Journal of Food Protection, Vol. 79, No. 3, 2016, Pages 454–462
doi:10.4315/0362-028X.JFP-15-013

Effect of Nonthermal, Conventional, and Combined Disinfection Technologies on the Stability of Human Adenoviruses as Fecal Contaminants on Surfaces of Fresh Ready-to-Eat Products

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MS 15-013: Received 8 January 2015/Accepted 29 June 2105

ABSTRACT

Over one-half of foodborne diseases are believed to be of viral origin. The ability of viruses to persist in the environment and fresh produce, as well as their low infectious dose, allows even a small amount of contamination to cause serious foodborne problems. Moreover, the consumer's demands for fresh, convenient, and safe foods have prompted research into alternative food disinfection technologies. Our study focuses on viral inactivation by both conventional and alternative nonthermal disinfection technologies on different fresh ready-to-eat food products. The use of chlorine, as well as that of nonthermal technologies such as UV light and ultrasound (US), was tested for different treatment times. UV nonthermal technology was found to be more effective for the disinfection of human adenoviruses (hAdVs) compared with US, achieving a log reduction of 2.13, 1.25, and 0.92 for lettuce, strawberries, and cherry tomatoes, respectively, when UV treatment was implemented for 30 min. US treatment for the same period achieved a log reduction of 0.85, 0.53, and 0.36, respectively. The sequential use of US and UV was found to be more effective compared with when the treatments were used separately, for the same treatment time, thus indicating a synergistic effect. In addition, human adenoviruses were inactivated sooner, when chlorine treatment was used. Therefore, the effect of each disinfection method was dependent upon the treatment time and the type of food.

Key words: Fresh produce; Human adenoviruses; Nonthermal technologies; Ultrasound; UV

Fruit and vegetables are considered important components of a healthy and balanced diet and recognized as an important source of nutrients for humans *(63, 66)*. Their consumption is encouraged in many countries by government health agencies to protect against a range of illnesses, such as cancers and cardiovascular diseases *(11, 71)*. In recent decades, the public health promotion of healthier lifestyles has led to an increased demand for minimally processed, prepacked, ready-to-eat fruit and vegetables *(31, 41)*. Thus, sales of fresh produce have significantly increased during the last decade, as consumers become increasingly concerned with healthy food and nutrition *(68)*.

Ready-to-eat products in contact with contaminated surfaces are among high-risk food products. The prevalence of viruses on different food surfaces has been linked to their high stability in the environment. In a food preparation setting, surfaces can be contaminated by food handlers with poor personal hygiene, which can lead to the transfer of the virus, such as norovirus (NoV), to various food products *(24)*. Numerous foodborne viral outbreaks have been linked to the consumption of contaminated fresh produce *(21, 22, 41, 42, 45, 51, 55, 87, 89)*. For instance, recent foodborne outbreaks in Europe were caused by NoVs present in lettuce *(31)* or hepatitis A virus (HAV) in semidried tomatoes *(36)*. The pathogens associated with environmental transmission routes, including water and food, encompass a wide diversity of bacteria, protozoa and viruses, such as NoV, HAV, hepatitis E virus, and human adenoviruses (hAdVs). Thus, the use of indicators is essential for investigating food safety and quality *(48)*. There is no strict evidence indicating which stage of the production process is the most vulnerable for virus contamination. However, in the majority of contamination cases, produce becomes contaminated on the farm during growing or harvesting *(41, 48)*. Viral contamination as a consequence of human handling can occur at any stage of food production or processing. The postharvest wash process in fresh produce processing is considered a critical control point for removing field-acquired contamination *(3, 11)*. In 2012, the number of reported outbreaks caused in Europe by the pathogenic enteric viruses increased compared with the previous year (9.3 to 13.8%). Human NoVs and HAV, transmitted by the fecal-oral route, are the main cause of nonbacterial gastroenteritis and enteric hepatitis, respectively *(28)*.

