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Research article

SARS-CoV-2 pseudotyped virus persists on the surface of multiple produce but can be inactivated with gaseous ozone

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ABSTRACT

Due to the immense societal and economic impact that the COVID-19 pandemic has caused, limiting the spread of SARS-CoV-2 is one of the most important priorities at this time. The global interconnectedness of the food industry makes it one of the biggest concerns for SARS-CoV-2 outbreaks. Although fomites are currently considered a low-risk route of transmission for SARS-CoV-2, new variants of the virus can potentially alter the transmission dynamics. In this study, we compared the survival rate of pseudotyped SARS-CoV-2 on plastic with some commonly used food samples (i.e., apple, strawberry, grapes, tomato, cucumber, lettuce, parsley, Brazil nut, almond, cashew, and hazelnut). The porosity level and the chemical composition of different food products affect the virus's stability and infectivity. Our results showed that tomato, cucumber, and apple offer a higher survival rate for the pseudotyped viruses. Next, we explored the effectiveness of ozone in deactivating the SARS-CoV-2 pseudotyped virus on the surface of tomato, cucumber, and apple. We found that the virus was effectively inactivated after being exposed to 15 ppm of ozone for 1 h under ambient conditions. SEM imaging revealed that while ozone exposure altered the wax layer on the surface of produce, it did not seem to damage the cells and their biological structures. The results of our study indicate that ozonated air can likely provide a convenient method of effectively disinfecting bulk food shipments that may harbour the SARS-CoV-2 virus.

1. Introduction

As new prevention protocols and tools are developed, the emergence of new variants makes the fight against the COVID-19 pandemic more challenging (Walensky et al., 2021). New variants are likely harbingers for future SARS-CoV-2 mutants that will display increased transmissibility and contagiousness. Due to this risk, it is important to revisit preventative measures against COVID-19. While there are several studies that investigate the transmission of the virus through aerosol droplets and physical objects, less attention has been paid to contaminated food products as potential carriers of SARS-CoV-2 (Yekta et al., 2020; Marques & Domingo, 2020).

Viruses have recently been recognized as a significant cause of foodborne diseases in the world (Miranda and Schaffner, 2019). In the 2002-2003 coronavirus outbreak, SARS-CoV-1 was transmitted through both respiratory secretion and the fecal-oral route (O'Shea et al., 2019). Upon infecting humans, the virus spreads through respiratory droplets and contaminated surfaces (Wang et al., 2005). The principal mode of SARS-CoV-2 transmission is through exposure to respiratory particles carrying the infectious viruses. However, a lower risk of transmission occurs through contacting contaminated fomites. Research shows that the virus can remain active in the air for up to 3 h and on non-organic surfaces such as plastic for up to 72 h (Ong et al., 2020; Van Doremalen et al., 2020; Hu et al., 2021).

In the food supply chain, contamination by foodborne viruses can occur at any step, starting from pre-harvest, during the post-harvest process, throughout product transportation or distribution, and even before consumption (Yekta et al., 2020; Khaneghah et al., 2020). Indeed, food-derived fomites are a major route of transmission for at least two hepatitis viruses: Hepatitis A virus, which is responsible for the most foodborne viral infections per year, and Hepatitis E virus, which is known to be a zoonotic virus. (Di Cola et al., 2020). So far, food consumption has not been recognized as a route for SARS-CoV-2 transmission (Ma et al., 2021). But there is still ambiguity about the foodborne transmission of

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SARS-CoV-2, which requires more investigation. Unlike bacteria, mold, and yeast, viruses (including coronaviruses) are not capable of replication and growth on food surfaces (Raj et al., 2015; Rodriguez et al., 2020). However, they can survive on food surfaces or packages depending on the virus stability. Other environmental factors such as temperature, pH, relative humidity, and the presence of nearby oxidizing agents affect the virus half-life (Farahmandfar et al., 2021; Miranda and Schaffner, 2019). Potentially, foods can act as a vehicle to facilitate the virus's transmission either locally or at the global level. The issue of food cross-contamination puts agricultural workers in general and food handlers in particular at higher risk of viral infections. Applying standard disinfection procedures on food products can prevent the potential transmission of SARS-CoV-2 and other foodborne diseases.

SARS-CoV-2 belongs to the betacoronavirus (βCoV) genus from the Coronaviridae family (Fecchi et al., 2020). SARS-CoV-2 is an enveloped virus, meaning that the genomic RNA is packaged within a fatty or lipid membrane (Blanco et al., 2021). There are four proteins that form the main structure of the virus; spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Manjunath et al., 2021) (Figure 1a). The lipid membrane or envelope contains the virus proteins such as spike (S) which promotes the viral attachment to the host cell (Huang et al., 2020) and acts as a natural shield that protects the RNA genetic material. The trade-off between the stability of the outer membrane and the ability to release the RNA after entry into the host cell constrains the virus to be susceptible to oxidation and disinfection.

Chemicals such as chlorine (Thurston-Enriquez et al., 2005), chlorine dioxide (ClO_2) (Hirneisen et al., 2010), hydrogen peroxide (H_2O_2) (Khadre et al., 2001), and ethanol (Kampf, 2020) can be used for virus inactivation and disinfection purposes. However, the use of chemicals as antiviral compounds may leave residue on food and food surfaces, cause irreversible changes in the food product, affect the physio-chemical properties of food, and adversely affect workers' health. For example, chlorine may react with organic materials in food and produce potentially harmful by-products such as chloroacetic acids and trichloromethanes (Marin et al., 2020; Fan and Sokorai, 2015). Moreover, worker safety is a serious concern when working with chlorine dioxide (Karabulut et al., 2009). Physical methods such as ultraviolet (UV) radiation (Cutler and Zimmerman, 2011; Quevedo et al., 2020) thermal processing (Hirneisen et al., 2010), and high-pressure processing (Kingsley et al., 2004) are also reported to be efficient in viral inactivation. However, high-pressure processing is a limited and expensive method, and thermal processing is not suitable for heat-sensitive products such as fresh produce.

Ozone, the triatomic form of oxygen, is a natural gas with a high oxidation/reduction potential (Afsah-Hejri et al., 2020). It is a suitable alternative for chemical disinfectants in the food industry, and a residue-free fumigant for microbial control and shelf-life extension of fresh produce (Fan, 2021; Afsah-Hejri et al., 2021). However, inhaling ozone at high concentrations can cause severe health problems. Using protective equipment is strictly necessary when ozone is used at concentrations higher than the safety limits. The National Institute of Occupational Safety and Health (NIOSH) suggests the ozone exposure limit of 0.10 ppm that should not be exceeded at any time. Several studies have proved the efficacy of ozone in viral inactivation (Emerson et al., 1982; Shin and Sobsey, 2003; Thurston-Enriquez et al., 2005; Predmore et al., 2015; Brié et al., 2018; Zhou et al., 2018; Cristiano, 2020; Hu et al., 2021). For example, application of 6.25 ppm ozone for 5 min resulted in a 1.6 $\log_{10}$ reduction in Murine norovirus MNV-1on the surface of green onions, and a 2.91 $\log_{10}$ reduction on the surface of lettuce samples (Hirneisen et al., 2011). Brié et al. (2018) showed that a low concentration of ozone (3 ppm for 1 min) inactivated Murine norovirus on the surface of raspberries (>3.3 $\log_{10}$ reduction) while hepatitis A virus (HAV) on raspberries was inactivated after 3 min exposure to 5 ppm ozone.

