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Comparison of pressure inactivation of caliciviruses and picornaviruses in a model food system ☆

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ABSTRACT

Human enteric viruses are a major cause of foodborne illnesses. The objective of this study was to determine the high pressure processing parameters necessary to inactivate hepatitis A virus (HAV), Aichi virus (AiV), and the human norovirus surrogates, feline calicivirus (FCV) and murine norovirus (MNV) in fresh salsa. Samples were treated at 250, 400 and 500 MPa for 1, 5, and 10 min at 9 °C. AiV was additionally HPP-treated at temperatures of 50, 60, 70, and 80 °C. In salsa, HAV and FCV were inactivated beyond the limit of detection after 1 min at 400 and 250 MPa, respectively. MNV was more pressure resistant than FCV, whereby 400 MPa for 1 min was needed to achieve the same degree of inactivation. AiV titers were not reduced after a 500 MPa treatment. When heat was combined with high pressure unique pressure-assisted heat stabilization was observed associated with AiV only.

Industrial relevance: The manuscript *Comparison of Pressure Inactivation of Caliciviruses and Picornaviruses in a Model Food System* is focused on the nonthermal processing technology high pressure processing (HPP), which is a highly used technology and one that is readily accepted by consumers. This manuscript discusses the use of HPP for the inactivation of foodborne viruses from two different families on a fresh produce item that may be widely contaminated during preharvest or post-harvest. Viruses are difficult to detect and therefore technologies that can be used to inactivate them without organoleptic changes to the product are advantageous. This manuscript includes consumer acceptance of the treated product. This manuscript will be useful to industry to show the continued important of HPP in ready-to-eat food products, like salsa.

1. Introduction

High pressure processing (HPP) is a nonthermal method of pathogen inactivation that maintains the raw character of foods, and is currently used on products such as oysters, salsa, guacamole, ready-to-eat deli meats and juices. Foods treated with HPP taste fresher, look better, retain normal textures, and have no loss of nutrition compared to thermally processed foods (Ramaswamy, Balasubramaniam, & Kaletunc, 2004). HPP products are currently available in retail markets of the United States, Europe, and Japan (Ramaswamy et al., 2004). It is important that both consumers and industry understand how HPP can be used to inactivate viruses as well as other pathogens.

The effects of pressure on the inactivation of many foodborne pathogens has been demonstrated to varying degrees on many foodborne pathogens (as reviewed by Black et al., 2007; Hirneisen et al., 2010; Patterson, 2005). Pressure inactivation of viruses and other pathogens is affected significantly by several factors, including the pressure magnitude, time, temperature at which the pressure treatment occurs, as well as the food matrix in which the pathogen is suspended (Chen, Hoover, & Kingsley, 2005; Hirneisen, Hoover, Hicks, Pivarnik, & Kniel, 2012; Kingsley, Guan, Hoover, & Chen, 2006, Kingsley, Holliman, Calci, Chen, & Flick, 2007; Sharma et al., 2008). The amount of fat, salt, and acidity can all influence the degree of inactivation (Calci, Meade, Tezloff, & Kingsley, 2005; Chen, Joerger, Kingsley, & Hoover, 2004; Hirneisen et al., 2012; Kingsley, Hoover, Papafragkou, & Richards, 2002, Kingsley et al., 2007). The four viruses analyzed in this study represent important foodborne pathogens and are representatives of two virus families: Picornaviruses and Caliciviruses. Hepatitis A virus (HAV) is a human enteric virus of the picornavirus family and causes fever, malaise, nausea, anorexia, and abdominal pain, followed by jaundice 15–50 days post-infection (Krugman & Giles, 1970) . Water, shellfish, and salads are the most commonly implicated food sources in HAV outbreaks. HAV is the cause of approximately 35,770 cases annually in the US (Scallan et al., 2011) with the estimated cost/case of HAV infection being $35,907 USD (Scharff, 2012). The largest HAV outbreak in the US occurred in 2003 and was caused by contaminated green onions that were used in cooking as garnish or in salsa. This outbreak

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resulted in approximately 1000 illnesses and 4 deaths. The green onions implicated in this outbreak were grown in Mexico and were believed to have been contaminated in the pre-harvest environment and not by food handlers. The green onions were chopped and stored in bulk, allowing for cross-contamination of clean green onions and may explain the large size of the outbreak (Chancellor et al., 2006).

