Nonthermal Inactivation of Norovirus Surrogates on Blueberries using Atmospheric Cold Plasma

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24 **Key Words:** Cold plasma, inactivation, Norovirus, surrogate, blueberries, food safety

ABSTRACT

Viruses are currently the leading cause of foodborne outbreaks, most of which are associated with foods consumed raw. Cold plasma (CP) is an emerging novel nonthermal technology that can be used to surface decontaminate foods. This study investigated CP technology for the nonthermal inactivation of human norovirus surrogates, Tulane virus (TV) and murine norovirus (MNV), on the surface of blueberries. Blueberries (5g) were weighed into sterile 4 oz. glass jars and inoculated with TV, 5 log PFU/g. Samples were treated with atmospheric CP for 0, 15, 30, 45, and 60s at a working distance of 7.5 cm with 4 cubic feet/minute (cfm) of CP jet. Temperature readings were taken with an infrared camera prior to, and immediately following, CP treatments. In order to establish the impact of air flow during CP treatment (4 cfm), an additional 7 cfm jet of room temperature air was introduced from a separate nozzle. The experiment was repeated with 90 and 120 seconds as additional treatment time points. Viral titers were measured immediately after each treatment with a plaque assay using LLC-MK2 cells (TV) or RAW 264.7 cells (MNV). TV was significantly reduced 1.5 PFU/g compared to the control after treatment time of 45s, which was achieved regardless of temperature conditions. With the addition of 7 cfm of ambient air, the maximum log reduction for TV was 3.5 log PFU/g after 120s of treatment. MNV was significantly reduced by 0.5 log PFU/g compare to the control at 15s, and further treatment of MNV with ambient air brought the log reduction to greater than 5 log PFU/g at 90 s of treatment (Fig 3). These results demonstrate that CP viral inactivation does not rely on thermal inactivation, and is

therefore nonthermal in nature. With further optimization, CP may be used by food processors as a means of nonthermal inactivation of foodborne viruses.

1. Introduction

Consumption of blueberries has increased significantly in recent decades due to their perceived health benefits (Zheng & Wang, 2003). However, blueberries are susceptible to contamination by biological hazards from pre-harvest to post-harvest stages and are most commonly consumed raw. Human norovirus (HuNoV) is one of the most common etiological agents that contaminates food and cause foodborne illness (Scallan et al., 2011). A European Food Safety Authority (EFSA) panel has identified that there have been 27 Norovirus outbreaks associated with berries between 2007 and 2011 (EFSA, 2014). While it is not possible to determine precisely when contamination occurred in these outbreaks, it is believed that the greatest risks are likely to occur during primary production as a result of environmental factors, use of sewage-contaminated water, worker hygiene issues, or through cross-contamination (EFSA, 2014). Currently, the preventative measures for viral outbreaks in the food supply chain primarily rely on good agricultural practices, chemical washes, and increased awareness on good hygienic practices in food handling areas (Scallan et al., 2011). However, few optimized nonthermal intervention processes are available for fresh and fresh-cut produce; hence, new intervention technologies are urgently needed.

Cold plasma (CP) is an emerging nonthermal technology that offers the advantage of being chemical- and water-free, in addition to being able to operate openly and continuously at atmospheric pressure (Niemira, 2012a). In the context of food processing and food microbiology, although this technology may contribute thermal energy to

treated commodities, it is commonly referred to as “cold”, “cool”, or “nonthermal” plasma, based on the nonthermal nature of the antimicrobial modes of action observed, rather than using terms based on plasma physics, such as “non-equilibrium” or “non-Maxwellian”; however, the terminology associated with this relatively new class of antimicrobial technologies is still being developed (Niemira, 2012a). Currently CP technologies are utilized in other industries to modify the surface energy of polymers, enhancing adhesion and printability. Recent studies have investigated the feasibility of applying cold plasma to food surfaces (Lacombe, 2015). CP has demonstrated the reduction of spoilage bacteria on the surface of blueberries with minimal effect on the organoleptic properties (Pankaj et al., 2013; Lacombe, 2015).

