Title:

High Hydrostatic Pressure Inactivation of Murine Norovirus and Human Noroviruses on Green Onions and in Salsa

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Abstract

In this study, high hydrostatic pressure (HHP) was evaluated as an intervention for human noroviruses (HuNoVs) in green onions and salsa. To determine the effect of water during HHP treatment on virus inactivation, a HuNoV surrogate, murine norovirus 1 (MNV-1), was inoculated onto green onions and then HHP-treated at 350 MPa with or without water at 4 or 20 °C. The presence of water enhanced HHP inactivation of MNV-1 on green onions at 4 °C but not at 20 °C. To test the temperature effect on HHP inactivation of MNV-1, inoculated green onions were HHP-treated at 300 MPa at 1, 4 and 10 °C. As the temperature decreased, MNV-1 became more sensitive to HHP treatment. HHP inactivation curves of MNV-1 on green onions and salsa were obtained at 300 or 350 MPa for 0.5 – 3 min at 1 °C. All three inactivation curves showed a linear relationship between log reduction of MNV-1 and time. *D* values of HHP inactivation of MNV-1 on green onions were 1.10 and 0.61 min at 300 and 350 MPa, respectively. The *D* value of HHP inactivation of MNV-1 in salsa at 300 MPa was 0.63 min. HHP inactivation of HuNoV GI.1 and GII.4 on green onions and salsa was also conducted. To achieve > 3 log reduction of HuNoV GI.1, HHP treatments for 2 min at 1 °C should be conducted at 600 MPa and 500 MPa for green onions and salsa, respectively. To achieve > 3 log reduction of HuNoV GII.4, HHP treatments for 2 min at 1 °C should be conducted at 500 MPa and 300 MPa for green onions and salsa, respectively.

Keywords: High hydrostatic pressure; norovirus; green onions; salsa

1. Introduction

Human norovirus (HuNoV), a leading cause of foodborne illness in the United States, affects millions of people each year and is often linked to fresh produce (Hall et al., 2012). Green onions and fresh salsa (which typically contains green onions) are commonly consumed raw and without significant food safety interventions beyond washing (Wheeler et al., 2005). From 1973-2008, HuNoV accounted for 24% of foodborne disease outbreaks associated with either salsa or guacamole in the U.S (Kendall et al., 2013). In 2003, green onions and fresh salsa containing green onions were identified as the source of an outbreak of hepatitis A virus (HAV) in Pennsylvania which led to 601 illness (Wheeler et al., 2005). In 2009, salsa was confirmed as the food vehicle for a HuNoV outbreak in California, leading to 14 illnesses (Centers for Disease control and prevention, 2016).

Currently no suitable cell culture systems or practical small animal models are available for HuNoV. The use of human subjects and gnotobiotic pig model for evaluating of a food processing technology on HuNoV inactivation is not practical due to the high cost involved (Leon et al., 2011; Lou et al., 2015). Recently an *in vitro* cell culture system for HuNoV was proposed by Jones et al. (2015) which still needs further development due to the modest viral output. There was an *ex vivo* model of human intestinal enteroids derived from stem cell recently developed for replication of HuNoV GII.3 and GII.4 strains. The study found that replication of HuNoV happened in enterocytes in human intestinal enteroids and some strains needed bile for replication (Ettayebi et al., 2016). Therefore, HuNoV surrogates, such as murine norovirus 1 (MNV-1), feline calicivirus (FCV) and Tulane virus (TV), have been commonly used to evaluate the efficacy of a food processing technology on HuNoV inactivation (Cannon et al., 2006; Chen