Ozonation is also an ideal method for disinfection of packaging materials and preservation of heat-sensitive foods (Sridhar et al., 2021). Both gaseous and aqueous forms of ozonation can effectively inactivate SARS-CoV-2 (Morrison et al., 2021; Criscuolo et al., 2021). Ozone attacks the lipid envelope and spike proteins of SARS-CoV-2 (Figure 1b), disrupts the viral attachment process, and inhibits replication (Tizaoui, 2020). Clavo et al. (2020) showed that ozone can successfully eliminate SARS-CoV-2 from the surface of personal protective equipment (PPE). In another recent study, 95% inactivity was reported after SARS-CoV-2 was exposed to 0.1 ppm ozone gas for 10 h (Murata et al., 2021). Murata et al. (2021) showed a 3-log reduction for SARS-CoV-2 after 10 s of exposure to 2 mg L^{-1} aqueous ozone, meaning that a low concentration of ozone can be used as an alternative to chemical disinfectants in the handwashing process. Their study indicated that handwashing and rubbing under ozonated water for 10 s inactivated SARS-CoV-2 in large droplets of about 1μl in size produced by sneezing. They also recommended the use of low doses of ozonated water for disinfection of office areas, schools, restaurants, and food rinsing.

Through the food supply chain, fresh produce such as fruits and vegetables and bulk nuts can be contaminated with viral particles and transmit viral contamination to individuals. Given the credible possibility that the virus survives on a variety of surfaces, this study aims to assess the propensity of different food products acting as vehicles to spread SARS-CoV-2. Next, we investigate the effectiveness of ozonated air (OA) on the deactivation of SARS-CoV-2 pseudovirus present on the surface of selected fresh produce. We assess how OA may damage food surfaces and viral structures. The food samples were observed by scanning electron microscopy (SEM) and viral particles were evaluated on a capture Enzyme-Linked Immunosorbent Assay (ELISA).

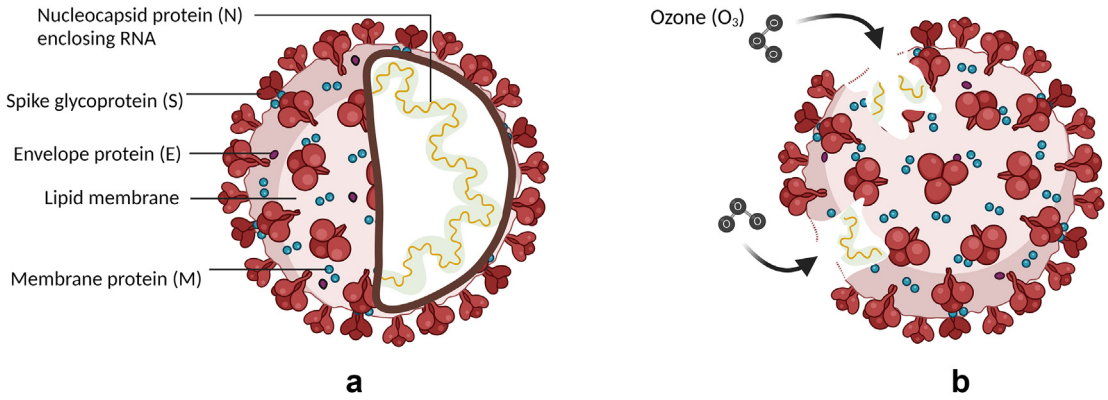


Figure 1. (a) Cartoon rendition of the SARS-CoV-2 structure (b) SARS-CoV-2 oxidized and inactivated by ozone. While the exact etiology behind ozone-based neutralization of SARS-CoV-2 and similarly structured viruses is unresolved, gaseous ozone is known to damage viral proteins, RNA, and lipids (Brié et al., 2018; Tizaoui, 2020; Bayarri et al., 2021). We aimed to depict how it is likely that ozone damages these different molecules of the virus concertedly, which leads to inactivation.

2. Experimental setup

2.1. Pseudovirus and plasmids creation

In this study, virus-associated experiments are performed in a biosafety level 2 (BSL-2) laboratory which only allows for pseudotyped SARS-CoV-2 research. Pseudovirus plasmids were obtained through BEI Resources, NIAID, NIH. SARS-CoV-2 spike pseudotyped HIV virions were produced in a protocol inspired by (Crawford et al., 2020). Briefly, 293Ft cells (Invitrogen Cat # R70007) were maintained in 293 Media (Gibco DMEM Cat # 11965-092 supplemented with 2 mM L-Glutamine, 250 μg mL^{-1} G418, 25 mM HEPES, and 10% Fetal Bovine Serum) and passaged once cells reached 70–100% confluency. The day prior to transfection, 293Ft cells were seeded at a density of approximately 2.5×10^6 cells in 8–10 mL of 293 Media into a 100 mm tissue culture Petri dish. Cells were allowed to recover for 12–16 h in a humidified incubator at 37 °C and 5% CO_2.

Petri dishes were then withdrawn from incubators and cells were assessed for confluency and adhesion. If the cells in petri dishes were 70% confluent and adherent, media was gently replaced with 8 mL of fresh 293 Media. The Petri dish was returned to the incubator for approximately 45 min while the plasmids and transfection reagent were prepared. The following plasmids were mixed in 900 μL of serum-free commercial DMEM (Gibco DMEM Cat # 11965-092) in a sterile 1.5 mL centrifuge tube: 1 μg of lentiviral backbone Luciferase-IRES-ZsGreen (BRI Resources NR-52516) vector, 0.22 μg each of vectors HDM-Hgpm2 (BEI Resources NR-52517), pRC-CMV-Rev1b (BEI Resources NR-52519) and HDM-tat1b (BEI Resources NR-52518), and 0.34 μg of vector pCMV14-3X-Flag-SARS-CoV-2 S (Addgene Cat # 145780).

After adding all plasmids, the solution was mixed by pipetting up and down approximately 10 times before 30 μL of XtremeGENE HP Version 9 (Millipore Sigma, Burlington, MA, USA) was added directly to the solution. The contents of the tube were again mixed by pipetting up and down approximately 10 times before being allowed to incubate at room temperature for 25–30 min. The Petri dish was then retrieved from the 37 °*C incubator* and the DNA- XtremeGENE HP mixture was dripped over the 293Ft cells. The Petri dish was then returned to the incubator and allowed to recover for 12–18 h overnight. After 12–18 h, petri dishes were again removed from the incubator and the cell media was gently replaced with 10 mL of fresh prewarmed 293 Medium before petri dishes were returned to the incubator. After an additional 48 h (60–66 h total post-transfection), petri dishes were removed from the incubator and the medium was gently removed and transferred to a 15 mL tube. This 15 mL tube was then briefly centrifuged at 750 RPM (105 RCF) for 3 min to pellet any large cell clumps. The supernatant was filtered through a 0.45 μm syringe filter and stored as 200 μL–600 μL aliquots in low-binding 1.5 mL tubes (ThermoFisher Cat # 90410, Waltham, MA, USA). Aliquots were stored at −75 °C until use in future assays.