Aichi virus (AiV) was first recognized in Japan in 1989 as the cause of oyster-related gastroenteritis (Yamashita et al., 1991). Aichi virus has since been found around the globe including isolation from fecal samples mostly in Southeastern Asian countries including Japan, Indonesia, Thailand, Malaysia, Pakistan, Bangladesh and Vietnam but AiV has also been detected in Germany and Brazil (Oh et al., 2006; Pham et al., 2007; Yamashita, Sakae, Ishihara, Isomura, & Utagawa, 1993; Yamashita et al., 1991; Yamashita et al., 2000). AiV is a picornavirus, like hepatitis A. As a member of the *Kobuvirus* genus, AiV causes a self-limiting gastroenteritis. Oysters are the most common vehicle of AiV transmission; however, it has been suggested that there are other vehicles for AiV transmission, but they have yet to be identified (Yamashita et al., 2000). The spread of AiV to European and South American countries may indicate the potential presence of the virus in the U.S. or the possibility for the virus to spread to the US in the near future. It is likely that AiV is not documented in the US due to the mild symptoms and the lack of virus testing performed in the US. Few studies have assessed the effects of processing treatments on AiV, but studies with high pressure have shown that AiV is uniquely pressure resistant (Black et al., 2010; Kingsley, Chen, & Hoover, 2004). AiV is being considered as a surrogate for other enteric viruses, including the non-cultivable norovirus, due to the resistance of AiV to many mitigation strategies.

Noroviruses (NoV) are the leading cause of foodborne illness in humans with an estimated 5.5 million illnesses each year in the United States (Scallan et al., 2011). It is likely that many of the cases of undiagnosed foodborne illness are caused by NoVs. NoV is a highly contagious virus causing gastroenteritis (Meehan & Grose, 1993). A cell culture model for human noroviruses currently does not exist, making assessment of norovirus infectivity difficult. Therefore, surrogates, including feline calicivirus and murine norovirus, are commonly used to predict human norovirus infectivity. Feline calicivirus (FCV) has been historically used as a surrogate for NoV because it is in the same family *Caliciviridae* and FCV can be grown in cell culture (Hutson, Atmar, & Estes, 2004). More recently, murine norovirus (MNV) was isolated and is used as a surrogate for human NoV (Wobus et al., 2004). MNV is more genetically related to human norovirus as it is a GV norovirus. MNV has been suggested as be a better surrogate for human noroviruses as it was found to be more acid tolerant than FCV (Cannon et al., 2006). Since the discovery of MNV, many studies assessing the environmental stability of human norovirus, have used MNV as a model; however, at this time organizations like the USEPA continues to use FCV for assessing the efficacy of antimicrobials.

Salsa was chosen as the model food to determine enteric virus inactivation in this study because there have been foodborne outbreaks caused by both bacterial and viral enteric pathogens involving green onions, tomatoes, and herbs, all ingredients found in a fresh salsa product (Fiore, 2004; Weissinger, Chantarapanont, & Beuchat, 2000). Contamination of these ingredients can occur throughout the food chain either in the pre-harvest environment, by foodhandlers in both the field and kitchen, and through cross-contamination. The objective of this study was to determine conditions to inactive human norovirus surrogates, FCV and MNV as well as two human picornaviruses, HAV and AiV, in a fresh salsa product using HPP.