et al., 2005; Li et al., 2013b). Li and Chen (2015) showed that a 2-min high hydrostatic pressure (HHP) treatment at 250 MPa and 21 °C reduce MNV-1 and FCV in cell culture media by 0.1 and 2.9 log, respectively. Chen et al. (2005) found that FCV in a cell culture medium was reduced by ~ 1.7 log with a 2-min HHP treatment at 250 MPa and 21 °C. These data demonstrated that MNV-1 was more resistant to HHP than TV and FCV. However, it is unclear which virus would be the best surrogate for HuNoV since direct comparison of surrogates with HuNoV is not possible. Molecular biology methods, mostly RT-qPCR, can be used for HuNoV quantification. However, these methods can only detect the presence of HuNoV RNA, but cannot distinguish between infectious and non-infectious virus particles. To overcome this limitation, Tian et al. (2007, 2008, 2010) pretreated HuNoV samples with porcine gastric mucin (PGM) before subjecting them to RT-qPCR quantification. PGM shares similar chemical and antigenic characteristics with histo-blood group antigens which are able to bind to HuNoV and are key factors in HuNoV infection of human B cells (Jones et al., 2014; Marionneau et al., 2002). The pretreatment of HuNoV with PGM was used in their studies to bind and collect potentially infectious HuNoV, and exclude presumably inactivated virions unable to bind to PGM. After the pretreatment with PGM, Tian et al. (2007, 2008, 2010) used RT-qPCR to quantify the potentially infectious HuNoV bound by PGM. The utility of the PGM pretreatment followed by RT-qPCR assay (referred to as PGM/PCR assay) for discriminating potentially infectious HuNoV was further demonstrated by Dancho et al. (2012) who showed that thermal, UV, and HHP treatments result in loss of PGM binding consistent with inactivation of the virus by damage to its capsid. Li and Chen (2015) later compared HHP inactivation results of MNV-1 and TV obtained through the PGM/PCR assay and plaque assay and concluded that the PGM/PCR assay would be very likely able to estimate HHP inactivation of HuNoV at ≤ 2 log-reduction levels. They also showed

that the order of HHP resistance for the virus strains used in their study was probably HuNoV GI.1 > MNV-1 > HuNoV GII.4 > TV, indicating that MNV-1 is probably a good surrogate for the GII.4 strain, but not a good surrogate for the GI.1 strain. A recent study by Lou et al. (2016) showed that even within the same GII genogroup, different HuNoV strains had different sensitivity to HHP with the order of GII.1 > GII.6 > GII.4, making it difficult to select an appropriate HuNoV surrogate for HHP.

HHP is a nonthermal processing technique which exposes foods to a high level of isostatic pressure, usually at 200 – 600 MPa (Cheftel, 1995). HHP has been used for a wide variety of food products, such as oysters, juice, jam, ready-to-eat meats, guacamole and salsa (Bermúdez-Aguirre and Barbosa-Cánovas, 2010; Oey et al., 2008; Torres and Velazquez, 2005). It has been shown that HHP can denature proteins while leaving small molecules, such as flavor and aroma compounds, intact (Bermúdez-Aguirre and Barbosa-Cánovas, 2010). HHP also has been identified as a potential intervention for HuNoV and HAV in various fresh produce foods, such as blueberries, strawberries and lettuce (Huang et al., 2014; Huang et al., 2016; Kingsley, 2013; Kingsley et al., 2005; Li et al., 2013b; Lou et al., 2011). Hirneisen and Kniel (2013) showed that a 2-min HHP treatment at 500 MPa and 20 °C could reduce MNV internalized in green onions and on the surface of green onions by > 4.7 and > 6.4 log, respectively. It was shown that a 5-min HHP treatment at 375 MPa could reduce 4.75 log of HAV on sliced green onions (Kingsley et al., 2005). Lou et al. (2011) showed that > 5 log reduction of MNV-1 on lettuce was achieved with a 2-min HHP treatment at 400 MPa and 4 °C. In salsa, it was shown that a 1-min HHP treatment at 400 MPa and 9 °C could reduce MNV by > 7.0 log in salsa (Hirneisen et al, 2014). Using the PGM/PCR assay, Li et al. (2013a), Ye et al. (2014) and Huang et al. (2016) demonstrated that HHP could inactivate HuNoV GI.1 and GII.4 in various foods.

For example, Li et al. (2013a) demonstrated that a 2-min HHP treatment at 600 MPa and 2 min could inactivate HuNoV GI.1 by > 3.0 log on blueberries with presence of surrounding water. Ye et al. (2014) showed that a treatment at 450 MPa for 5 min at 1°C achieved a > 4 log reduction of HuNoV GI.1 in both oyster and clam homogenates.

The objectives of this study was to determine the HHP inactivation effect of MNV-1, HuNoV GI.1 and GII.4 strains on green onions and in salsa and to identify practical HHP parameters for processing green onions and salsa.

