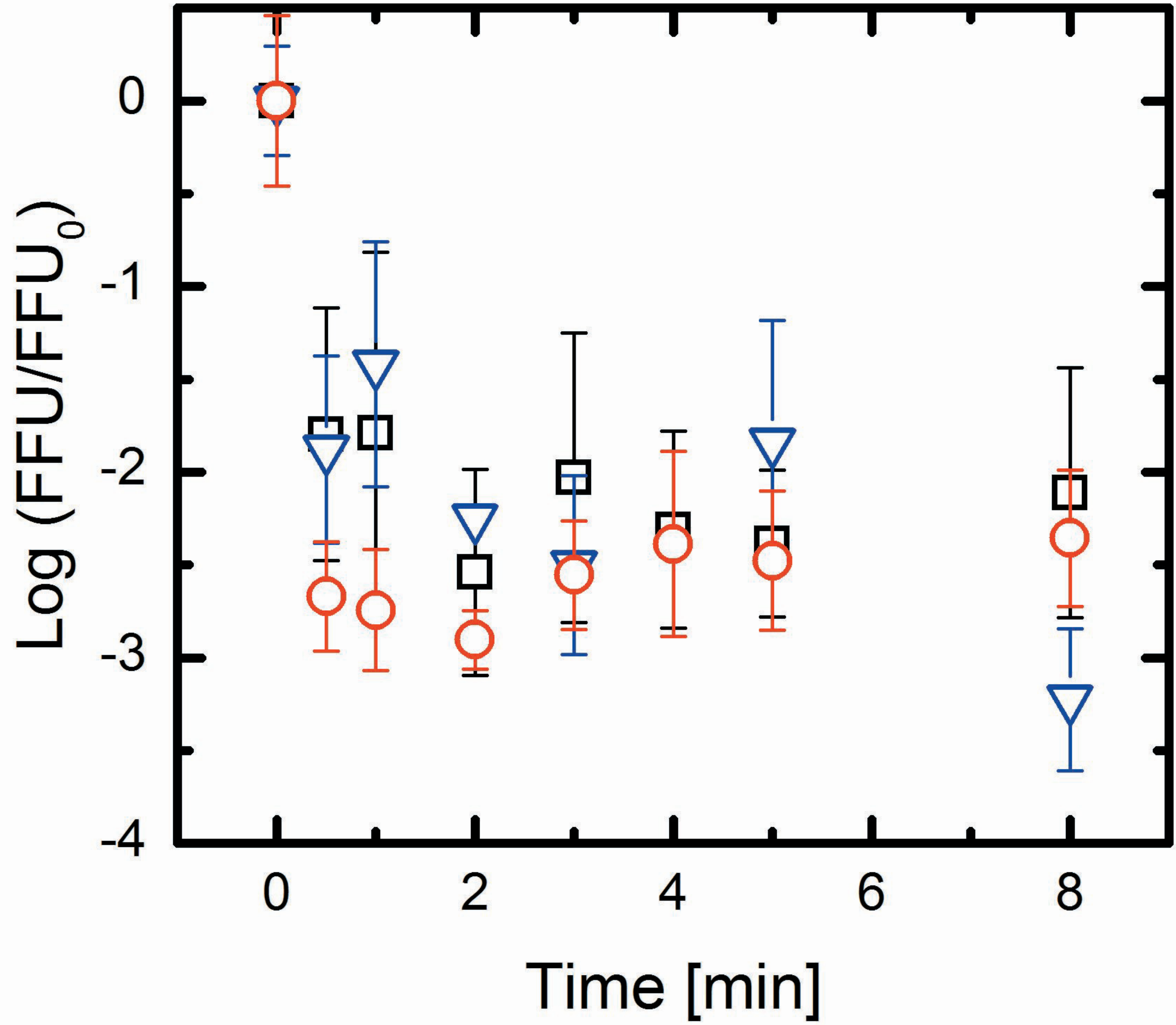

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