Due to the non-cultivability of HuNoVs, potential surrogate microorganisms that share pathological and/or biological features, such as feline calicivirus (FCV), murine norovirus (MNV), coliphage MS2, *Bacteroides fragilis* phage B40-8, etc. have been used (Cannon et al., 2012; Cannon, et al, 2006; D'Souza, 2010; Li, et al, 2013). FCV and MNV have been widely used as such surrogates (Cannon et al., 2006, Li et al., 2013). However, MNV has gradually been replacing FCV as a surrogate, due to its superior resistant to acid and heat, and its ability to persistent in the environment (Cannon et al., 2006; Li et al., 2013). The additional robustness of MNV may prove valuable to investigators, however, MNV does not bind to the same receptors needed for infection as HuNoV. For HuNoV, human histo-blood group antigens (HBGAs) are proven to be the receptors of the viruses (Huang et al., 2003) and play an important role as molecular targets for the prevention of infection. Recently, the Tulane National Primate Research Center reported the discovery of a new potential viral surrogate, Tulane virus (TV), that

recognizes the type A and B HBGAs (Farkas, 2010). Due to these characteristics and its closeness with HuNoV, TV could potentially serve as a surrogate for HuNoV. Recent studies demonstrated that TV and MNV are both stable under heat, acidic and basic conditions, and respond similarly to high pressure when inoculated on blueberries (Li et al., 2013).

Consumer demand for minimally processed fresh produce has created a challenge for processors which aim to provide safe and adulterant-free products. Conventional postharvest washing and sanitizers, such as chlorine-based treatments, can reduce cross-contamination, but are not highly effective for reducing contamination on produce, contributing less than 2 log unit reductions of pathogens, and may leave residues (Crowe, Bushway, & Bushway, 2005; Kingsley et al., 2014; Wu and Rioux, 2010). CP treatment was recently shown to inactivate potential spoilage microorganisms on blueberries without effecting firmness, color, or anthocyanin concentration (Lacombe, 2015). In addition to improving food safety, CP technologies are environmentally friendly and sustainable, as they do not require on-site storage of supply chemicals or large volumes of processing water, either for implementation or in post-treatment rinsing (Niemira, 2012a). The microbial inactivation effect of plasma treatment can be attributed to several synergic mechanisms, including the generation of UV radiation, ozone, charged particles, and oxygen radicals, in addition to other reactive species (Niemira, 2012a).

Little literature exists on CP application to reduce foodborne viruses. In studies with argon plasmas, FCV in liquid suspension was reduced by up to 5 log PFU/g after treatment times of up to 300s (Aboubakr et al., 2015). Virucidal activity was enhanced by addition of oxygen (Aboubakr et al., 2015). A study of the MS2 bacteriophage showed

that a helium/oxygen-based CP process reduced that virus by 3 log PFU/g at 120s (Alshraideh et al, 2013). Ahlfed et al. (2015) showed that HuNoV in liquid suspension was reduced by 1.69 log after 15 minutes exposure to the cold plasma of an atmospheric pressure mesh electrode system.

The objective of this work was to determine the effects of cold plasma processing parameters (*e.g.*, temperature and time) on the inactivation two HuNoV surrogates, TV and MNV.

2. Methods and materials

2.1 Blueberry Preparation

Blueberries were obtained from the local grocery store and held at 4°C until use. Prior to CP treatment, five blueberries per sample (~10g) were weighed out in sterile 4 oz. (120 ml) clear, straight-sided glass jars, 60 mm diameter, 67 mm height, and 3 mm glass thickness. Blueberries were inoculated with 500 µl of either TV or MNV and vortexed for 1 min to ensure uniform coverage. After vortexing, the blueberries were allowed to dry in a biosafety cabinet for 1 hour prior to treatment.