Preventive measures play an important role on an everyday basis in fresh produce industries. Good Agricultural Practices, *Codex Alimentarius,* and the Code of Good Practice for fresh fruits and vegetables are well-known for including requirements that must be fulfilled for the safe

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production of fresh produce. The implementation of disinfection technologies in the food industries' remains one of the most important aspects concerning the safety of ready-to-eat fresh products. Disinfection methods, such as chlorinated water, have been evaluated for their disinfecting capacity as far as bacteria are concerned *(8, 12, 20, 69)*. Washing tanks, filled with fresh clean water before washing starts, are common in fresh produce processing facilities. The organic matter content of the water increases during processing due to transfer of field soil, plant debris, and exudates from fresh produce *(38)*. The progressive accumulation of organic matter decreases the efficacy of chlorine by reacting with it, which makes necessary the use of higher chlorine doses to maintain a certain level of free chlorine (FC) available for microbial inactivation *(38)*. The current trend used on an everyday basis is to avoid hyperchlorination and use only the chlorine dose necessary to maintain a minimum FC level. However, even though chlorine is the most common sanitizer in the fresh-cut industry, there is a lack of information on the minimum FC residual level required for efficient water disinfection. Shen et al. *(77)* have proposed the use of less than 0.5 mg/liter of FC for over 30 s for the inactivation of *Salmonella, Escherichia coli* O157:H7, and non-O157 Shiga toxin–producing *E. coli* in lettuce. Van Haute et al. *(85)* proposed the level of 1 mg/liter of FC for the disinfection of wash water used for fresh-cut lettuce. The oxidation-reduction potential titration still remains an effective way of controlling chlorine residuals in wastewater.

However, cross-contamination during the industrial process can happen; thus, new promising methods are needed for fresh-cut industry *(89)*. Electrochemical treatment of water can reduce water consumption and wastewater discharge, as well as avoid, at the same time, cross-contamination *(52)*. Furthermore, nonthermal technologies, such as UV light and ultrasound (US), are promising alternative technologies for food disinfection *(10)*. The complete removal and inactivation of pathogens from the surfaces of fresh products are considered a challenge for the food industry.

Chlorine is the most commonly used disinfectant. The three forms of chlorine that can be used for disinfection are chlorine gas, sodium hypochlorite, and calcium hypochlorite. Sodium hypochlorite has been widely used for disinfection and is a low-cost and easily available disinfectant. Chlorine dosages of 50 to 200 ppm and contact times of 1 to 2 min on different fresh ready-to-eat products have been used to result in a 1- to 2-log reduction of different bacteria *(5, 13, 40)*.

The inactivation mechanism of UV is based on the formation of photoproducts causing lethal mutations in the DNA. Of these photoproducts, the most important is the pyrimidine dimer, which is formed between adjacent pyrimidine molecules on the same strand of DNA and can interrupt both DNA transcription and translation *(35)*. The DNA damage inflicted by UV radiation leads to lethality by directly altering microbial DNA through dimer formation between neighboring pyrimidine nucleoside bases in the same DNA strand *(89)*. UV radiation leads to direct photolysis of photolabile virus components, regardless of their solvent accessibility. A genome size–based approach to predict the sensitivity of virus strains to UV has been proposed *(57)*, although others have reported that genome size does not always correlate with virus susceptibility to UV disinfection *(80, 81)*. Enteric viruses are resistant to several processes and conditions found in the food industry, such as low pH *(62)*, treatment with UV light *(23)*, low temperatures *(7)*, and washing with and without disinfectants *(6)*.

US is defined as pressure waves, with a frequency of 20 kHz or more, which cause chemical and physical changes in biological structures (in a liquid medium) due to intracellular cavitation *(14)*. Microbial inactivation by US is mainly due to breakage of the cell walls, disruption and thinning of cell membranes, and DNA damage via free radical production *(46, 73)*. US leads to the production of cavitation bubbles, which generate high temperature and pressure at the heart of collapsing bubbles. However, microbial inactivation by cavitation is attributed to a combination of simultaneously acting mechanisms. Only mechanical effects (caused by turbulence generation, microstreaming, liquid circulation currents, and shear stress) are capable of disrupting the microbes' cell membranes *(19)*.

