HIGH PRESSURE INACTIVATION OF MURINE NOROVIRUS AND HUMAN NOROVIRUSES ON STRAWBERRIES, BLUEBERRIES OR RASPBERRIES OR IN THEIR PUREES

by

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ABSTRACT

The consumption of fresh and frozen strawberries, blueberries, raspberries and their purees is continuously growing. However, these berries and berry puree have been associated with human norovirus outbreaks. Therefore, interventions are urgently needed. High hydrostatic pressure (HHP) has been successfully used to process various foods since it has less detrimental effects on nutrients and sensory qualities compared with thermal processing. The overall goal of this study was to investigate high hydrostatic pressure inactivation effect of human norovirus on strawberries, blueberries, raspberries and in their purees. The study consisted of two parts: 1. Test influence of different parameters on HHP inactivation of murine norovirus 1 (MNV-1) on strawberries and in strawberry puree; 2. Determine the efficacy of HHP inactivation of human norovirus GI.1 and GII.4 on strawberries, blueberries, raspberries and in their puree.

MNV- 1, a common surrogate for human norovirus, was tested on strawberries and in strawberry puree to determine the inactivation effect of different parameters during high hydrostatic pressure processing. Strawberry puree inoculated with ~10^6 PFU/g of MNV-1 was treated at 350 MPa for 2 min at initial sample temperatures of 0, 5, 10 and 20 °C. MNV-1 became more sensitive to HHP at lower initial sample temperature. To determine the effect of pressure cycling on MNV-1 inactivation, inoculated puree samples were treated at 300 MPa and 0 °C with 1, 2 and 4 cycles. Pressure cycling offered no distinct advantage over continuous HHP treatment. Strawberries inoculated with ~ 4×10^5 PFU/g of MNV-1 were either pressure-treated

directly (dry state) or immersed in water during pressure treatment. MNV-1 was very resistant to pressure under the dry state condition, but became sensitive to pressure under the wet state condition. The inactivation curves of MNV-1 in strawberry puree and on strawberries were obtained at 300 and 350 MPa and 0 °C. Most curves were characterized by rapid reduction of titers at the beginning followed by tailing, indicating that increasing pressure level is a better way to enhance HHP inactivation of MNV-1 than increasing treatment time. The fate of MNV-1 in the un-treated and pressure-treated strawberries and strawberry puree during frozen storage was determined. MNV-1 was relatively stable during the 28-day frozen storage.

Human norovirus (HuNoV) GI.1 and GII.4 were tested on strawberries, blueberries, raspberries and in their puree to determine the HHP inactivation effect. Strawberry puree inoculated with HuNoV GI.1 strain was treated at 450, 500 and 550 MPa for 2 min at initial sample temperatures of 0, 4 and 20 °C. HuNoV GI.1 strain showed less sensitiveness to HHP at higher temperature at all three pressure levels. As for GI.1 strain, 2 min HHP treatment at 550 MPa and 0 °C achieved > 2.9 log reduction in strawberry puree, blueberry puree and raspberry puree. As for GII.4 strain, 2 min HHP treatment at 0 °C achieved > 4 log reduction at 500, 550 and 400 MPa in strawberry puree, blueberry puree and raspberry puree, respectively. HuNoV GI.1 strain showed more resistance to HHP treatment than HuNoV GII.4 strain. Strawberry quarters, blueberries and raspberries were spot-inoculated with HuNoV and HHP-treated with water. Two min HHP treatments of 650 MPa at 0 °C could reduce HuNoV GI.1 and GII.4 by 1.7 and 3.1 log respectively on strawberry quarter. As on blueberry, 2 min HHP treatments at 0 °C achieved > 3.2 log reduction of GI.1 strain at 550 MPa and > 4 log reduction of GII.4 strain at 300 MPa. As for raspberry, 2 min

HHP treatments at 0 °C achieved 2.48 log reduction of GI.1 strain at 650 MPa and 4 log reduction of GII.4 strain at 600 MPa. pH of surrounding water of blueberries was higher than strawberry quarters and raspberries and HHP treatment turned out to have better HuNoV inactivation effect on blueberries than strawberry quarters and raspberries.

Chapter 1

INTRODUCTION

Berries and berry products, such as strawberries, blueberries and raspberries are growing popular. From 1990 to 2012, the production of strawberries, blueberries and raspberries in United States of America increased from 568,940 tons to 1,366,850 tons, 79,940 tons to 214,708 tons and 23,650 tons to 100,775 tons, respectively (Food and Agricultre Organization of United Nations, 2014). Berries are healthy fruits containing many micronutrients, like vitamin C and folic acid. Other than those, high content of phytochemicals along with other polyphenols have been found in berries, which has a wide range of potential anti-cancer and heart disease properties (Beattie et al., 2005).