2. Material and methods

2.1 Virus and cell lines

Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA kindly provided the HuNoV GII.4 strain in fecal suspension used in this study. The information of the HuNoV GI.1 strain (8FIIb) used in this study was described by Leon et al. (2011). Fecal suspensions were centrifuged at 4000 ×g for 20 min, filtered through a 0.22-μm filter, aliquoted, and stored at −80 °C until use.

MNV-1 and murine macrophage cell line RAW 264.7 were generously provided by Dr. Jianrong Li at the Ohio State University. RAW 264.7 cells were cultured in DMEM (Gibco, ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS (Gibco, ThermoFisher Scientific, Waltham, MA) at 37 °C under a 5% CO₂ atmosphere. To prepare MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h incubation at 37 °C under a 5% CO₂ atmosphere, 25 ml of DMEM supplemented with 2% FBS was added. MNV-1 was harvested 2 days after post-inoculation by three freezing–thawing cycles and centrifugation. The virus was stored at − 80 °C until use.

2.2 Inoculation of MNV-1 and HuNoVs on green onions and in salsa

Fresh green onions were obtained from local markets, washed and dried before use. The green part of the green onions was removed using a sterile knife and the remaining white part was sliced into 3-mm sections. The cut surfaces of white part sections (3 g) were inoculated with 100 μ l of MNV-1 stock ($\sim 10^7$ PFU/ml) or 50 μ l of HuNoV GI.1 ($\sim 10^6$ genome copies/ml) or GII.4 ($\sim 10^7$ genome copies/ml) stocks. The inoculated samples were dried in a biosafety hood for 2 h for viral attachment and then placed in sterile stomacher bags (3 g of sample/bag) with or without 1 ml of sterile dd-H₂O in the bag. The bags were sealed and double-bagged. To prepare salsa for HHP treatment, Tostitos restaurant style salsa (medium heat; major ingredients are diced tomatoes in tomato juice, water, unpeeled ground tomatoes in tomato puree, onions, jalapeno peppers) was bought from local market, supplemented with 5% (w/w) 3-mm sections of washed green onions and stomached at 260 RPM for 2 min to reduce the size of vegetable chunks. pH of the salsa samples was ~ 4.0 and water activity of salsa samples was ~ 0.967 . Salsa samples (2.5 g) were inoculated with 100 μ l of MNV-1 stock or 50 μ l of HuNoV GI.1 or GII.4 stocks. Samples were placed in sterile stomacher bags, sealed and double-bagged.

2.3 HHP treatment

Pressure treatments were conducted using an Avure PT-1 pressure unit (Avure Technologies, Kent, WA) with temperature control and water as a hydrostatic medium. A circulating water bath surrounded the pressure vessel (50 ml) to control the temperature. The pressure come-up rate was approximately 22 MPa/s. The pressure-release was almost immediate (< 4 s). Pressurization hold time reported in this study did not include the pressure come-up or release times. To determine whether the presence of water surrounding green onions would affect the HHP inactivation of MNV-1, inoculated green onions samples with and without water in the bags were pressurized at 350 MPa for 2 min at initial temperatures of 4 and 20 $^{\circ}$ C. To

determine the temperature effect on HHP inactivation of MNV-1 on green onions, green onion samples with water in the bags were treated at 300 MPa for 2 min at initial temperatures of 1, 4 and 10 °C. To obtain the HHP inactivation curves of MNV-1 on green onions and in salsa, samples were treated at 300 or 350 MPa for 0.5 – 3 min at an initial temperature of 1 °C. The *D* value (decimal reduction time; time required at a given condition to reduce target microorganism by 1 log) of MNV-1 under difference conditions were also calculated. To determine the HHP inactivation of HuNoV GI.1 and GII.4 on green onions and in salsa, samples were treated at 100 - 600 MPa for 2 min at an initial temperature of 1 °C. MNV-1 and HuNoVs were extracted from the pressurized samples and untreated samples as described in Section 2.4. MNV-1 was quantified by plaque assay as described in Section 2.5 and HuNoVs were quantified by PGM-MBs binding assay and RT-qPCR as described in Section 2.6 and 2.7.