Nonthermal technologies are perceived to be safe, nontoxic, and environmentally friendly *(29, 49)*. Moreover, they have high efficiency, low instrumental requirements (such as UV and US), and a significantly reduced process time, and their performance is economically viable *(83)*. Many studies have been conducted for disinfection of NoV and HAV on food contact surfaces, such as stainless steel and polyvinyl chloride, and lettuce and strawberries *(33, 47, 58)*. Note that hAdVs have been proposed as indicators to enhance the control of the microbiological quality of water and food and to reduce the microbiological risks *(32, 70, 73)*. The use of hAdVs as indicators of fecal contamination has been evaluated, and its presence has been linked to that of other enteric pathogenic viruses, such as human NoV *(48)*. The feasibility of using hAdVs as indicators of human enteric viruses in environmental and food samples has also been suggested because these viruses are easily detected and seem to be more abundant and stable in the environment *(70)*.

A prolonged shelf life of fresh produce complying with microbiological regulations can be achieved by reducing the microbial load. The efficacy of washing fresh produce with tap water and water supplemented with chlorine or other chemical agents to decrease the level of viruses is known *(4)*. Typically, after harvesting by hand, fruits and vegetables can be sprayed with 50 to 200 ppm of chlorine solutions on a continuous belt *(4)*. However, currently, new nonthermal technologies have emerged. UV is a simple, reliable, and economical treatment that has the potential to be applied to whole or cut fruits and vegetables *(44, 88)*. US is based on the production of cavitation bubbles that generate high temperature and pressure *(30, 82)*. Microbial inactivation by cavitation is attributed to a combination of simultaneously acting mechanisms (mechanical and thermal effects) *(37, 71, 76)*. To the author's knowledge, there is only one study that exhibits the effect of US, as well as of combined US+UV on bacteriophages X174 and MS2 *(19)*.

The main objective of the present study was to evaluate the effect of conventional (sodium hypochlorite solutions) and alternative disinfection technologies (UV and US), as well as their combination on hAdV inactivation, on a series of ready-to-eat fresh products, to identify critical scenarios for the potential foodborne transmission of viral pathogens. The outcomes of these experiments may provide valuable information that might be considered to improve food safety regarding foodborne viral pathogens. In this study, changes in hAdV titers were determined by quantitative real-time PCR (qPCR) and infectivity assays.

MATERIALS AND METHODS

Food samples. Romaine lettuce (*Lactuca sativa* L. var. *longifolia*), strawberries (*Fragaria ×ananassa*), and cherry tomatoes (*Solanum lycopersicum* var. *cerasiforme*) were selected to study the level of viral decontamination. The fresh ready-to-eat products were purchased from a local supermarket (Patras, Greece) on the day of the experiment and stored under refrigerated conditions (4°C) until the time of the experiment.

Cell lines and viral stocks. HAdV serotype 35 stocks were cultivated in the human lung carcinoma cell line (A549). A549 cells were then cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 4,5 g/liter of D-glucose, L-glutamine, and pyruvate supplemented with 10% heat-inactivated fetal bovine serum (Gibco). A549 cells were cultured confluent (80 to 90%) in 175-cm^2 flasks at 37°C and 5% CO_2 and infected with hAdV serotype 35 (kindly donated by Dr. Annika Allard, University of Umea, Umea, Sweden). HAdVs were released from cells by freezing and thawing the culturing flasks three times. A centrifugation step at 3,000 × *g* for 20 min was applied to eliminate cell debris. The obtained supernatant was ultracentrifuged for 1 h at 34,500 × *g* and finally resuspended in phosphate-buffered saline (PBS), quantified, and stored in 10-ml aliquots at –80°C until use. The initial concentrations of hAdV stock suspensions were quantified by qPCR and calculated to be 10^8 to 10^9 genomic equivalents (GE) per ml.