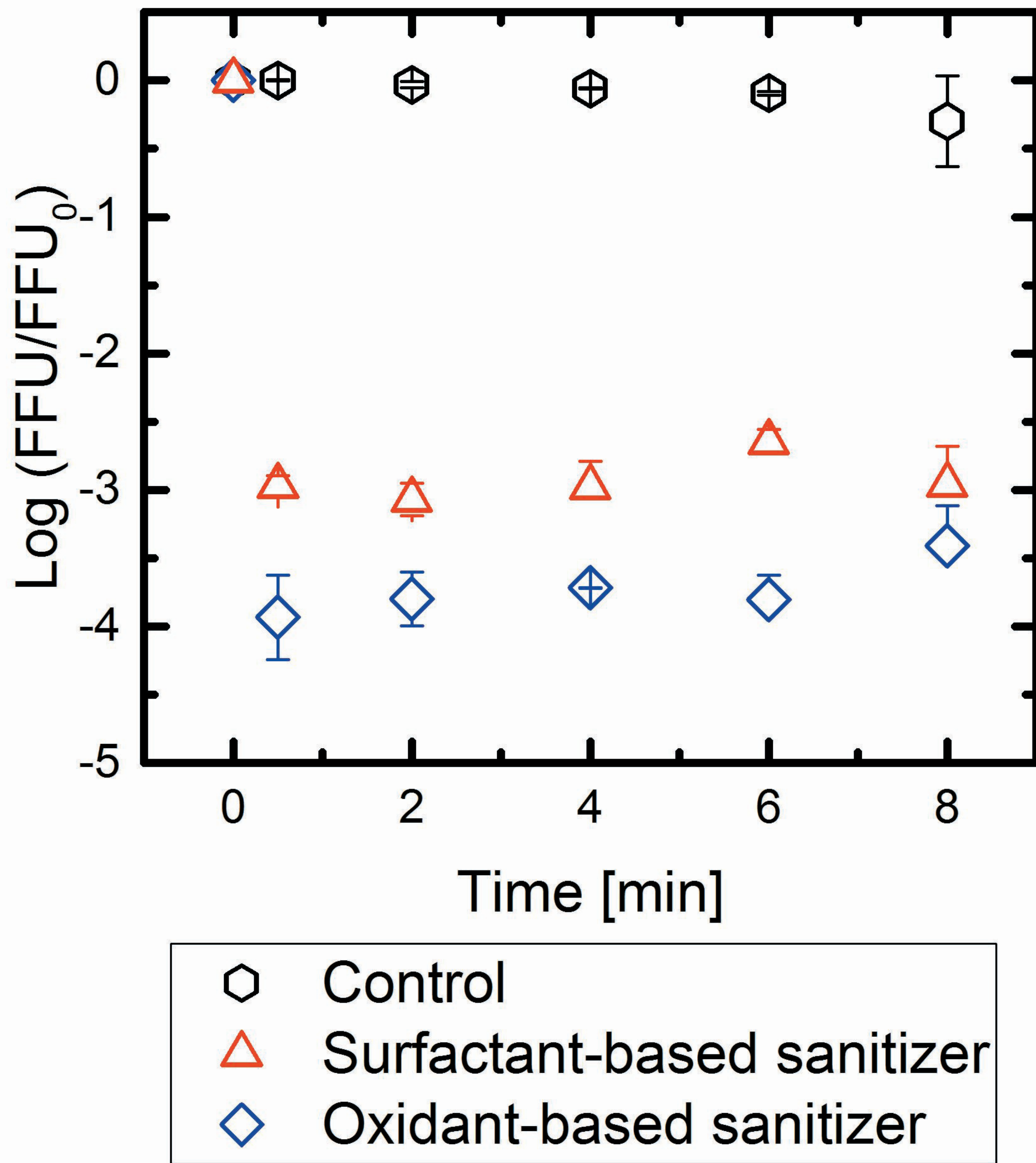

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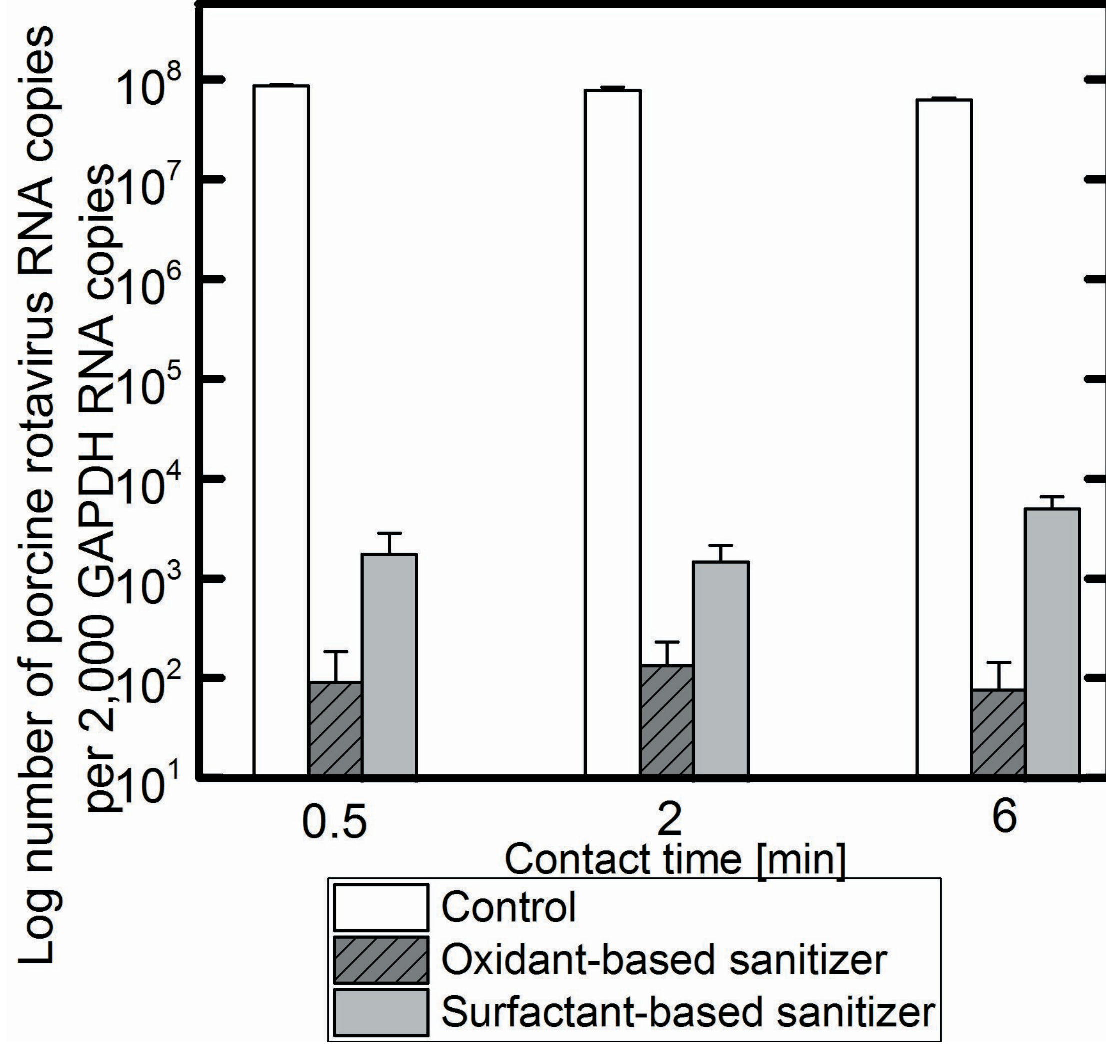