2. Materials and methods

2.1. Viruses and quantification

HAV (ATCC VR-1402) was propagated in fetal Rhesus monkey kidney cells (FRhK-4) (ATCC CRL 1688) using Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA). FCV (ATCC VR-651) was propagated in Crandell Reese feline kidney cells (CrFK) (ATCC CCL-94) using minimal essential medium (MEM) (Mediatech). MNV was propagated in RAW 264.7 cells cultured in RAW DMEM. AiV (strain A846/88) was propagated in African green monkey kidney cells (Vero) (ATCC CCL-81) using MEM. Media was supplemented with 2% fetal bovine serum (FBS) (Mediatech) for maintenance and 10% FBS for cell growth. MEM media was also supplemented with 1% penicillin/streptomycin/amphotericin B (Mediatech), 1% sodium bicarbonate (Mediatech), 1% sodium pyruvate (Mediatech), and 1% MEM non-essential amino acids (Mediatech). DMEM media was supplemented with 1% penicillin/streptomycin/amphotericin B and 1% sodium bicarbonate. RAW DMEM media was also supplemented with 1% glutamate (Mediatech). Virus infected lysates were purified by three cycles of freeze-thaw, and the supernatant was recovered after centrifugation at 2,500 x *g* for 15 min, and stored at -80 °C until used. Viral titers for all but MNV were determined by $TCID_{50}$ (tissue culture infectious dose for 50% of the cultures) and calculated using the Reed Muench method (Brown, 1964). In this method, cell monolayers were grown in 96-well cell culture plates for 24 h containing media with 10% FBS. Confluent cell monolayers were inoculated with serially diluted virus in Hank's balanced salt solution (HBSS) (Mediatech) and incubated (37 °C) for 2 h. After a 2 h incubation, media containing 2% FBS was added to the plates (Deng & Cliver, 1995). Plates were incubated for a specific amount of time and at that point cytopathic effects were observed microscopically and virus titer calculated. HAV was read for cytopathic effect 14 d post-inoculation (dpi), and FCV and AiV were read 3–5 dpi. For MNV, plaque assays were performed as described previously (Wobus et al., 2004). Briefly, virus was diluted and inoculated onto confluent monolayers of RAW cells grown in twelve well plates for 24 h. After 2 h of agitation at 37 °C the inoculums were aspirated and the cells overlayed with 1 ml of 1.5% Seaplaque agarose in 2X DMEM containing 2% FBS. Plates were incubated at 37 °C with 5% CO_2 for 48 h and plaques were visualized by staining with 0.5 ml complete MEM with 0.5% neutral red per well for 6–8 h.

2.2. Salsa preparation and HPP

A commercially prepared refrigerated "fresh" salsa product was purchased at a local grocery store in Newark, DE. The pH of the salsa was measured using a Beckman Φ350 pH/Temp/mV meter (Beckman Coulter, Inc., Fullerton, CA). Water activity (a_w) was measured using an AquaLab Water Activity meter (Decagon Devices, Inc., Pullman, WA).

Salsa samples of 2 g each were inoculated with approximately 10^8 $TCID_{50}$/ml HAV, AiV, or FCV or 10^5 PFU/ml MNV, mixed thoroughly, and placed in sterile polypropylene sampling pouches (VWR International, West Chester, PA) for HPP. The pouches were heat-sealed and then sealed in a secondary pouch. The pressure unit (PT-1 unit, Avure Technologies Inc., Kent, WA) was a laboratory scale unit with a pressure chamber having a capacity of 50 ml. Water was the pressure transmitting medium. The come-up rate was approximately 600 MPa/ 30 s and the pressure release time was <4 sec. Compression of the water causes adiabatic heat which depends on initial water temperature and rate of compression (Cheftel, 1992). Adiabatic heat of water is 3 °C for every 100 MPa increase in pressure (Solomon & Hoover, 2004). Samples were treated at 250, 400, and 500 MPa for 1, 5, and 10 min at 9 °C. The treatment temperature of 9 °C allows for mild temperature fluctuations or abuse which could occur under refrigeration during industrial pressure processing. AiV suspended in salsa was also exposed to 400 and 500 MPa for 5 min at 50, 60, 70, and 80 °C and AiV suspended in HBSS was treated at 400 and 500 MPa at 20, 50, 60, 70 and 80 °C.

2.3. Virus recovery

After HPP, samples were removed from the sterile pouches and then pouches were washed with 1 ml of HBSS to recover any remaining virus.