Sample inoculation. All samples were rinsed with sterile water to remove some of the natural flora or any other matter before treatment. For the inoculation of the samples, the method of Birmpa et al. *(10)* was used. A spot inoculation method was applied to inoculate the hAdVs on the ready-to-eat food products. Briefly, 100 μl (10 drops) of Adeno-35, corresponding to a concentration of 10^8 to 10^9 GE/ml was spotted with a micropipette on 10 different areas of the surface of each produce. The samples weight was 25 g. After spiking, the samples with inoculum were dried in a class II biosafety cabinet (Cytair 155, FluFrance, Wissous, France) for 20 min at 22 ± 2°C to allow viral attachments prior to treatments.

Disinfection technologies. For the chlorine treatment, inoculated samples were immersed in a beaker containing 100 ml of chlorinated water (200 ppm of NaOCl, pH 6.5) and hand agitated for 1, 3, and 5 min at 22 ± 2°C. After that, samples were transferred to a beaker with sterile water and left for another 3-min period. Finally, they were placed on sterile paper for drying before analysis. At least two replicates of each treatment were performed. Selected significant inactivation ($P > 0.05$) treatment times are presented in Table 1.

TABLE 1. *Single and combined disinfection treatments*

	Treatment	Time (min)
Single-step disinfection treatments		
Nonthermal	UV	30
Nonthermal	UV	60
Nonthermal	US	30
Nonthermal	US	60
Conventional	NaOCl	3
Conventional	NaOCl	5
Conventional	NaOCl	10
Combined disinfection treatments		
Nonthermal + conventional	UV+NaOCl	30 + 3
Nonthermal + conventional	US+NaOCl	30 + 3
Nonthermal + nonthermal	US+UV	10 + 20
Nonthermal + nonthermal	US+UV	20 + 10

For UV treatment, a UV cabinet with four UV-C (Osram Germicidal G5, Munich, Germany) lamps was used. The peak emission of the lamps was 254 nm. The UV dose was the output irradiance and was delivered on one surface of the samples of the lettuce and strawberries and on both surfaces of the cherry tomatoes. The inoculated samples were placed in sterile petri dishes, at an 8 cm distance from the lamps, and treated for 30 and 60 min. The treatment was conducted at an intensity of 2 mW/cm^2 at dosages of 3.6 and 7.2 J/cm^2. Throughout the experiments, the UV-C light intensity was kept constant, and the applied doses varied by altering the exposure time at the fixed distance *(53)*. Strawberries were cut in such a way as only their equatorial zone was exposed to the UV source, and their flesh was hidden. This was chosen as the purpose of the disinfection method to disinfect the skin of the strawberry *(10)*. Lettuce and strawberries were irradiated on just one side, whereas cherry tomatoes were irradiated on both sides. At least two replicates of each treatment were carried out. Sample temperature was monitored using a K-type thermocouple attached to a Grant Data Logger (Squirrel 2040; Grant Instruments, Cambridge, UK) to ensure that at the end of the longest exposure time, the temperature was 25 ± 2°C. Selected treatment times are presented in Table 1.

For the US treatment, a 5.75-liter US tank (Elmasonic, Singen, Germany) was filled with 3 liters of distilled water and used at an operating frequency of 37 kHz and a power up to 30 W/liter. A glass beaker (600 ml) was placed in the US tank and filled with ninefold dilution of sterile water, according to the method described by Birmpa et al. *(10)*. The treatment times were 30 and 60 min. At least two replicates of each treatment were performed. The temperature was monitored and kept constant throughout the experiments to ensure that the maximum reached temperature was nonlethal to the viruses under the treatment times investigated (<50°C). Treatment times that were selected are presented in Table 1.

The combined treatments involved sequential treatments of alternative and conventional technologies (UV+NaOCl and US+NaOCl) and treatments of alternative technologies (US+UV; Table 1).