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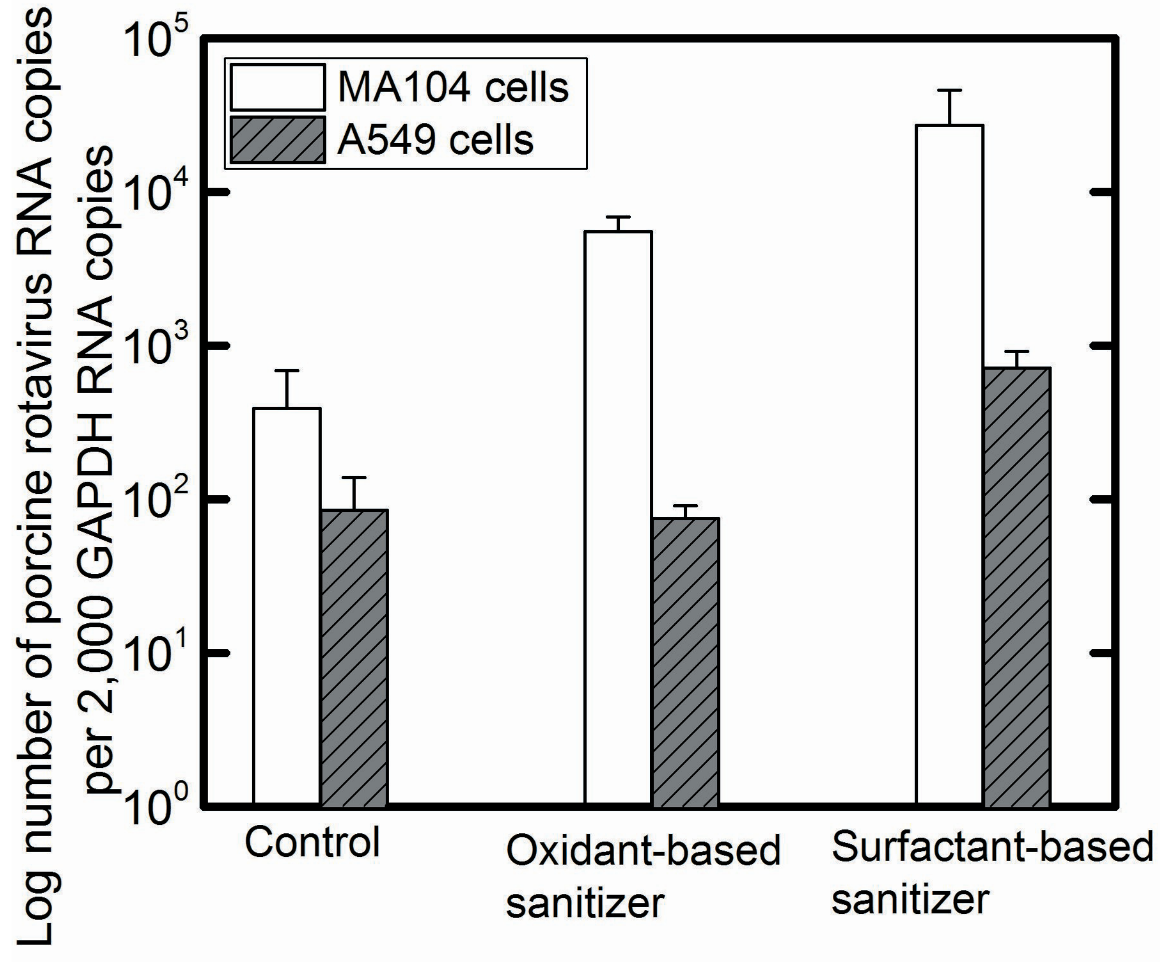