2.4 Extraction of viruses from green onion and salsa samples

Extraction of MNV-1 and HuNoVs from samples followed procedure described by Li et al. (2013a) with slight modifications. Briefly, each green onion sample was transferred into a 50 ml sterile centrifuge tube and 20 ml of PBS was added into the tube. To release the viruses from the green onion samples, each tube was vortexed for 30 s with a Vortex-Genie 2 vortexer (Scientific Industries, Bohemia, NY) with speed set at “10” and 10 ml of supernatant was removed from the tube for further analysis. Each salsa sample was transferred to a 50 ml sterile centrifuge tube and 7.5 ml of PBS was added into the tube. Each sample was then centrifuged at 5000 × *g* for 10 min at 20 °C. Eight milliliters of supernatant was removed for further analysis.

2.5 Plaque assay of MNV-1

MNV-1 was quantified using the procedure described by Li et al. (2013b) with slight modifications. RAW 264.7 cells were seeded into 6-well tissue culture plates (Becton, Dickinson

and Company, Franklin Lakes, NJ) at a density of $\sim 2 \times 10^6$ cells per well. After 24 h of incubation, cell monolayers were infected with 400 μ l of a 10-fold dilution series of the virus and the plates were incubated for 1 h at 37 °C and 5% CO₂ with gentle agitation every 10 min. After incubation, the samples were removed and cells were overlaid with 2.5 ml of Eagle minimum essential medium (MEM) supplemented with 5% (v/v) FBS, 1% (w/v) sodium bicarbonate, 10 mM HEPES (pH 7.7), 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B, 2 mM l-glutamine (ThermoFisher Scientific, Waltham, MA) and 0.5% (w/v) low-melting agarose (Lonza Group Ltd., Switzerland). Plates were incubated at 37 °C in 5% CO₂ for 48 h, fixed in 3.7% formaldehyde for 30 min and plaques were then visualized by staining with 0.05% crystal violet (w/v in 10% aqueous ethanol).

2.6 Preparation of PGM-MBs and PGM-MBs binding assay

PGM-MBs were prepared as described by Li et al. (2013a). Before PGM-MB binding assay, each sample was treated with 100 μ g of RNase A (Life Technologies, Carlsbad, CA) at 37 °C for 30 min. The PGM-MB binding assay was conducted as described by Li et al. (2013a) with slight modifications. Two hundred and 100 μ l of PGM-MBs were added to green onion samples and salsa samples, respectively. The samples were then incubated at room temperature for 15 min on the Labquake shaker rotisserie and PGM-MBs were separated from the liquid using the bead attractor. The PGM-MBs were then washed 3 times with 1 ml of PBS for each wash, suspended in 140 μ l ddH₂O and stored on ice.

2.7 RNA extraction and RT-qPCR detection of HuNoVs

RNA of HuNoV was extracted using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol with slight modifications. For each sample, 560 μ l buffer AVL containing 5.6 μ l carrier RNA was added, pulse-vortexed for 15 s and incubated at

room temperature for 10 min. PGM-MBs were then separated by the bead attractor. The liquid was transferred to a new 1.5-ml sterile centrifuge tube and 560 μ l of ethanol was added followed by a 15 s pulse-vortex. The liquid was then transferred to the QIAamp Mini column and centrifuged at $6000 \times g$ for 1 min. The column containing the RNA was then washed by 500 μ l buffer AW1 and 500 μ l buffer AW2. Finally, the RNA was eluted in $2 \times 40 \mu$ l AVE elution buffer.

The RT-qPCR quantification, including the primers, the TaqMan probes and their concentration, followed the protocol described by (Li et al., 2013a). All primers were synthesized by Life Technologies and all probes were synthesized by Integrated DNA Technologies. RT-qPCR was performed using the Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Waltham, MA). For each sample, 10 μ l RT-qPCR system was adopted, containing 2.5 μ l master mix, 0.5 μ l forward primer, 0.5 reverse primer, 0.5 μ l probe and 6 μ l sample RNA template. The following condition was used for the PCR process: 50 °C for 5 min for reverse transcription, followed by 95 °C for 20 s for initial denaturation, 45 cycles of 95 °C for 3 s and 60 °C for 30 s. RNA standard template was prepared to determine the reduction of HuNoV after the HHP treatment. Viral RNA of HuNoV GI.1 or GII.4 virus stock (280 μ l) was extracted as RNA standard template and eluted in 200 μ l of AVE buffer. The RNA standard template was aliquoted and store at -80°C until use. The RNA standard template was 10-fold diluted for RT-qPCR. Serial 10-fold dilutions of GI.1 RNA and GII.4 RNA were used as standard for RT-qPCR. Reduction of HuNoV was calculated using the difference between untreated samples and treated samples corresponding to the RNA standard. When quantity of HuNoV detected by RT-qPCR was below the most diluted RNA standard, the result was considered as below detection limit. The maximum reduction levels that could be detected were ~ 3.0 logs for GI.1 and ~ 4.0