Recovery of viruses from surfaces of fresh ready-to-eat products. Samples of 25 g were processed by the method of Dubois et al. *(25),* with slight modifications, as described by Kokkinos et al. *(48)*. Approximately 25 g of treated sample were placed in a sterile beaker. The final pellet was compacted by centrifugation at 10,000 × *g* for 5 min at 4°C before suspension in

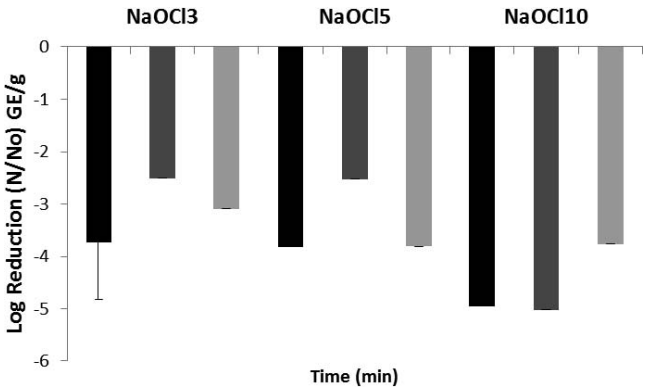

FIGURE 1. *Log reduction of lettuce (black bars), strawberries (dark grey bars), and cherry tomatoes (light grey bars) and single-step conventional disinfection treatments.*

PBS (Invitrogen, Waltham, MA) and chloroform-butanol. Finally, the aqueous phase was transferred to a clean tube and stored at –20°C until nucleic acid (NA) extraction.

Evaluation of hAdV35 infectivity. Briefly, A549 monolayers were incubated overnight in 12-well plates (Cellstar, Greiner Bio-One, Radnor, PA) at 37°C in 5% CO_2 until they reached 90 to 100% of confluence. Thirty microliters of direct and diluted samples was inoculated into each well and incubated for 90 min at 37°C on a shaking incubator (Environmental Shaker Incubator ES-20, Biosan, Riga, Latvia). Consequently, the media with inoculate were discarded and 1% fetal bovine serum supplemented with DPH (Dulbecco's modified Eagle's medium-Hepes) was added. The flasks were incubated for 3 to 4 days at 37°C in 5% CO_2. Finally, cells were observed under an epifluorescence microscope for cytopathic effect. All assays were performed in triplicate, and negative and positive controls were included.

DNase treatment. An enzymatic digestion treatment was applied in this study to reduce false-positive results by detection of free DNA when using qPCR analysis *(64)*. Each analyzed sample was treated with DNase I (RNase-Free; DNase I, Molecular Grade, Invitrogen), before DNA extraction to degrade DNA released from damaged viral capsids, according to the manufacturer's instructions.

NA extraction. NA from viral concentrates derived from fresh produce samples were extracted using a NucliSENS miniMag kit (bioMérieux, Marcy l'Etoile, France), according to previous published protocols *(48)*. A negative control was included in all NA extraction procedures. Finally, the NA eluates (100 μl) were stored at –70°C until use.

qPCR. The initial and final hAdV concentrations were quantified by qPCR, as mentioned previously. Additional quality control analyses were performed by a cultivation method *(15)*. For hAdV detection, the conserved region of the hexon gene was used as the target area. The presence of hAdV was evaluated by using qPCR. In all cases, neat and 10-fold dilutions of the virus NA extract were tested; all samples were tested in duplicate (two neat and two diluted). The qPCR assays were performed by using TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA) and a carry-over contamination prevention system, uracil *N*-glycosylase *(43)*. In each assay, 10-μl sample of NA extract was added to make a final reaction volume of 25 μl. For each plate, the GE per milliliter was measured. Ultrapure water was used as the nontemplate control for each assay. Virus assays were performed by using the conditions (primers, probes, and amplification conditions) presented by Hernroth et al. *(43)*.

Data analysis and statistics. Experiments were performed in triplicate on different days. Statistical analysis was performed with IBM SPSS 21 (IBM Corp., Armonk, NY). Analysis of variance and a pairwise *t* test were performed to compare different disinfection treatments. Results with values of $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of conventional treatments. The results obtained in the present study indicate that the type of disinfection treatment plays an important role in the stability of hAdVs in different fresh products. The initial concentrations of hAdVs inoculated on lettuce, strawberries, and cherry tomatoes were 7.82, 7.13, and 6.93 log GE/g respectively.

In the present study, uninoculated food surfaces served as negative controls. However, the log reduction of hAdVs when only tap water was used was less than 1 log GE/ml for romaine lettuce. Other studies with iceberg lettuce have found a virus log reduction of 0.69 to 1.29 log *(7)*. For this reason, washing would probably be a basic step in reducing virus numbers in food preparation.