2.2. Food samples

Some of the most popular food samples in California were used in this study. Food samples were chosen from three categories of 1) fruits (apple (*Malus domestica*), strawberry (*Fragaria* × *ananassa*), and grapes (*Vitis vinifera*)), 2) vegetables (tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), broccoli (*Brassica oleracea* var. *italica*), and parsley (*Petroselinum crispum* L.)), and 3) nuts (Brazil Nut (*Bertholletia excelsa*), almond (*Prunus amygdalus*), cashew (*Anacardium occidentale* L.), and hazelnut (*Corylus avellana* L.). All produce was purchased from local grocery stores. Before use, produce was rinsed thoroughly with tap water before a final deionized water rinse. Produce was then wiped down with lint-free wipes. Large produce (such as tomato, cucumber, and apple) was then sectioned into approximately 2 cm by 2 cm by 0.5 cm slices in the presence of a sterile flame. Thin produce (lettuce, parsley) was cut into approximately 2 cm by 2 cm squares. Produce not suitable for sectioning (Brazil Nuts, almonds, cashews, hazelnuts, grapes, strawberries) were split in half -or in the case of almonds, purchased as slivers-to ensure that samples did not tip over during the course of experimentation. Sections of produce were then sprayed thoroughly with 70% Ethanol and allowed to dry. For viral infectivity assays, produce was placed into a BSL2 Biosafety Cabinet (Thermo Fisher Scientific, Marietta, OH, USA) before ethanol was fully dry. The biosafety cabinet interior was sterilized with 70% ethanol and 30 min of UV-C irradiation prior to use.

2.3. Artificial contamination of produce and treatment with ozone

Sections of produce were spotted with 75 μL of SARS-CoV-2 pseudotyped virus (from frozen aliquots as described above in section 2.1). Virus solution was allowed to dry in a biosafety cabinet with an air downflow of 63 ft min^{-1} (0.320 m s^{-1}) and an air inflow of 103 ft min^{-1} (0.523 m s^{-1}) for 1 h. After samples were dry, virus was collected from surfaces by resuspending droplets in 90 μL of 293 Media. For ozone treatment experiments, produce with dried virus sample were placed into plastic Ziploc bags in preparation for transport to the gaseous ozone generator. Each sample that was treated with ozone had a corresponding matched negative control sample that was enclosed in a bag but not exposed to ozone. After samples were exposed to ozone (or solely kept in containment for control samples), samples were returned to biosafety cabinet and resuspended in 90 μL of 293 Media as described above.

Statistical analysis was performed to assess whether pseudovirus was, a) able to retain enough activity for a piece of produce to continue to be considered contaminated, and b) whether there was variability between pseudoviral survival on different surfaces. To assess whether a piece of produce is still considered contaminated, we looked for a 2-log reduction in viral activity, as inspired by existing literature (Brié et al., 2018; Tizaoui et al., 2022). We performed a bootstrapped One-tailed t-test with a significance level $\alpha = 0.05$ and 10000 re-sampling simulations. The null hypothesis asserted the mean normalized pseudoviral infectivity for the surface is greater than or equal to 0.01. To gauge whether there was a difference in the retention of virus infectivity, we performed a One-Way ANOVA with a significance level $\alpha = 0.001$. Results were then assessed with a Tukey-Kramer Multiple Comparison Test (run at an $\alpha = 0.05$ and $\alpha = 0.001$). Since a Tukey-Kramer Multiple Comparison Test is more conservative, we ran two different α values to judge the relationship between samples. High viral retention produce exhibited significant similarity to Positive Control viral infectivity for both $\alpha = 0.05$ and $\alpha = 0.001$.

2.4. Generation of ozone

A commercial corona discharge ozone generator MP-8000 110V (A2Z Ozone Inc., Louisville, KY, USA) was used to provide gaseous ozone for exposure to the samples. To control the ozone concentration, samples were kept inside a 30 L Sterilite weathertight gasket box. The ozone concentration in the box was then monitored by a BH-90A portable ozone detector (BOSEAN Inc., Zhengzhou, Henan, China). Throughout all experiments, the ozone concentration inside the box was kept within 15 ppm ± 1 ppm. For safety, we performed our experiment in a well-ventilated environment. The ozone generator along with the weathertight box were all placed under an F-103 5 Feet Benchtop Chemical Fume Hood (Jamestown Metal Products, Jamestown, NY, USA).

2.5. Virus infectivity assays

Each well of a clear 96-well cell-culture plate was coated with 25 μL poly-L-lysine (ScienCell Research Laboratories, Cat # 0413, Carlsbad, CA, USA) according to the manufacturer's instructions. The plate was returned to the 37 °*C incubator* for 1–36 h. After this time, the 96-well plate was removed from the incubator and the poly-L-lysine solution was pipetted out. Wells were then rinsed twice with 45 μL of sterile, ultrapure deionized water before being set aside in preparation for seeding. Wells were then immediately seeded with 1.5×10^4 293T-

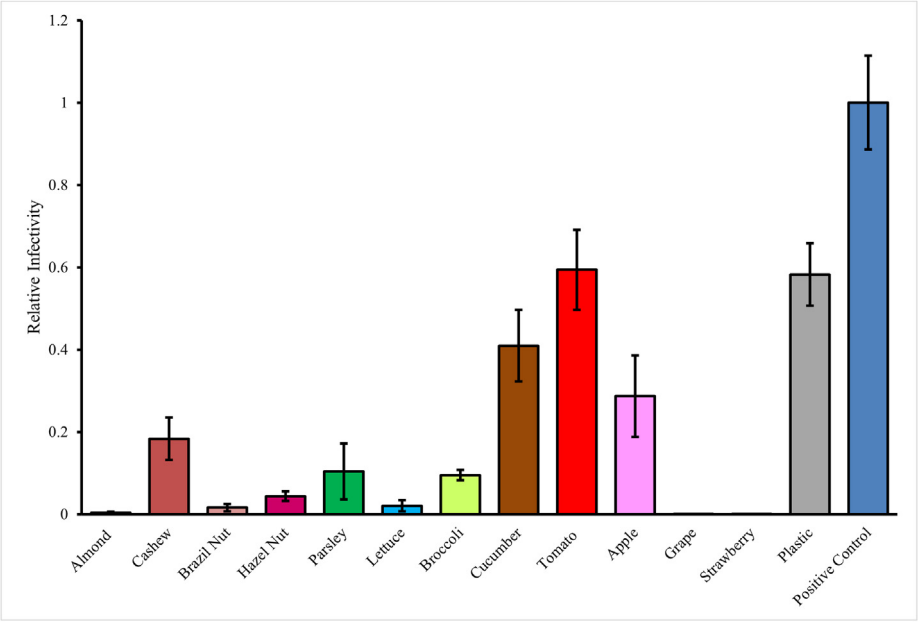


Figure 2. The relative retention of infectivity of SARS-CoV-2 pseudotyped virus after being spotted onto the surface of food samples without further eradication measures. Here, the results are normalized based on the positive control. The positive control was 25 μL of single-round virus directly added to infect susceptible cells without spotting or drying on a surface. Tomato, cucumber, and apple showed a higher rate of infectivity compared to other samples.

hACE2 cells (BEI NR-52511, Manassas, VA, USA) and the plate was returned to the humidified incubator so that cells may recover. 293T-hACE2 cells were passaged in identical conditions as 293Ft cells. After 12–16 h, the plate was removed from the incubator and the wells were verified for cell adhesion. Supernatant media was gently pipetted out and immediately replaced with 30 μL of 293t Infectivity Media (Gibco DMEM Cat # 11965-092 supplemented with 2 mM L-Glutamine, 250 μg mL^{-1} G418, 25 mM HEPES, and 1% Fetal Bovine Serum). 25 μL of virus (either directly from frozen stocks for positive control or from resuspended virus for produce tests) was then added to wells. The plate was then returned to the incubator. Each experimental infectivity condition was tested in triplicate per 96 well plate.