logs for GII.4. The difference in maximum reduction levels between the two strains was due to the different initial virus titers in the fecal samples.

2.8 Statistical analysis

At least 3 independent replicates were conducted for each experiment. Log reductions were the differences between the untreated samples and treated samples. One-way analysis of variance following Tukey's HSD (for comparisons of more than two groups involving one factor), two-way analysis of variance following Tukey's HSD (for comparisons of more than two groups involving two independent factors) or an independent t test (for comparisons between two groups) was used for statistical analysis with JMP version 11.2 (SAS Institute Inc., Cary, NC). A P-value of < 0.05 was considered statistically significant.

3. Results

3.1 Effect of water presence on HHP inactivation of MNV-1 on green onions

As shown in Fig. 1, under the dry state condition (without water surrounding green onion sample during pressure treatment), 2-min HHP treatments at 350 MPa reduced MNV-1 on green onions by < 2.0 log at either initial sample temperature of 4 or 20 °C. At 20 °C, no significant difference was found between the dry state and wet state (with water surrounding green onion sample during pressure treatment). However, when the initial sample temperature was reduced from 20 °C to 4 °C, MNV-1 became significantly more sensitive ($P < 0.05$) to HHP treatment under the wet state condition. A 2-min HHP treatment at 350 MPa and 4 °C reduced the MNV-1 on green onions by 4.3 log under the wet state condition, which was significantly higher than the 1.7 log reduction under the dry state condition.

3.2 Effect of initial sample temperature on HHP inactivation of MNV-1 on green onion

As shown in Fig. 2, MNV-1 became more sensitive to HHP treatment as the initial sample temperature decreased from 10 to 1 °C, though no significant difference was found among the 3 treatment groups. This is probably due to the small temperature range tested. A 2-min HHP treatment at 300 MPa reduced MNV-1 on green onions by 1.2 log at 10 °C while 2.8 log reduction was achieved at 1 °C. Similar initial sample temperature effect on HHP inactivation of MNV-1 was also found in the water presence effect study (Fig. 1).

3.3 HHP inactivation curves of MNV-1 on green onions and salsa

The HHP inactivation curves of MNV-1 on green onions were obtained at 300 and 350 MPa and an initial sample temperature of 1°C under wet state condition and the HHP inactivation curve of MNV-1 in salsa was obtained at 300 MPa and an initial sample temperature of 1 °C (Fig. 3). The degree of MNV-1 inactivation on green onions and salsa showed linear relationship with the HHP treatment time with R^2 values of > 0.9 . For green onions, the degree of MNV-1 inactivation increased as the pressure level and time increased. The calculated D values of green onion curves were 1.10 and 0.61 min at 300 and 350 MPa, respectively and the D value of salsa curve at 300 MPa was 0.63 min. The D value results indicated that MNV-1 was more resistant to pressure on green onion than in salsa.

3.4 HHP inactivation of HuNoV GI.1 and GII.4 on green onion and in salsa

HHP inactivation studies of HuNoV GI.1 and GII.4 were conducted at 100 - 600 MPa for 2 min at an initial temperature of 1 °C to determine optimum HHP processing conditions to achieve ≥ 3 log reduction of these strains. These conditions were designed according to both detection limits of HuNoV GI.1 and GII.4 and the likely HuNoV contamination level of ~ 3 log genomic copies/g (Baert et al., 2011). To achieve > 3 log reduction of HuNoV GI.1, HHP treatment should be conducted at 600 MPa and 500 MPa for green onions and salsa respectively.