In the evaluated experimental conditions, hAdVs were reduced when the samples were immersed in sodium hypochlorite solutions, and significant log reductions ($P < 0.05$) were observed as the treatment time of immersion to 200 ppm of sodium hypochlorite was enhanced (Fig. 1). Significantly higher reductions were recorded for lettuce and strawberries compared with cherry tomatoes. For example, after the longest exposure time, reductions of 4.95 and 5.02 log GE/g have been recorded for lettuce and strawberries, respectively, whereas a reduction of 3.76 log GE/g has been recorded for cherry tomatoes (Fig. 1). Many researchers have compared the chlorine disinfection with tap water disinfection. A treatment of 200 ppm of chlorine resulted in an additional 1.0-log reduction of murine norovirus 1 present on lettuce compared with washing in tap water *(6)*. On the contrary, the application of 200 ppm of chlorine to treat strawberries and lettuce did not result in an additional reduction of feline caliciviruses (FCV), compared with washing with tap water *(39)*. Leafy vegetables (lettuce and cabbage) treated with 200 ppm of chlorine enabled a reduction of 2.9 log of MS2 and FCV, whereas *E. coli* was reduced by 5.5 log *(2)*. Reductions of at least 1.7 log of both MS2 and HAV on strawberries, tomatoes, and lettuce treated with 20 ppm of chlorine have been observed *(16)*. Factors, such as the produce:treatment solution ratio, the presence of organic matter, inoculation method and produce type, are reported to influence the efficacy of chlorination towards viral pathogens *(9, 34, 50)*.

In general, small fruits, e.g., strawberries and raspberries destined for the fresh market are not washed because they deteriorate rapidly. Nevertheless, berries can be disinfected if they are used for further processing *(59)*.

Disinfection by-product formation in chlorine disinfection is normally associated with hours of contact time. The formation of disinfection by-products associated with chlorination has been widely studied, but little information is available on the unique high dose and short contact time that would occur *(86)*. Thus, the scope of the present study was not only to disinfect the soft fruits but also, at the same time, achieve an adequate concentration and contact time with sodium hypochlorite solution. Soft fruits, such as strawberries have the ability to internalize bacteria into their skin and are possibly less affected by disinfection methods, due to their surface structure and topography *(10)*.

Adequate chlorine levels (up to 200 ppm) would be required to achieve a 2- to 3-log reduction of viruses on fresh ready-to-eat products. The application of higher concentrations (more than 200 ppm) is unacceptable due to sensorial aspects. According to other studies, it seems inadequate to increase the efficacy of chlorination because it has been shown that a contact time beyond 10 min had little effect on antiviral activity toward FCV *(26, 39)*.

In the present study, the treatment time of 3 min significantly reduced hAdVs, as far as cherry tomatoes were concerned. An additional 0.79-log reduction was achieved after another 2-min immersion in sodium hypochlorite solution. For strawberries and lettuce, the inactivation efficiency between two treatment time intervals of 3 and 5 min was not found to be statistically significant ($P > 0.05$). However, when a 10-min treatment time was implemented, the inactivation rate was significantly enhanced ($P < 0.05$). In strawberries, for instance, a double inactivation of hAdVs (log GE per gram) was achieved, whereas, an additional 1.23-log reduction (GE per gram) of hAdVs was reported for lettuce when the treatment time was increased from 3 to 10 min. All of the previously mentioned results were confirmed by evaluating hAdV infectivity (cytopathic effect), and the results were similar to the results obtained by real-time PCR (data not shown).