2.2 Cold plasma treatment

The equipment used was described by Niemira et al. (2012b). Briefly, a modified version of a Dyne-A-Mite HP AC plasma jet device (Enercon Corp., Menomonee Falls, Wis., USA) was used with an ionizing potential of 1 cm between two shaped electrodes to generate a plasma arc within a Teflon cowling (Niemira, 2012b). Ambient air feed gas set at 60 psi drove the plasma arc outward, allowing it to expand and cool. Based upon

results from previous experiments, the plasma jet was adjusted to a pulse frequency of 47 kHz, with a power consumption of 549 W. The jars (5 berries per sample) were under the CP apparatus with a working distance of 7.5 cm measured from the base of the Teflon cowl to the outside base of the jar. The jars were treated either with 4 cubic feet/minute atmospheric CP alone for 0, 15, 30, 45, 60, 90, and 120 sec, or with the CP jet and 7 cfm of ambient-temperature air, introduced via side nozzles. Average surface temperature readings for the berries were taken with an infrared camera (Fluke Ti32, Everett, WA, USA) immediately after CP treatment (Fig. 1). Limit of detection post-treatment was approximately 1.5 log PFU/g.

2.3 Virus propagation and cell lines

MNV strain (MNV-1), murine macrophage cell line RAW 264.7, TV, and monkey kidney cell line LLC-MK2 were used in these studies. MNV was propagated in RAW 264.7 cells and were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 30% fetal bovine serum (FBS, heat-inactivated, <0.05 EU/ml endotoxins), GlutaMax, and 1% penicillin/streptomycin (100 U/ml) (Life Technologies). MNV stock (approximately 6 log PFU/ml) was prepared by infecting (MOI =1) a RAW 264.1 cell monolayer (90% confluence over 150 cm²). After 1 h of incubation at 37°C under a 5% CO₂ atmosphere, 25 ml of DMEM supplemented with 30% FBS was added. MNV-1 was harvested 2 days after post-inoculation by three freeze–thawing cycles, centrifugation, and 0.22 µm filtration (Li et al., 2013).

TV was propagated in MK2-LLC cells that were cultured in M199 medium (Life Technologies) supplemented with 10% FBS (Life Technologies) and

penicillin/streptomycin (100 U/ml). The same procedures were followed to grow TV except that cells were infected with TV at a MOI of 0.1 and M199 supplemented with 10% FBS was used after 1 h incubation period. Both cells were cultured at 37 °C under a 5% CO₂ atmosphere. Virus was stored at – 80°C until use.

2.4 Viral plaque assay

After treatment with CP, the virus samples were recovered from the 5 berries per sample in 10 ml of either M199 (TV) or DMEM (MNV) through vortexing agitation on high for one min. This method ensures a 90% recovery and that the berries stay whole so that the native polyphenols do not interfere with the plaque assay. The liquid was recovered using a pipette and filtered through a 0.22 µm filter (EMD Millipore, Billerica, MA). Limit of detection post-treatment was approximately 1.5 log PFU/g. MNV-1 and TV were quantified by serial dilution plaque assay procedures by Li et al. (2013) with slight modifications. For MNV-1, 24 hour prior to infection RAW 264.7 cells were seeded into six-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of 2×10^6 (6.3 log) cells per well. After incubation, cell monolayers were infected with 400 µl virus samples in duplicate. After a 1 hour incubation at 37°C and 5% CO₂, cells were overlaid with 4 ml of minimum essential medium (MEM) containing 0.5% agarose, 30% FBS, 0.12% sodium bicarbonate, penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), 25 mM HEPES (pH 7.7), and 2 mM l-glutamine (Life Technologies). TV was quantified using a monolayer of LLC-MK2 cells at a density of 4×10^5 cells per well. After incubating for 24 hours, cell monolayers were infected with 400 µl virus samples in duplicate. Cells were overlaid with 2 ml of M199 medium, 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 µg/ml), and

amphotericin B (0.25 µg/ml), and 0.5% agarose. Plates infected with MNV were incubated for 2 days and plate containing TV were incubated for 4 days, both at 37°C and 5% CO₂ before enumeration. After incubating, plates were fixed in 3.7% formaldehyde (Fisher Scientific, Pittsburg, PA) and the plaques were visualized by staining with 0.05% (w/v in 10% ethanol) crystal violet.