To achieve > 3 log reduction of HuNoV GII.4, HHP treatment should be conducted at 500 MPa and 300 MPa for green onions and salsa respectively. For both green onions and salsa, HuNoV GI.1 showed less sensitivity to HHP treatment than HuNoV GII.4. For green onions, HHP treatment could reduce HuNoV GI.1 by > 3.0 log at 600 MPa while > 3.87 log reduction was achieved for HuNoV GII.4 under the same condition. Similar results were also found with salsa which showed 1.39 log reduction of HuNoV GI.1 and 3.52 log reduction of HuNoV GII.4 at 400 MPa. Food matrices also influenced HHP inactivation of HuNoV GI.1 and GII.4, with HuNoV showing higher sensitivity to HHP treatment in salsa than on green onions. For example, the HHP treatment of 300 MPa reduced HuNoV GII.4 by 3.31 log in salsa and 2.57 log on green onions.

4. Discussion

In this study, MNV-1, a HuNoV surrogate, was used to evaluate the HHP inactivation of HuNoV on green onions and in salsa. Several studies have shown that MNV-1 was a good surrogate for HuNoV for HHP treatment (Cannon et al., 2006; Huang et al., 2014; Li et al., 2012; Wobus et al., 2006). Not only did MNV-1 share biomedical features and a genomic size similar to HuNoV, it also showed behaviors similar to HuNoV when exposed to HHP under different treatment conditions such as pH, temperature and presence of water (Li et al., 2012; Li et al., 2013a). Therefore, it was considered as a suitable surrogate for HuNoV and used in this study. Examining the HHP inactivation data in this study, it can be concluded that MNV-1 was more sensitive to HHP than HuNoV GI.1, but had HHP sensitivity similar to HuNoV GII.4 either on green onions or in salsa.

Since there is an uncertainty regarding the use of MNV-1 data to establish HHP processing parameters, we also used the PGM/PCR assay to further increase our confidence

level. This assay has been found to be likely able to quantify HHP inactivation of HuNoV GI.1 and GII.4 at ≤ 2 log reduction levels and likely to correctly estimate or underestimate their inactivation at > 2 log reduction levels (Li and Chen, 2015; Li et al., 2013a). It should be noted that the data obtained from this study can only be used as a general guideline due to the uncertainty regarding the suitability of MNV-1 as a HuNoV surrogate and the accuracy of the PGM/PCR assay.

Our results showed that at initial sample temperature of 4 °C, HHP achieved better inactivation effect of MNV-1 on green onion under wet state condition than under dry state condition (Fig. 1). Several studies have shown that the presence of water during HHP treatment can improve the HHP inactivation of HuNoV and its surrogates (Huang et al., 2014; Huang et al., 2016; Li et al., 2013a; Li et al., 2013b). For example, Li et al. (2013b) demonstrated that HHP inactivation of MNV-1 and TV on blueberries was much more efficient with water surrounding the blueberries. In that study, a 2-min HHP treatment at 400 MPa and 4 °C achieved < 1 log and > 5 log reduction without or with the presence of water, respectively. Huang et al. (2014) demonstrated that a 2-min HHP treatment at 300 MPa and 0 °C reduced MNV-1 on strawberry quarters by 2.9 log under wet state condition while same HHP condition only achieved 1.0 log reduction under dry state condition. The effect of water presence during HHP was also observed for HuNoV. Li et al. (2013a) showed that > 3.0 log reduction of HuNoV GI.1 on blueberries was achieved with a 2-min HHP treatment at 500 MPa and 1 °C under wet state condition comparing to merely 0.9 log reduction under dry state condition. The mechanism for this phenomenon might be that HHP was able to force water into the solvation cage surrounding the virus capsid protein, resulting disruption of the virus capsid (Kingsley, 2013; Li et al., 2013a).