Effect of alternative nonthermal treatments. As it was expected, higher UV doses resulted in higher reductions of viruses on romaine lettuce, strawberry pieces, and cherry tomatoes. Despite the known limited ability of UV light to penetrate rough food surfaces, such as lettuce surfaces, this study demonstrated that UV light has the potential to reduce viral contamination on the previously mentioned food surfaces and therefore can be used as a postlethality treatment to control pathogens in ready-to-eat foods. The type of produce treated with UV-C represents an important factor because the product surface may greatly affect the penetration and reflection of UV-C light. The UV-C irradiation is difficult to insert through thin leafy vegetable tissues, such as lettuce *(60)*. However, UV was less effective compared with other methods used in this study, such as treatment with sodium hypochlorite solutions or combined methods at reducing viral populations in lettuce. It was observed, that when the time was doubled (from 30 to 60 min), the mean reduction of hAdVs was also doubled for strawberries (from −1.26 to −3.98 log GE/g) and cherry tomatoes (from −0.92 to −2.22 log GE/g). Similar to previous results, a confirmation was performed by evaluating the cytopathic effect, and the results were similar to that obtained by real-time PCR (data not shown). Moreover, it has been shown that more irregular and complicated food surfaces are less decontaminated *(56)*. Because UV-C light has limited penetration and depth, plant morphological characteristics, such as roughness and presence of wounds on fruit surfaces, impact microbial inactivation. Understanding these influences is needed if this technology is to be commercialized *(74)*. Meng and Gerba *(61)* found a 3-log inactivation of adenovirus type 40 at a UV dose of 90 mJ/cm^2 and a 4-log reduction at 120 mJ/cm^2, whereas Thurston-Enriquez et al. *(84)* found that adenovirus type 40 requires over 150 mJ/cm^2 for 3-log inactivation and over 200 mJ/cm^2 for 4-log inactivation. Adenoviruses 1, 2, and 6 require 120 mJ/cm^2 for 3-log inactivation *(65)*. High standard deviation values have been recorded (Fig. 2) in the use of single-step nonthermal disinfection treatments and, especially, in the case of lettuce. Variation between studies can occur as a result of viral preparation methods and complexity of the adenovirus capsid.

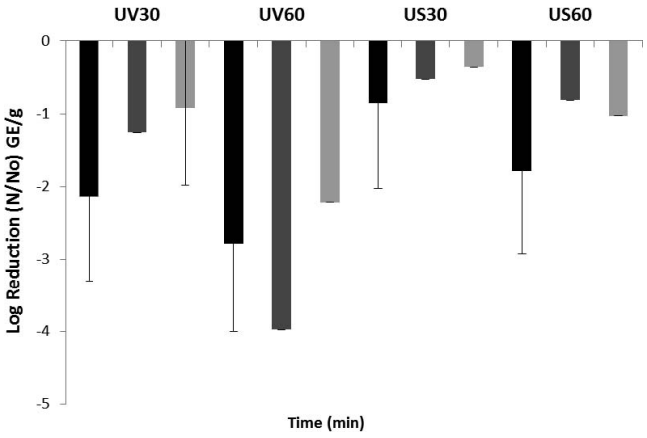

FIGURE 2. *Log reduction of lettuce (black bars), strawberries (dark grey bars), and cherry tomatoes (light grey bars) and single-step nonthermal disinfection treatments.*

Treatment with US was less effective ($P < 0.05$) compared with UV. After the longest exposure time, lettuce exhibited the greatest reduction (−1.79 log GE/g) compared with other fresh ready-to-eat products. However, the treatment time also played an important role, as far as virus reduction is concerned. Chrysikopoulos et el. *(19)* showed that the bacteriophages X174 and MS2, which were used as model viruses, were removed adequately when relatively high US frequencies (i.e., 582, 862, and 1,142 kHz) were used.

Effect of combined treatments. The synergistic or additive effect of disinfectants has been investigated in some studies *(17, 19, 54, 75, 78)* by carefully selecting the primary and secondary disinfectants and avoiding long contact times and high concentrations. However, the mechanism of action by which the combination of two disinfectants affects the disinfection action is still not clear.