12–16 h later, the 96-well plate was retrieved from the humidified incubator and 150 μL of fresh, pre-warmed 293 Medium was added over the top of each well. This was to ensure that cells remained alive and viable for the duration of the experiment. The 96 well plate was returned to the 37 °*C incubator* for an additional 36–48 h. After 36–48 h, the 96 well plate was retrieved from the humidified incubator. Bright-Glo Luciferase Reagent (Promega Corp., Cat #E2610, Madison, WI, USA) was thawed out and kept wrapped in foil until use. 200 μL of the medium in each of the infectivity plate wells was pipetted out, leaving about 30 μL of the medium in each well. Then 30 μL of Bright-Glo reagent was added over the top of the wells and given 2–4 min to lyse cells. The contents of each well were then transferred to a white-backed 96-well plate and luciferase signal was read on a ClarioStar Plus microplate reader (BMG Labtech, Ortenberg, Germany) with a 3600 gain and a 1 s normalization time. All samples were run in triplicate with at least two separate biological replicates for each condition.

2.6. Electron microscope imaging

There is a concern that ozone as a strong oxidizer may damage cells and cuticles and compromise cell membrane integrity. To investigate the effect of ozone on the fruit cuticles, the surface structures of tomatoes, apples, and cucumbers were observed with the scanning electron microscope (SEM) before and after ozone treatments. Thin layers of the fruit epidermis were cut from samples (control and ozone-treated), immediately ethanol fixed, gold-coated, and observed under Zeiss Gemini SEM 500 (Zeiss, Oberkochen, Germany).

2.7. Virion capture Enzyme-Linked Immunosorbent Assay

Nunc MaxiSorp™ high protein-binding capacity 96 well ELISA plates (ThermoFisher Scientific, Cat # 44-2404-21, Waltham, MA, USA) were coated overnight at 4 °C with 100 ng of anti-SARS-CoV-2 Spike monoclonal antibody (SinoBiological Cat # 40150-D001, Beijing, China). After overnight incubation, wells were then rinsed three times with 300 μL of commercial PBS each (Gibco, PBS, pH 7.2 Cat # 20012027). Wells were then blocked with 300 μL of 3% Bovine Serum Albumin (Sigma Chemical Company Cat # A-4503, St. Louis, MO, USA) dissolved in commercial PBS. The Nunc MaxiSorp capture plate was allowed to incubate at 37 °C for 1 h.

During this time, tubes of pseudovirus aliquot (see section 2.2) were thawed at room temperature and then exposed to 15 ppm of ozone (generated by corona discharge as described in section 2.4) for either 15 min or 45 min. As a positive control, an aliquot of the virus was thawed and left capped in the fume hood to act as a non-ozone exposed control. ELISA Nunc MaxiSorp capture plate was then retrieved from 37 °*C incubator* and wells were rinsed once more with 300 μL of commercial PBS. Pseudovirus samples were then diluted tenfold with 293 Media and 50 μL of these virus samples were dispensed into wells. The plate was then incubated at 37 °C for 1 h to allow for virus capture.

After 1 h of incubation, 50 μL of PBS with 0.5% Triton X-100 (Sigma Chemical Company Cat # X100, St. Louis, MO, USA) was added to each well and allowed to sit for 5 min at ambient temperature to allow pseudovirions to lyse and release p24. After 5 min, 100 μL of PBS was added over the top. Then 10 μL of 10x XpressBio Lysis Buffer (provided in HIV-1 p24 ELISA Kit, XpressBio Cat # XB-1000, Frederick, MD, USA) was added over the top and allowed to sit for 5 min to ensure complete p24 release. 145 μL of lysed solution from each well was then transferred from Nunc MaxiSorp capture plate to an XpressBio p24 ELISA kit plate (XpressBio Cat # XB-1000, Frederick, MD, USA). The p24 capture assay was then carried out according to the manufacturer's instructions, and the levels of p24 in each well were measured on a ClarioStar Plus microplate reader set to 450 nm (BMG Labtech, Ortenberg, Germany). Data were analyzed by performing a One-Way ANOVA ($\alpha = 0.10$) comparing experimental wells to each other. Results were verified with a Tukey-Kramer Multiple Comparison Test ($\alpha = 0.10$).

3. Results and discussion

3.1. Virus survival on various produce surfaces

To evaluate the virus survival on different produce surfaces without any eradication measures, single-round virus with the SARS-CoV-2 spike protein on its surface was spotted on a variety of food items: dry rosids (almond, cashew, Brazil nut, hazelnut), asterid leaves (parsley, lettuce), cruciferous vegetables (broccoli), rosid fruits (cucumber, tomato, apple, grape), and an aggregate accessory fruit (strawberry). 75 μL of the single-round virus was spotted onto the surface of these foods and incubated for 1 h. In general, this led to the evaporation of the liquid. The spot was then rehydrated with buffer and the rehydrated virus was used to infect hACE2-bearing 293t cells to determine viral vitality. We also included an assessment of the survival of this pseudovirus on an inert, non-biological surface (plastic) so we could evaluate the degree to which the virus is solely affected by the evaporation process. As a positive control, the virus was added directly to the susceptible hACE2 transfected 293t cells from frozen aliquots without being spotted or dried.

The recovery of viral infectivity after incubation on the surface of the fruit was found to be highest on tomato, at 59.4% relative to the positive control (Figure 2). High rates of retention of viral infectivity were also observed with cucumber, apple, and cashew at 40.9%, 28.7%, and 18.3% infectivity respectively. Retention of viral infectivity of pseudovirus above 1% (the ISO-inspired recommended standard) was also found for cashews, Brazil nuts, hazel nuts, parsley, lettuce, and broccoli. As expected, the plastic surface also showed high retention of infectivity at 58.2% after 1 h of drying. (Van Dormaelen et al., 2020; Brié et al., 2018). Meanwhile, the recovery rate of the virus on the surface of almond, grape, and strawberry was under 1% (determined by a bootstrapped One-tailed t-test). The significant differences in pseudovirus survival on different produce surfaces (determined by a One-Way ANOVA) indicate that the food matrix itself affects the infectivity of pseudovirus.