As the initial sample temperature decreased, the degree of HHP-inactivation of MNV-1 on green onions increased. Similar temperature effects were also reported for HHP inactivation of MNV-1 on strawberries, strawberry puree and lettuce (Huang et al., 2014; Lou et al., 2011). This result was also consistent with those reported for other HuNoV surrogates, TV and FCV (Chen et al., 2005; Li et al., 2013b). As for HuNoV GI.1 and GII.4, Li et al. (2013a) also demonstrated that HuNoV GI.1 and GII.4 suspended in PBS became more sensitive to HHP treatment as the initial sample temperature decreased from 35 to 1 °C. They showed the degree of a 2-min HHP at 250 MPa inactivation of HuNoV GII.4 was reduced from 2.9 log to 0.0 log as the temperature increased from 1 to 21 °C. Similar results were found for HHP inactivation of HuNoV GI.1 and GII.4 in food matrices, such as berries, berry puree and oyster (Huang et al., 2016; Ye et al., 2015). It was suggested that the cooler temperature in HHP treatment might increase the density of water in the solvation cage of the capsid, which led to more severe capsid protein denaturation and higher inactivation effect (Balny et al., 2002; Kingsley et al., 2007; Kunugi and Tanaka, 2002).

All three HHP inactivation curves of MNV-1 on green onions and salsa were almost linear in the logarithmic plot. Some previous studies showed different findings about HHP inactivation kinetics of FCV and MNV (Buckow et al., 2008; Chen et al., 2005; Kingsley et al., 2007). In those studies, the FCV and MNV had concave upward HHP inactivation curves, which showed a better fit for nonlinear models such as the Weibull model rather than for the linear model. This difference in the shape of the curves might be attributed to different properties of the surrounding environment since numerous factors might affect the inactivation efficacy of HHP treatment, like salt content, pH and sugar content (Kingsley, 2013).

Our results showed that MNV-1, HuNoV GI.1 and GII.4 were more resistant to HHP treatments on green onions than in salsa. It has been well documented that food matrixes or compositions and pH could influence the HHP inactivation of viruses. For example, Kingsley and Chen (2008) showed that increasing the NaCl or sucrose concentration would reduce the HHP inactivation of FCV. Li and Chen (2015) showed that pH influenced the HHP inactivation of HuNoV GI.1 and GII.4, TV, and MNV-1. The viruses were more resistant to HHP at pH 4 than at neutral pH. Previous study showed that lower water activity resulted in greater resistance of FCV and HAV to HHP treatment which corroborated our result (Kingsley et al., 2008; Kingsley et al., 2009; Kovač et al., 2010). Ye et al. (2015) and Huang et al. (2016) reported that HuNoV GI.1 and GII.4 showed different HHP sensitivity when they were inoculated in oysters, berry purees and on berries.

HuNoV GII strains were the leading cause for foodborne outbreaks while both GI and GII strains showed similar prevalence for waterborne outbreaks of HuNoV (Bitler et al., 2013; Vega et al., 2014). From 2009 to 2013, HuNoV GII.4 was the most prevalent strain among all HuNoV outbreaks (Vega et al., 2014). Therefore, a GII.4 strain and a GI strain were chosen for this study. One of the main goals in this study was to identify proper HHP processing parameters for green onions and salsa as an intervention for HuNoV contamination. It was generally agreed that relative low dose (10 – 100 virions) of HuNoV was enough for human infection (Hutson et al., 2004). It was reported that the HuNoV titer level of majority of contaminated leafy greens fell in the range between 0 – 3.1 log genomic copies/g (Baert et al., 2011). Therefore, achieving > 3 log reduction of GI.1 and GII.4 was our targeted level of HHP inactivation. Using this criterion, our results indicated that a 2-min treatment of 600 MPa and 1 °C and a 2-min treatment of 500 MPa and 1 °C would be adequate for decontamination of HuNoV GI.1 on green onion

and in salsa, respectively. To achieve > 3 log reduction of HuNoV GII.4 on green onion and salsa, a 2-min treatment of 500 MPa and 1 °C and a 2-min treatment of 300 MPa and 1 °C would be needed, respectively. Since it is almost impossible to decide what contaminating genotype is present, it is logical to use HHP processing parameters that target the most resistant HuNoV strain. It should also be noted that these conclusions were drawn based on the assumption that the PGM/PCR assay was able to correctly estimate the HHP inactivation of HuNoVs. In addition, since the efficacy of HHP inactivation of HuNoV is influenced by different food matrices, validation study needs to be conducted for salsa with formulations different from those used in this study.

Based on our visual observation, color and smell of salsa and green onion did not show much change after HHP treatments. The texture of green onion became softer after HHP treatments. Neetoo et al. (2011) found that after a 2-min HHP treatment at 450 MPa and 40 °C, little adverse impact was shown on the overall appearance, color and aroma of chopped green onion while texture was partly affected. As for salsa, a commercial HHP-processed salsa product produced by Fresherized Foods under the brand name of Wholly Salsa® is currently available in the United States.