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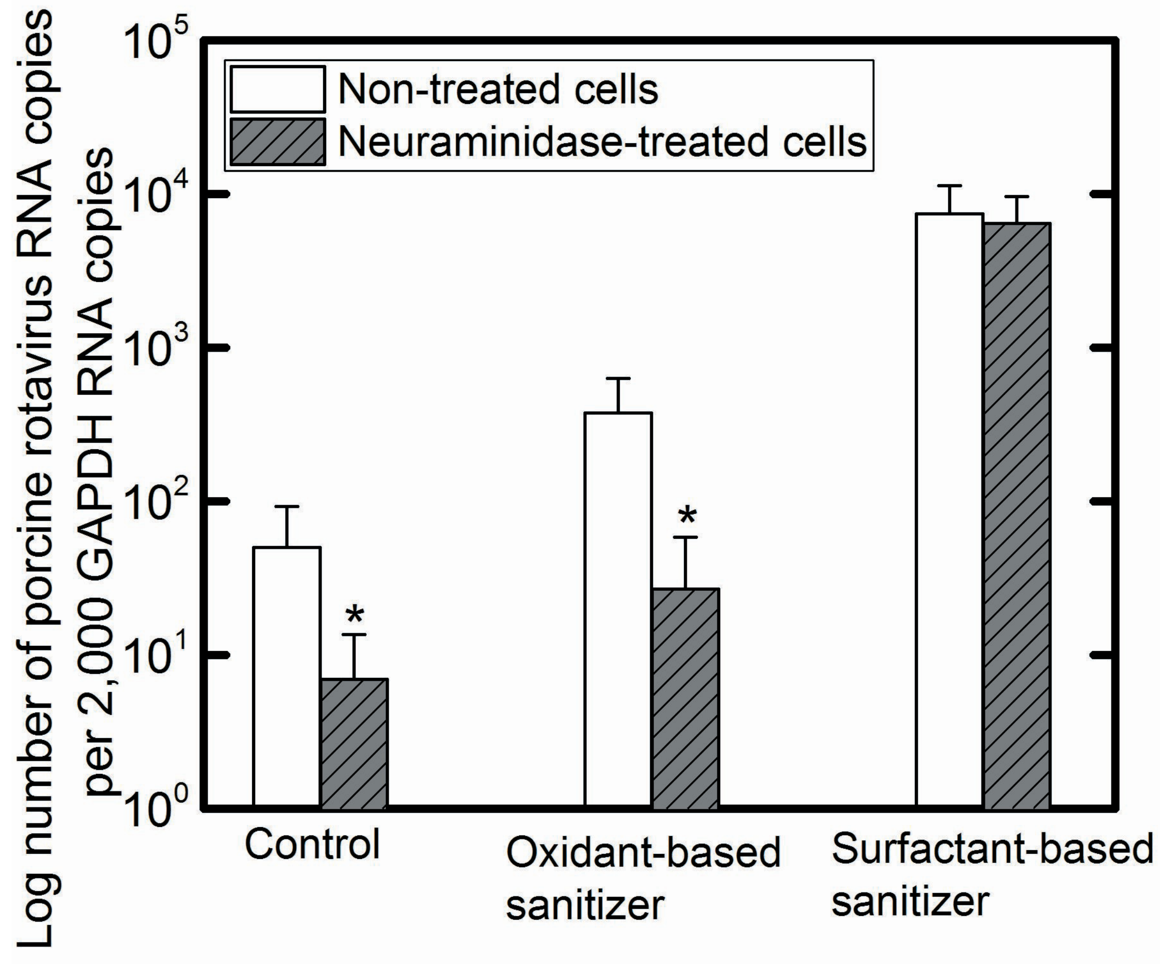

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