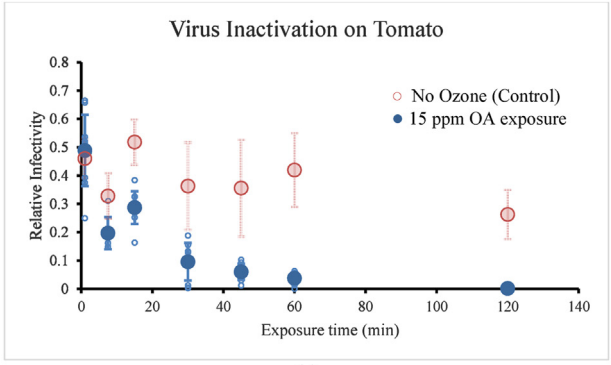
Based on our results from a Tukey-Kramer Multiple Comparison Test, we found that cucumber, tomato, and apple were the food samples with the greatest similarity in signal to the Positive Control. They had the highest retention of viral infectivity after spotting on the surface (more than 20%), and also share the trait of having a very high moisture content (96%, 94.78%, and 83.6%, respectively) (Valverde-Miranda et al., 2021; Perveen et al., 2015; USDA, 2020). The high retention of infectivity in cucumber, tomato, and apple could also be explained by the presence of the wax layer that is added to these items for retail sale. Among all produce samples, only cucumber, tomato, and apple are normally covered with a thick layer of wax to increase etheir shelf life. The wax layer may protect SARS-CoV-2 from the effects of drying and air inactivation. This wax, much like plastic, may hinder viral droplet adsorption into the matrix beneath the protein surface, which may help retain the integrity of the virion. Due to the pandemic, we did not have access to freshly harvested produce samples during the course of experimentations. Therefore, future studies should also include freshly harvested produce that does not have a wax surface.

The cashew nut also showed a high rate of retention of viral infectivity following incubation on the item's surface (Figure 2), which could be related to the high oil, protein, and carbohydrate content of the nut (48.3%, 21.3%, and 20%, respectively) (Rico, Bulló & Salas-Salvadó, 2016). According to Muniz et al. (2013), the cashew nut (cotyledon) surface is covered with starch grains and oil bodies distributed across parenchymal cells. Possibly, SARS-CoV-2 can be trapped in these oil and starch structures and be preserved from inactivation. The floral apices or buds in the broccoli head and the shriveled cuticle structure of parsley (Díaz-Maroto, Viñas and Cabezudo, 2003; Mukherjee, 2012) also seem to serve as traps for SARS-CoV-2 and reduce the inactivation induced by incubation/drying on the surface (Figure 2).

3.2. Effect of gaseous ozone on the surface survival of SARS-CoV-2

It is very important to develop an environmentally friendly and residue-free method to destroy active viruses on food samples. Chemicals such as chlorine destroy some enzymes in plant products and affect the organoleptic properties of food (Khadre et al., 2001; Praeger et al., 2017). However, ozone is a very promising compound for this purpose since it can easily be produced on-site at a low cost at almost any point in the production process. It is easily dissipated and rapidly rendered non-toxic when left in the open air. Also, several comparative studies indicated that ozone inactivates viruses faster than chlorine (Malka and Park, 2022).

The virus inactivation on the food surfaces depends on several factors, such as food surface structure, food compounds, temperature, relative humidity, and ozone concentration (Herbold et al., 1989; Pascual, Llorca, & Canut 2007; Hierneisen et al., 2011). In low organic matter foods or liquids, viruses will be inactivated rapidly by ozone (Wang et al., 2018). However, most foods are high in organic matter. Due to the high amount



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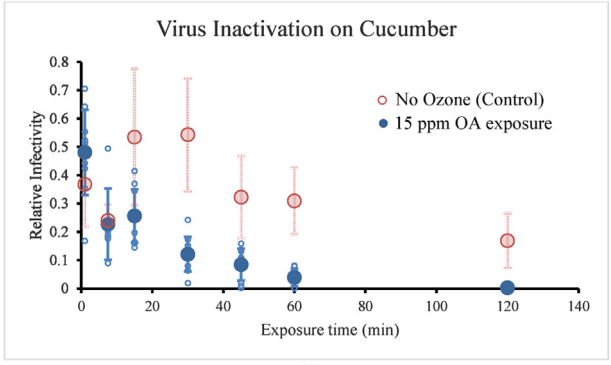

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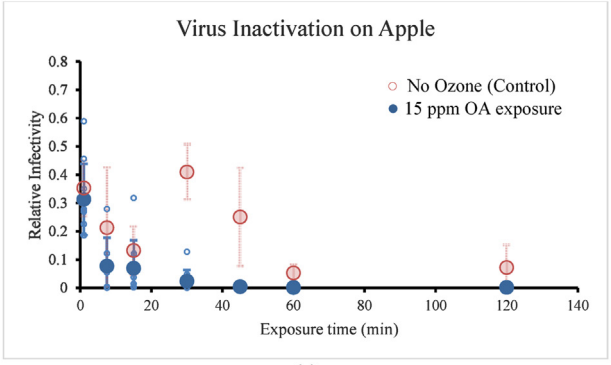


(c)

Figure 3. The relative infectivity of SARS-CoV-2 pseudotyped virus on the surface of (a) tomato, (b) cucumber, and (c) apple, in the presence (blue) and the absence (red) of 15 ppm of ozonated air (OA). Relative infectivity was obtained by normalizing values with the positive control. The positive control was 25 μL of virus from a frozen aliquot directly onto susceptible cells (i.e., virus was not spotted onto any surfaces). Samples consistently show inhibition of virus infectivity after 60 min of OA exposure.

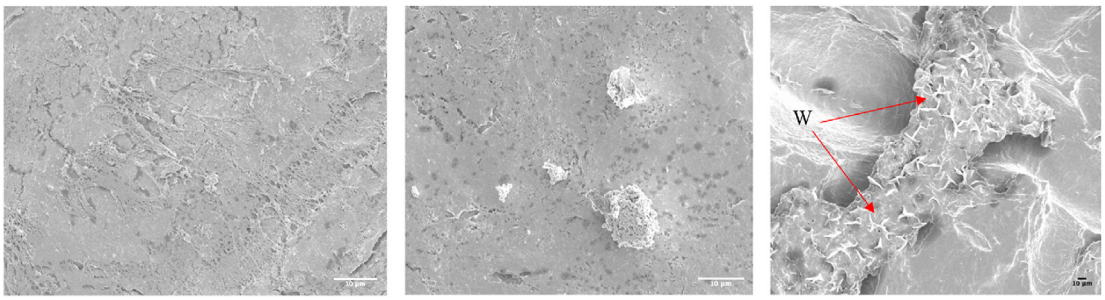


Figure 4. SEM images of apple epidermis: (a) control, (b) 20 min exposure to OA, and (c) 60 min exposure to OA resulting in irregular shaped wax platelets with undulated edges (W).

of oxidizable organic matter in food, higher ozone concentrations or longer exposure times might be required for virus inactivation. The combined effect of ozonation and other techniques such as sonication, hydrogen peroxide (H_2O_2), and pulsed electric field might increase the virus inactivation rate due to the synergistic effect.

Among our experimental food samples, cucumber, tomato, and apple provided a high pseudoviral survival after 1 h of incubation on their surface. To understand the effect of gaseous ozone on SARS-CoV-2 infectivity, we restricted the ozone exposure to these three food samples. In these experiments, 75 μL of pseudovirus was spotted onto the surface of the sample and then exposed to ozone. Figure 3 shows the relative infectivity of the SARS-CoV-2 pseudotyped virus on the surface of the three products with and without 15 ppm ozonated air over the course of 2 h of exposure. The virus was spotted onto the surface of both an experimental group and a control group of produce. The control group (red) was kept in containment, while the experimental groups (blue) were exposed to 15 ppm of ozone for the time depicted on the x-axis of each graph. The experiments were conducted under ambient conditions with the relative humidity of 50% ± 10% and a temperature of 22°C ± 2 °C. To collect the virus from the surface of both the controlled and the ozone-treated samples, viral spots were resuspended with 90 μL of 293 Media. As shown in Figure 3, for all produce samples, the ozone exposure significantly reduced the SARS-CoV-2 infectivity, with relatively no retention of infectivity after 60 min.