5. Conclusion

The overall goal of this study was to identify suitable HHP conditions for HuNoVs inactivation on green onions and in salsa. It should be noted that conclusions made in this study was based on the assumption that MNV-1 was a good surrogate for HuNoV and the PGM/PCR assay could reliably estimate the HHP inactivation of HuNoV. Cooler initial sample temperature and presence of water could enhance the HHP inactivation of MNV-1 on green onions. HHP inactivation curves demonstrated a positive relationship between inactivation of MNV-1 and

pressure level and treatment time. Our results also suggested that a 2-min HHP treatment at 600 MPa and 1 °C could achieve > 3.0 log reduction of HuNoV GI.1 and GII.4 on green onions and in salsa.

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Fig. 1. Effect of water presence on HHP inactivation of MNV-1 on green onions. Green onions with an initial MNV-1 inoculation level of $\sim 3.3 \times 10^6$ PFU/g were either placed into bags (dry state) or bags containing water (wet state). They were then treated at 350 MPa for 2 min at initial sample temperatures of 4 and 20 °C. Error bars represent one standard deviation. Data with different lowercase letter are significantly different ($P < 0.05$).

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526 Fig. 2. Effect of initial sample temperature on HHP inactivation of MNV-1 on green onions.

527 Green onions with an initial MNV-1 inoculation level of $\sim 3.3 \times 10^6$ PFU/g were treated at 300

528 MPa for 2 min at initial sample temperatures of 1, 4 and 10 °C under wet state condition. Error

529 bars represent one standard deviation. Data with different lowercase letter are significantly

530 different ($P < 0.05$).

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533 Fig. 3. HHP inactivation curves of MNV-1 on green onions and in salsa. Green onions with an
534 initial MNV-1 inoculation level of $\sim 3.3 \times 10^6$ PFU/g were treated at 300 and 350 MPa and 1 °C
535 for 0.5 - 3.0 min under wet state condition. Salsa with an initial MNV-1 inoculation level of ~ 4
536 $\times 10^6$ PFU/g were treated at 300 MPa and 1 °C for 0.5 - 2.5 min. Error bars represent one
537 standard deviation and data were fitted with linear function. *, data marked with asterisk
538 represents some replicates were below detection limit. Number in fraction represents the number
539 of replicates below detection limit out of total number of replicates.

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541

542 Table 1.

543 HHP inactivation of HuNoV GI.1 and GII.4 on green onions and salsa. Green onions and salsa

544 samples were inoculated with HuNoV GI.1 or GII.4 and HHP-treated at an initial sample

545 temperature of 1 °C for 2 min. Data are the means of log reductions of genome copies/g $\pm$ 1

546 standard deviation from at least 3 replicates. Number in fraction represents the number of

547 replicates below detection limit out of total number of replicates. Under the same HuNoV strain,

548 data with different lowercase letters in the same row are significantly different ($P < 0.05$). Data549 with different uppercase letters in the same column are significantly different ($P < 0.05$). ND: not

550 done.

	HuNoV GI.1		HuNoV GII.4	
Pressure (MPa)	Green Onion	Salsa	Green Onion	Salsa
100	ND	ND	ND	0.03 $\pm$ 0.06 A
200	ND	0.08 $\pm$ 0.22 A	0.76 $\pm$ 0.27 Ab	0.14 $\pm$ 0.10 Aa
300	ND	0.23 $\pm$ 0.03 A	2.57 $\pm$ 0.34 Ba	3.31 $\pm$ 0.20 Ba
400	ND	1.39 $\pm$ 0.35 B	2.93 $\pm$ 0.25 Ba	3.52 $\pm$ 0.27 Ba
500	> 2.89 $\pm$ 0.18 (2/3) Aa	> 3.00 $\pm$ 0.00 (3/3) Ca	3.37 $\pm$ 0.15 Ba	3.52 $\pm$ 0.34 Ba
600	> 3.00 $\pm$ 0.00 (2/3) A	ND	> 3.87 $\pm$ 0.22 (2/3) Ba	3.46 $\pm$ 0.32 Ba

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