Food Microbiology 63 (2017) 1–5

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Nonthermal inactivation of norovirus surrogates on blueberries using atmospheric cold plasma☆

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ARTICLE INFO

Article history:
Received 24 September 2015
Received in revised form
13 October 2016
Accepted 14 October 2016
Available online 19 October 2016

Keywords:
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 (5 g) 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 60 s 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 s 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.

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1. Introduction

Consumption of blueberries has increased significantly in recent decades due to their perceived health benefits (Zheng and 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 (Huang et al., 2005; 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

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http://dx.doi.org/10.1016/j.fm.2016.10.030
0740-0020/© 2016 Published by Elsevier Ltd.

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 et al., 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 et al., 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 et al., 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 et al., 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 et al., 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 (Alshraiedeh et al., 2013). Ahlfeld et al. (2015) showed that HuNoV in liquid suspension was reduced by 1.69 log after 15 min 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 (~10 g) 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 h 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 cowling 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 s, 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, Evrett,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 cm^2). After 1 h of incubation at 37 °C under a 5% CO_2 atmosphere, 25 ml of DMEM supplemented with 30% FBS was added.

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