As shown in Figure 3, the viral infectivity tended to drop with prolonged exposure to ozone. While there is some fluctuation in the trend at ~7.5 min–30 min, the observed data variability is within the expectations for biological model systems. The expression of large mammalian proteins and the efficient packaging of viruses are known to fluctuate, even when cell lines and culture conditions are kept constant (Masters 2019; Popova et al., 2015; Tait et al., 2013). Furthermore, proteins have fluctuating lifespans within cells, which may further explain some of the observed variability in the signals for replicates of our luciferase-based assays (Toyama et al., 2013). However, even with these caveats, the differences for our results fall inside of the twofold range of data that is considered acceptable for serological assays (Reed et al. 2002; Wood and Durham 1980).

Gaseous ozone dosages of 3–300 ppm·min have been found to be effective in neutralizing several families of viruses from sterile surfaces (Brié et al., 2018; Maier et al., 2016; Hudson et al., 2009). But other viruses exhibit resistance to gaseous ozone treatment, with some retaining up to 80% infectivity after over 400 ppm·min of ozone. (Volkoff et al., 2021). In comparison, we found SARS-CoV-2 pseudovirus was moderately susceptible to ozone treatment, with 225 ppm·min leading to 48–74% inactivation. Furthermore, doses of 675 ppm·min ozone led to at least 85% viral inactivation and 1800 ppm·min guaranteed 100% inactivation of our pseudovirus. While this is a higher dose than several other viruses, it is not a prohibitively large amount of ozone, as we could

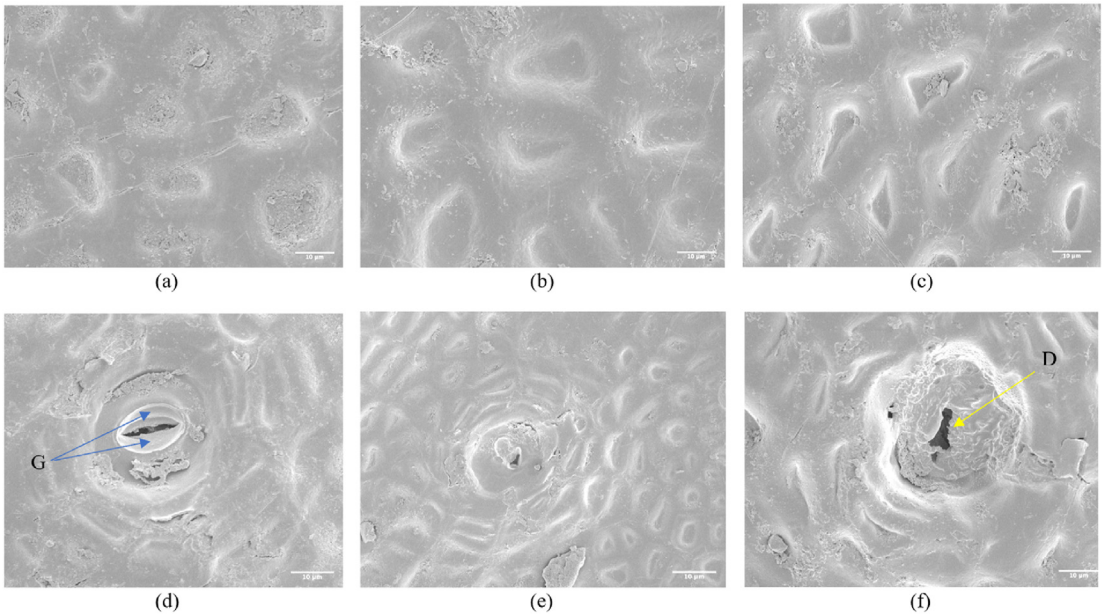


Figure 5. SEM images of cucumber epidermis: (a) control, (b) 20 min exposure to OA, (c) 60 min exposure to OA, (d) control sample open stomata and guard cells (G), (e) clogged stomata after 20 min exposure to OA, and (f) deformed stomata (D) after 60 min exposure to OA.

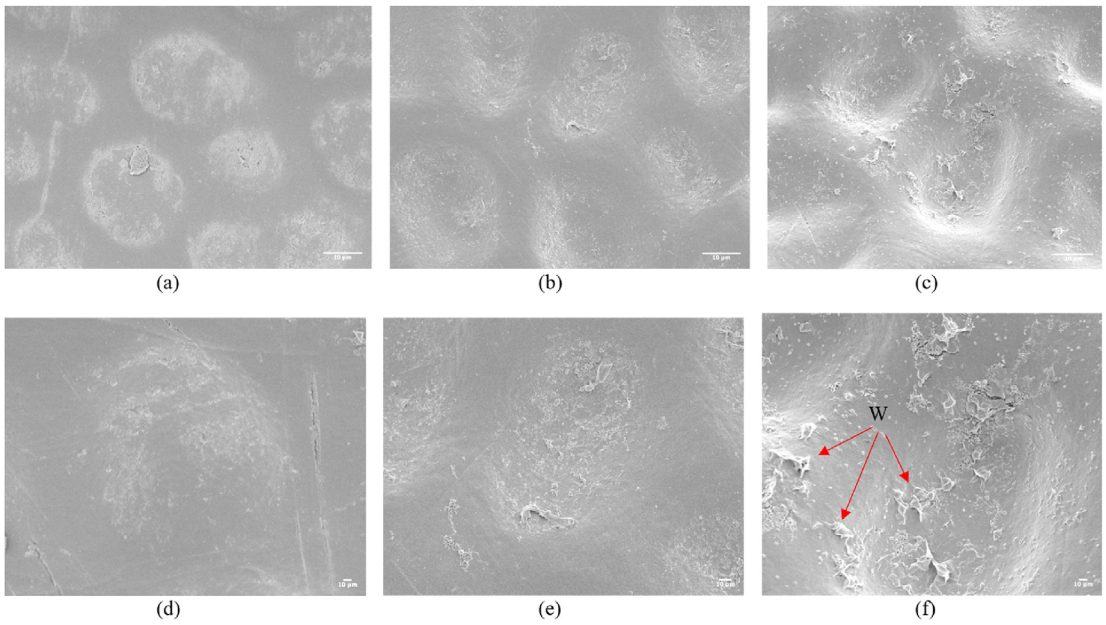


Figure 6. SEM images of tomato epidermis: (a) control, (b) 20 min exposure to OA, (c) 60 min exposure to OA, (d) closer view of the control sample, (e) closer view after 20 min exposure to OA, and (f) closer view after 60 min exposure to OA resulting in irregular shaped wax platelets with undulated edges (W).

reliably obtain 15 ppm of ozone within 3 min of turning on our commercial ozone generator.

Ozone can alter membrane permeability (Khadre et al., 2001; Zhang et al., 2011), damage the viral RNA (Roy, Chian, & Engelbrecht, 1981; Volkoff et al., 2021) or viral envelope (Herbold et al., 1989; Bayarri et al., 2021) and inactivate the virus. Due to the protective lipid layer, encapsulated (enveloped) viruses are more resistant to oxidants (such as ozone) than nonenveloped ones (Fan, 2021). In this study, 60 min of 15 ppm OA exposure inactivated SARS-CoV-2 pseudotyped virus (below 10% of initial infectivity) on selected produce. Other combinations of time and OA concentration might be needed for different food samples depending on the type and structure of the food.