2.5 Statistical analysis

Treatments were performed in three independent replications of five berries each, with two subsamples taken from each replication. Reductions of viral surrogates (log PFU/g) by CP were calculated based on the pooled data (n= 6). Mean comparisons were made between the untreated control and each treatment using ANOVA, with Fisher's least significant difference to determine significance (P<0.05).

3. Results and discussion

This is the first study that investigates the inactivation of HuNoV viral surrogates on blueberries by CP while considering the effect of temperature on reduction. The results from this study indicate that CP has the potential as a postharvest decontamination step for viruses. Previous research, using the device described herein, showed that where the plasma plume was allowed to expand, the temperature of the treated product remained well below the minimal level of thermal kill for enteric pathogens and HuNoV (viz., ca. 58-60°C) (Niemira 2012b; Bozkhurt et al, 2013). In contrast, for berries treated in the enclosed vessel used in the present study, temperatures rose above this level (Fig 2). For berries treated with CP alone, thermal damage to the berries occurred after treatments of

60s, when temperatures reached $>70^{\circ}\text{C}$. As this resulted in significant loss of mechanical integrity, the CP-alone treatments were discontinued for treatments longer than 60s. In contrast, however, introduction of ambient-temperature air via flanking nozzles effectively mitigated this thermal accumulation effect. The process, conducted at temperatures well below the level of thermal kill for norovirus surrogates (Bozkhurt et al, 2013), nevertheless, achieved significant ($p<0.05$) inactivation of viral titers. The CP process is, therefore, shown to be nonthermal in nature. TV was significantly reduced by 1.5 PFU/g compared to the control after treatment time of 45s, which was achieved regardless of temperature conditions (Fig 2). With the addition of 7 cfm of ambient air, the maximum log reduction for TV was 3.5 log PFU/g after 120s of treatment. MNV was significantly reduced by 0.5 log PFU/g compare to the control at 15s, and further treatment of MNV with ambient air brought the log reduction to greater than 5 log PFU/g at 90 s of treatment (Fig 3).

The inability to propagate HuNoV in vitro precludes direct determination of inactivation, therefore propagable genetically-related surrogates (MNV and TV) with similar biophysical properties were utilized to assess surface decontamination and virocidal efficacy. The majority of chemical disinfection studies and studies on processing interventions have been carried out using FCV and MNV (Ahlfeld, 2015; Baert, 2009). However, the susceptibility of FCV and MNV to different treatments varies considerably. FCV is less tolerant of low and high pH when compared to MNV (Cannon et al., 2006). MNV demonstrated more susceptibility to alcohol based hand sanitizers than FCV (Sattar, 2011). Also, in studies with argon/oxygen plasmas, FCV demonstrated notably higher sensitivity to CP than the surrogates used herein (Aboubakr et al., 2015).

Therefore, inferences drawn from surrogates regarding the response of HuNoV to CP are contingent on a clear understanding of mechanism of inactivation, and should be made judiciously. The present study demonstrates that MNV was more susceptible to CP treatments than TV, with MNV demonstrating significant reduction after 15 sec in comparison to TV demonstrating significant reduction at 45 sec. TV's HBGA binding properties, which are similar to HuNoV, may be important for its increased resistance to CP treatment. The inactivation mechanisms for virus disinfectants are not well-defined and, for enteric virus, there are two potential inactivation targets – the virus genome and the capsid. CP presents a complex mode of action, as it generates a combination of photoinactivating light, gaseous antimicrobials, and reactive species (Niemira 2012a). Each of these can act on both the viral capsid and genome (Niemira, 2012b). In addition, intrinsic factors such as water activity, texture, and protein and fat content can dramatically alter the antimicrobial effects of CP and the sensory properties of the treated product. The proposed mechanism in which ozone and ROS inactivate viruses involves first the damage of polypeptide chains causing a perforation in the viral capsid (Kim, and Sproul, 1980; Roy, 1981). The inactivation of viruses by ozone is rate limited by the diffusional step of ROS through the protein coat into the nucleic acid core, where damage to the RNA ultimately prevents infection (Roy et al., 1981; Kim et al., 1980).