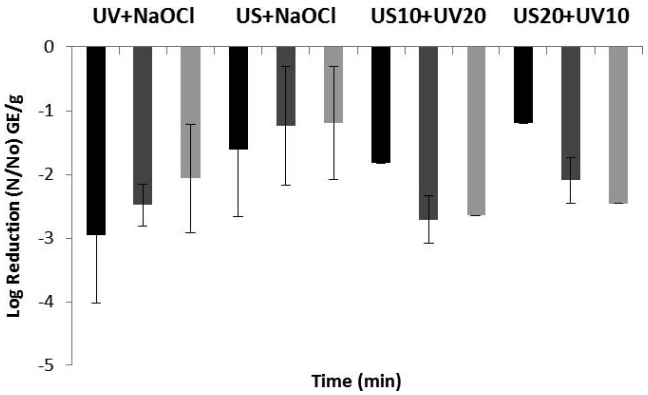

FIGURE 3. *Log reduction of lettuce (black bars), strawberries (dark grey bars), and cherry tomatoes (light grey bars) and combined disinfection treatments.*

For instance, the sequential application of ozone (or ozone and H_2O_2), followed by FC, has led to a higher level of inactivation of *Bacillus subtilis* spores than the sum of the inactivation level achieved with individual ozone (or ozone and H_2O_2) and FC application *(18)*. This enhanced inactivation is referred to as a synergism, which is beneficial because it leads to a reduction in the amount of disinfectant and reaction time, as well as to a potential decrease in the formation of disinfection by-products *(72)*.

However, there are few reports in the literature regarding the synergism involved in sequential disinfection processes employing UV or US, followed by FC *(17, 19, 79)*. Chrysikopoulos et al. *(19)* found that US+UV treatment was more effective compared with US alone for the inactivation of MS2. For X174, US and UV did not provide any synergistic effects; on the contrary, the inactivation of X174 was hindered. Therefore, the combined use of US and UV should be employed only in specific cases.

In the present study, a synergistic effect was observed when UV and US treatments were followed by immersion in sodium hypochlorite solutions. This effect was enhanced more when UV was followed by sodium hypochlorite, rather than when US was followed by sodium hypochlorite ($P < 0.05$) treatment (Fig. 3). Moreover, the sequential treatment of alternative methods exhibited more promising results compared with the combination of an alternative and a conventional treatment in strawberries and cherry tomatoes. In all cases, the sequential application of two alternative technologies depended on the time used for each method. Selected combined experiments were also confirmed by evaluating the cytopathic effect under an epifluorescence microscope, and the results were found to be similar to that obtained by real-time PCR (data not shown). The analytical approach was designed to contain a step of an enzymatic treatment by DNase I, aimed at reducing the detection of false positives by qPCR. DNase I should degrade any viral DNA that is no longer protected by the viral capsid. It could be concluded that there is a need for alternative sanitizers to be used for fresh-cut produce not only for the organic food sector but also for conventional food processors *(67)*. Thus, nonthermal technologies, alone or in combination, could offer attractive benefits to food disinfection.

Foodborne viruses are not considered in current regulations; however, contamination by viral pathogens through different steps in the production chains has been identified. Thus, viruses must be considered carefully when defining critical control points in food safety plans.

The effectiveness of all disinfection methods tested in this research was shown to be influenced by the dose of the agent, the exposure time, and the surface of the food product. Disinfection by chlorine still remains an effective method, and more experiments can be carried out by taking into account the nature of the process water (e.g., high organic matter, high turbidity, and high microbial load). The results of the present study showed that chlorine is an effective treatment; however, it can be substituted by nonthermal disinfection technologies, as these not only disinfect the food products but also do not burden human health. On the other hand, the use of chlorine is not recommended because it may generate the formation of disinfection by-products, such as trihalomethanes, and other potential carcinogenic disinfection by-products. The use of alternative disinfection technologies can also offer promising results if adequate combinations of dosage and treatment time can be achieved. However, the food appearance after 30 min of treatment with nonthermal technologies was degraded. Thus, it can be concluded that the combined nonthermal treatments (with treatment times less than 30 min) not only achieved an adequate inactivation but also resulted in a better food appearance, compared with the food appearance achieved when nonthermal treatments were used. To the author's knowledge, this study is one of the first studies that includes both conventional and alternative disinfection methods for virus inactivation in fresh ready-to-eat products. More studies are needed to better understand and clarify the mechanisms of virus disinfection on food products.

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