3.3. Effect of gaseous ozone on the surface of produce

The fruit skin is composed of both polymeric and cellular components. The cuticle is a lipophilic polymer film constituted of wax and cutin that forms the outermost layer of the cells covering the epidermal surface of the fruit (Khanal and Knoche, 2014; Martin & Rose, 2014). This waxy cuticle provides a protective barrier against water loss, infection, and mechanical injury. Cuticular waxes consist of a mixture of very long-chain fatty acids and their derivatives (Jetter, Kunst & Samuels, 2008). To provide extra protection, the surface of some fruits and vegetables can be coated with natural waxes (such as sugarcane wax, bee wax, candelilla wax, and carnauba wax) or petroleum-based wax (such as paraffin). The wax layer used for fruits is mainly derived from plant sources (such as Carnauba wax from Brazilian palm, Candelilla wax from Candelilla shrub, and Jojoba wax from Jojoba seeds). Tomatoes are typically waxed with a paraffin-edible oil mix, while cucumbers are mainly waxed with paraffin and apples are coated with carnauba wax (de Freitas, 2019). The type of wax crystal and their level of crystallinity is different in each of these earlier-mentioned waxes.

Pores in the epidermis, called stomata, control the gas exchange and water loss in leaves and fruits by regulating the extent to which they are open or closed. Apples also have porous surface structures called lenticels that contribute to gas exchange and their high transpiration rate (Veraverbeke et al., 2003). The stomatal structure consists of the peristomatal rims and guard cells (Figure 5d). The relative rate of cuticular water loss from the surfaces around the stomatal pore (known as peristomatal transpiration) plays a significant role in the overall water loss in plants (Maier-Maercker, 1983). The fruit will retain its quality if the water loss through the stomata can be controlled. To assess whether OA damages the surface of produce, Scanning Electron microscopy (SEM) was used to observe the skin surface of the apple, cucumber, and tomato after ozone exposure.

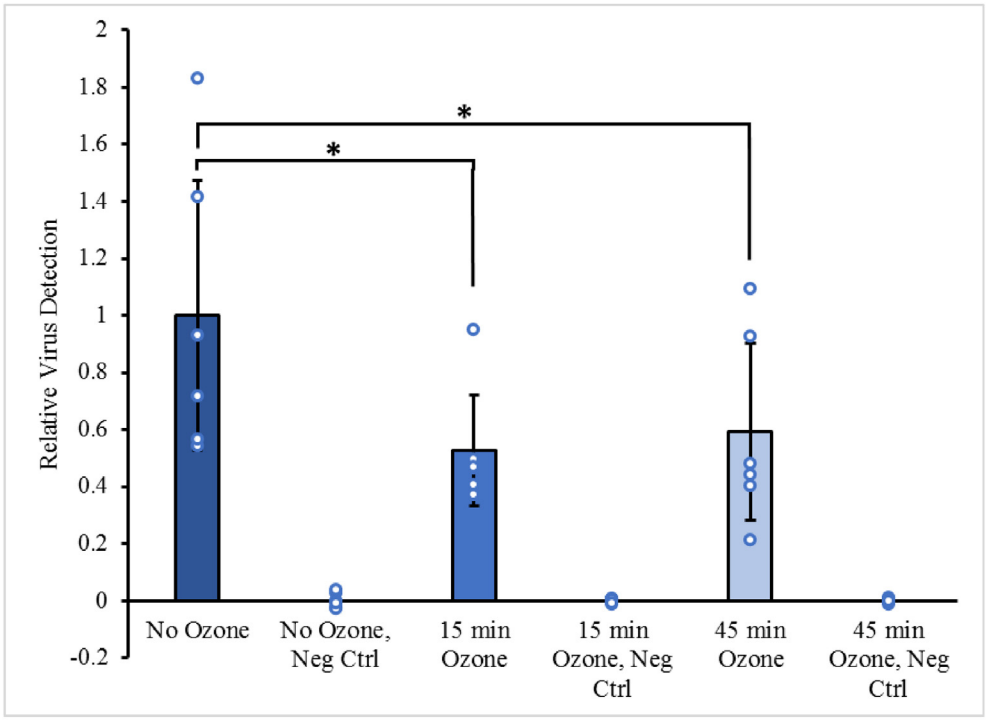
No significant visual changes were observed on the OA-treated food samples. As shown in Figures 4, 5, and 6, OA treatment did not damage cells nor affect cell membrane integrity. However, ozone treatment affected the wax layer on the skin surface of apples and to a lesser extent on tomatoes (Figures 4 and 6). Apples and tomatoes were covered with a thin layer of wax. Wax platelets with irregular shapes and undulated edges appeared on the apple epidermis after 60 min of exposure to OA (Figure 4c). Although tomato had the thickest initial wax layer (Figure 6a), the level of wax crystals deformation (Figure 6d) was less than the one in apple (Figure 4c). This could be related to the type of wax applied to each produce and describes the difference in the level of wax deformation on produce (Figures 4c, 5c, and 6c) at a specific OA treatment.

It should be mentioned that the changes in the wax layer structure are not permanent and fruit skin will resemble the original shape in the absence of ozone. Afsah-Hejri et al. (2021) showed that the irregular-shaped wax layer of OA-treated fig samples was recovered through a self-assembly process. They explained that the wax layer has a crystalline structure with recrystallization and self-assembly behavior (Barnes, Davison & Booth, 1988; Afsah-Hejri et al., 2021).

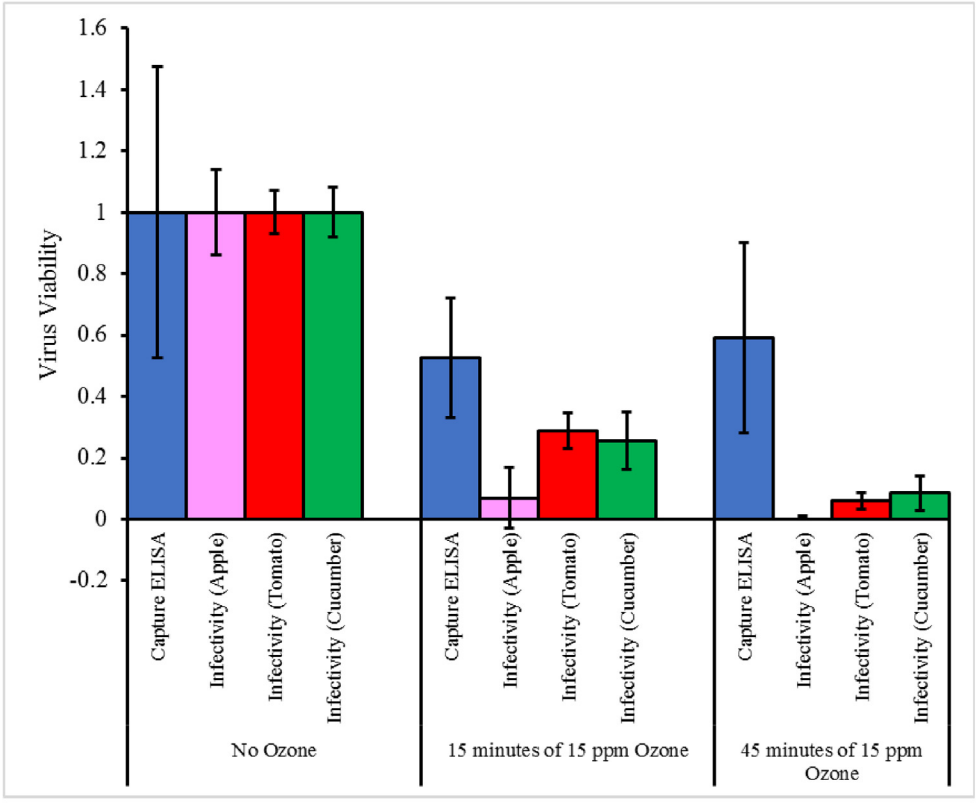
OA treatment also affected the stomata on the OA-treated cucumbers. Ozone treatment resulted in occluded or deformed stomata (Figures 5e and 5f), which could potentially reduce the water loss and weight loss in OA-treated samples. Lowering the water loss in food samples can increase their shelf-life and improve their marketability. Thus, in addition to the disinfection property of ozone, the shelf-life improvement is an additional benefit from OA treatment.