CP can efficiently kill or inactivate yeasts and molds and other hazardous microorganisms, even spores and biofilms that are generally difficult to inactivate (Niemira, 2012a). The logarithmic reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium biofilms on agar surfaces and on food products has been demonstrated after short treatments with CP (Niemira, 2012a, 2012b). Recent studies

254 demonstrated the capability of CP as a postharvest sanitation process. When applied to
255 blueberries, native microflora were reduced immediately after treatment, and were
256 suppressed during refrigerated storage of up to 7 days (Lacombe et al., 2015). Treatment
257 conditions in that study which effectively reduced aerobic bacteria on the surface of
258 blueberries did not adversely affect texture or cause significant loss of anthocyanins
259 (Lacombe et al., 2015).

260 Contact times of 60s and 120s are standard industry practice for chemical washes during
261 blueberry line processing, and CP demonstrated virucidal effect well within those
262 parameters, regardless of surrogate (Crowe et al., 2005). The capability of CP as a
263 postharvest sanitation process was demonstrated by reducing human pathogens such as *E.*
264 *coli* O157:H7, *Salmonella*, *L. monocytogenes*, *S. aureus*, and *Shigella* spp. (Niemira,
265 2012a). Many methods for removing pathogens as well as minimizing their effect on the
266 quality of treated produce have been investigated. The efficacy of an electrostatic spray
267 of electrolyzed oxidizing water, UV light, ozone, and a combination of ozone and UV
268 light in inactivating *Escherichia coli* O157:H7 was studied on blueberries (Kim & Hung,
269 2012). Combinatorial treatments of ozone and UV had demonstrated more efficacy in
270 killing microorganisms on the calyx of blueberries than UV alone or ozone treatments
271 alone indicating a synergistic effect (Kim & Hung, 2012). CP has the potential of being a
272 more economic delivery mechanism of antimicrobial treatments, especially if ambient air
273 is used as its feed gas (Niemira, 2012a). As this technology moves closer to commercial
274 viability, further research is required to more fully elucidate the mode(s) of action of this
275 type of cold plasma system. Detailed chemical analyses, based on emission spectroscopy,
276 absorption spectroscopy, and related methods, will assist in optimization of CP for a

wider range of fresh and fresh-cut fruits and vegetables (Niemira, 2012a; Min et al, 2016). The present study demonstrates a lab-scale CP system to inactivate viruses on the surface of blueberry in conjunction with force air cooling. Previous studies demonstrated that, when using the injected cooling air, this system had no significant impact on the firmness, color, or anthocyanins concentration following minimum treatment times necessary to inactivate the viral surrogates. Optimization and scale-up to commercial treatment levels will require a more complete understanding of these chemical processes and mechanism of viral inactivation. The short time of treatment necessary for inactivation and limited deterioration of the product suggest that CP technologies may offer advantages with regard to adaptation for existing processing facilities for postharvest, prepackage, and in-package decontamination treatment of foods.

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Figure Legends:

Figure 1: Thermal images of cold plasma treatment: 4 cfm plasma jet plus 7 cfm air

Figure 2: Inactivation of Tulane virus (TV) using cold plasma (with or without 7cfm ambient air). Left axis represents viral titer. Right axis represents temperature immediately after treatment. Asterisk (*) for a given treatment time indicates significant ($P < 0.05$) difference from control. Error bars indicate standard error.

Figure 3: Inactivation of Murine norovirus (MNV) using cold plasma (with or without 7cfm ambient air). Left axis represents viral titer. Right axis represents temperature immediately after treatment. Asterisk (*) for a given treatment time indicates significant ($P < 0.05$) difference from control. Error bars indicate standard error.

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