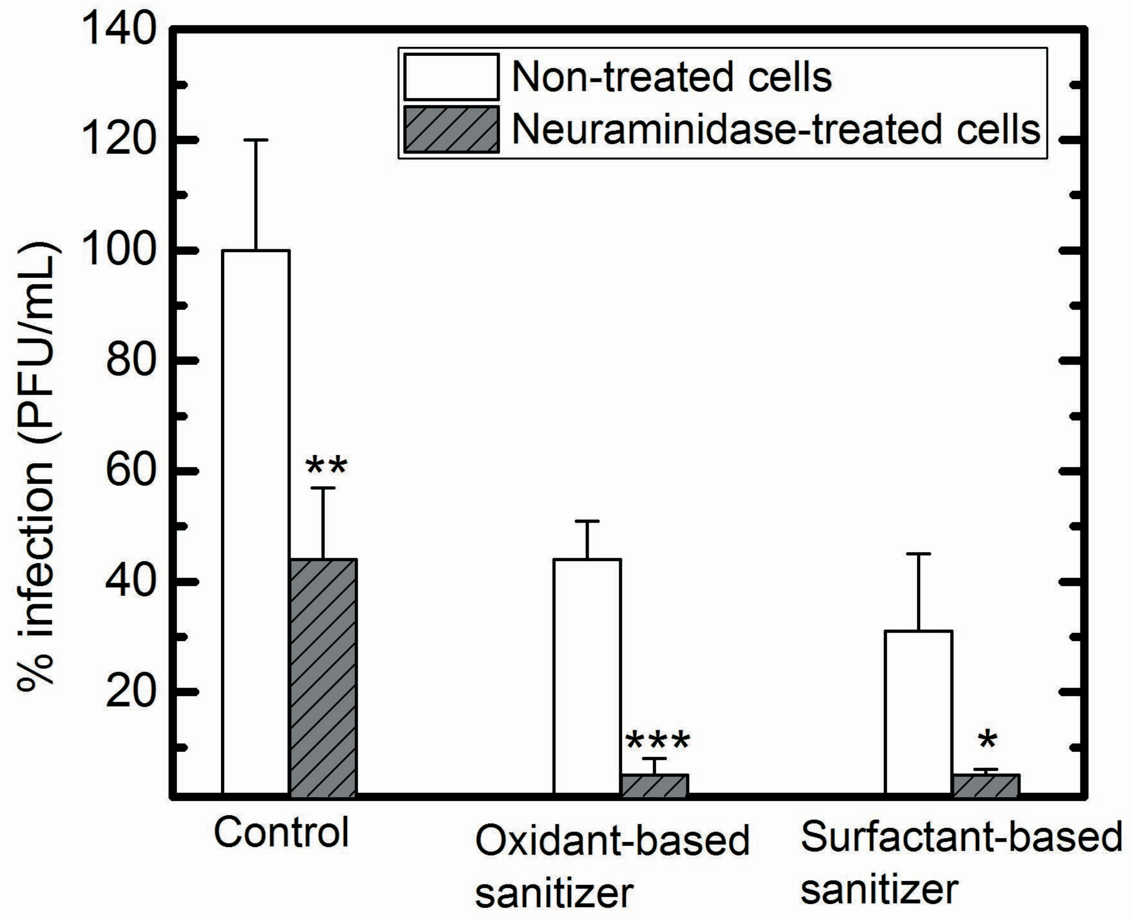

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