3.4. The effect of gaseous ozone on viral SARS-CoV-2 spike

We further investigated the etiology of ozone in virus load reduction of pseudotyped SARS-CoV-2 to understand whether ozone significantly damages the structure of virus-associated SARS-CoV-2 spikes. We performed a viral capture ELISA assay by coating a plate with an anti-spike antibody to determine whether that antibody was able to bind the virus



(a)



(b)

Figure 7. (a) SARS-CoV-2 spike damage as quantified by capture ELISA. Negative control wells were not coated with anti-SARS-CoV-2 spike antibody. This was done to ensure that the ozone exposure did not lead to virus samples nonspecifically interacting with plate wells or BSA. Significant differences in infectivity are shown as asterisks. We performed a One-Way ANOVA test comparing ozone-exposed samples to each other with an $\alpha = 0.10$ followed by a Tukey-Kramer Multiple Comparison Test. (b) SARS-CoV-2 spike damage (blue) versus infectivity on produce: apple (pink), tomato (red), and cucumber (green). Data show infectivity decreases to a greater extent than SARS-CoV-2 spike protein degrades during 15 ppm of OA exposure.

that had been treated with ozone. It was found that the ozone treatment had a moderate decrease in the ability of the antibody to bind the ozone-treated pseudovirus (Figure 7). After 15 min of ozone treatment, the assay shows a decrease of about 47.3% of binding by anti-spike antibody. However, continued ozone exposure doesn't appear to accumulate significantly more damage to the virus spike protein: at 45 min of ozone treatment, a time frame that almost wholly inactivates the virus on the surface of produce, the antibody is still able to successfully bind the spike protein at a level of about 40.8%. While the decrease in virus detection was statistically significant with an $\alpha = 0.10$, the rate and extent of SARS-CoV-2 spike protein inactivation were not commensurate with the ability of ozone to neutralize the virus on the surface of produce. Therefore, while the data indicate that ozone causes some damage to the viral spike or its presentation on the surface of the virus, this does not entirely explain the ability of ozone to completely inactivate the virus. This result is not unexpected, as previous studies on the effects of ozone on the RNA of virus found that viral inactivation did not necessarily correlate with damage to the virus genome (Brié et al., 2018). It is entirely possible that ozone damage to the SARS-CoV-2 spike did not entirely destroy the epitope detected by the antibody but rather resulted in a combination of effects that overall render the virus noninfectious.

3.5. Challenges

Ozone fumigation has some practical limitations despite its efficacy against viruses and the improved shelf-life that it confers to fresh produce. Ozone is not suitable for foods high in organic matter, might affect the sensory quality of food, is corrosive, and has a safety limit for safe working exposure levels. Ozonation is further affected by water purity and pH (if used in the aqueous form), and the optimum conditions vary for different food products (Afsah-Hejri et al., 2020).

The formation of unwanted and harmful compounds in food is another concern while using ozone as a disinfectant. Ozone treatment (both gaseous and aqueous) is not suitable for fatty foods as it reacts with Polyunsaturated Fatty Acids (PUFA) in food. The use of ozone in water can form hydrogen peroxide and aldehydes (Sagai and Bocci, 2011) that might react with food compounds and produce harmful products. Hence, food composition and their possible reaction with ozone must be considered before each ozone treatment.

4. Conclusion

Our results showed that several different kinds of produce could be effective media for SARS-CoV-2 pseudovirus transmission. While we tested only a few representative pieces of produce, it is likely that other popular foodstuffs could maintain significant survival of SARS-CoV-2 virions. Due to the potential connection between SARS-CoV-2 infection and food safety, it is crucial to impose specific disinfection and food handling strategies to reduce the risk of contamination. Ozone generators are relatively compact and can be easily reused, thereby providing an economical and time-efficient disinfection method suitable for large amounts of produce (Grignani et al., 2021). We tested this by selecting the three foods with the highest virus retention on our produce test (apples, tomatoes, and cucumbers) and subjecting them to neutralization tests with aerosolized ozone (OA). Our results suggest that 60 min of ozone exposure (15 ppm OA) inactivated SARS-CoV-2 on the surface of all selected food samples and did not damage cuticle cells nor affect cell membrane integrity. Virus-associated SARS-CoV-2 spike ELISA capture assays did not display ozone damage concomitant to the loss of infectivity as expected from ozone exposure infectivity assays. This was likely because ozone-mediated viral neutralization occurs by damaging a variety of biomolecules in the virions, including membrane lipids, RNA within the capsid, and other surface or capsid proteins. The accumulation of this damage to various aspects of the virus is likely what is responsible for successful disinfection and virus load reduction.

Gaseous ozone is diffusive in air and can penetrate through the underside of surfaces or areas that are obscured from direct light emission (Grignani et al., 2021). Ozone also rapidly decomposes into oxygen and leaves no toxic by-product, thereby making it a viable alternative to liquid and light-based disinfection methods (Batakliev et al., 2014; Tizaoui et al., 2020). These properties make ozone a particularly useful disinfectant for food shipments, where bulk crates of produce can be disinfected collectively with minimal need for follow-up washing or processing. Ozonation of produce can take place at the packaging plants, during storage, or transportation. We hope that the results of our study can provide some direction into developing safe food shipping and handling strategies in the context of the global SARS-CoV-2 pandemic.

Declarations

Author contribution statement

Mehrad Mortazavi, Arjan Bains: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Leili Afsah-Hejri: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Reza Ehsani: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Patricia J. LiWang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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The following reagents were obtained through BEI Resources, NIAID, NIH: Human Embryonic Kidney Cells (HEK-293T) Expressing Human Angiotensin-Converting Enzyme 2/HEK-293T-hACE2 Cell Line, NR-52511. Vector pHAGE2 Containing the Angiotensin-Converting Enzyme 2 Gene, NR-52512. SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike-Pseudotyped Lentiviral Kit, NR-52948. The following reagent was obtained from Addgene, Watertown, MA, USA: pCMV14-3X-Flag-SARS-CoV-2 S from Zhaohui Qian, Addgene plasmid # 145780. The authors would like to thank the Imaging and Microscopy Facility (IMF) and the Health Science Research Institute at the University of California, Merced.

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