However, several foodborne illness outbreaks are associated with berries associated with both bacterial and viral pathogens. *Escherichia coli* O157:H7 was reported to be involved in an outbreak due to consumption of fresh strawberries (Laidler et al., 2013; Stone and Oregon Health Authority, 2011). Imported frozen berry mix was suspected to be the source of a Hepatitis A virus (HAV) outbreak in Austria in 2013 (Wenzel et al., 2014). In 1990 and 1997, HAV caused two multistate outbreaks associated with frozen strawberries (Niu et al., 1992). Raw blueberries were linked with a HAV outbreak in New Zealand (Calder et al., 2003). In 1997, a large, foodborne outbreak of HAV was involved with consumption of frozen strawberries, which led to 213 illness (Hutin et al., 1999). In addition, frozen strawberries were implicated in a massive human norovirus gastroenteritis outbreak which affected about

11,000 people in Germany, 2012 (Mäde et al., 2013). In 2002, strawberries on wedding cake was linked to a HuNoV outbreak which resulted in 332 illness (Friedman et al., 2005). Raspberries have also been associated with some HuNoV outbreaks (Korsager et al., 2005; Le Guyader et al., 2004). It is estimated that 19–21 million cases of acute gastroenteritis were caused by HuNoV every year, leading to 56,000–71,000 hospitalizations including 570–800 deaths (Hall et al., 2013). Indeed, certain interventions are needed for HuNoV in fresh berries and berry products.

As there is no available in vitro cell culture system or a small animal model for HuNoV, studies of HuNoV usually have to rely on surrogate viruses or molecular biology techniques. Common HuNoV surrogates include MNV-1, feline calicivirus (FCV) and Tulane virus (TV). According to our lab's result, HuNoV GI.1 strain showed highest resistance to HHP treatment, followed by MNV-1, HuNoV GII.4 strain and FCV which made MNV-1 the best surrogate for HHP treatment (Chen et al., 2005; Huang et al., 2014; Li et al., 2013a; Li et al., 2013b).). Other than surrogates, molecular biotechnologies were used to quantify the RNA content to estimate the inactivation of HuNoV. However, methods are still needed for discrimination of infectious virus particles from those impaired virus which still have detectable RNA genomes. It has been demonstrated that histo-blood group antigens (HBGAs) in the human intestinal tract act as receptors of HuNoV (Marionneau et al., 2002). Further, porcine gastric mucin (PGM), which is antigenically-similar to HBGAs, can bind to HuNoV and norovirus-like particles (Tian et al., 2008; Tian et al., 2007; Tian et al., 2010). Dancho et al. (2012) showed that HHP treatment could abolish the ability of HuNoV to bind to PGM-MBs. Li et al. (2013a) demonstrated that a PGM-MB binding assay showed capability to discriminate infectious HuNoV from non-infectious and

reflect HHP inactivation of HuNoV GI.1 and GII.4 strains. Furthermore, comparison between results of the PGM-MB binding assay and results of HuNoV surrogate, human challenge study and original PGM-MB binding assay study results indicated that PGM-MBs could offer reliable discrimination of infectious HuNoV(Chen et al., 2005; Dancho et al., 2012; Leon et al., 2011; Li et al., 2013b).

In the last two decades, HHP has been successfully used to process a variety of foods such as oysters, guacamole, salsa, ready-to-eat meats and juices since it has less detrimental effects on nutrients and can better retain the fresh-like characteristics and flavors of foods compared with thermal processing. Recently, Lou et al. (2011) demonstrated that HHP treatment at 350 MPa and 4 °C for 2 min didn't affect the color, freshness and texture of strawberry puree. Huang et al. (2013) also confirmed that physical properties and visual appearance of strawberry puree could be well preserved during HHP treatments at 250 – 300 MPa and 21 °C for 2 min. It also has been demonstrated that high pressure treatment has a limited effect on pigments (e.g. chlorophyll, carotenoids, anthocyanins, etc.) at low and moderate temperature which made HHP treatment could well preserve the color of fresh produce (Oey et al., 2008).

The overall goal of this study is to investigate high hydrostatic pressure inactivation of human norovirus on strawberries, blueberries, raspberries and in purees and identify suitable HHP treatment condition for commercial use in the future. The project was mainly divided into two parts: First, investigating the effect of different parameters on HHP inactivation of MNV-1 in strawberry puree and on strawberry quarters to provide insights for HHP processing of human norovirus with optimized conditions. Second, verifying HHP inactivation effect of HuNoV GI.1 and GII.4 strains on strawberries, blueberries, raspberries and in purees to offer guidance for

commercial utilization of high pressure processing on fresh berries and berry puree in the future.

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Chapter 2

LITERATURE REVIEW

2.1 Human Norovirus

2.1.1 Characteristics

Human norovirus is a genetically diverse group of single-stranded RNA, non-enveloped viruses, belonging to the *Caliciviridae* family (Austrilian Government Depatment of Health, 2014). HuNoV was first found by Kapikian et al. (1972) from immune electron microscopic examination of stools from a group of elementary school students affected by an outbreak of gastroenteritis in Norwalk, Ohio in 1968 (Adler and Zickl, 1969). HuNoV was previously denoted as "Norwalk-like viruses" until their taxonomy was determined using modern molecular biotechnology. Four genera belonged to the *Caliciviridae* family, including *Norovirus*, *Sapovirus* (also a cause of human gastroenteritis), *Lagovirus*, and *Vesivirus* (Green et al., 2000).

The RNA genome of HuNoV is positive-sense with an approximate length of 7.5 kbp, encoding two proteins: a 58- 60 kDa major structural protein (VP1) and a minor capsid protein (VP2) (Clarke and Lambden, 2000). Prasad et al. (2001) described HuNoV as an amorphous surface structure and the size of it was between 27 – 38 nm. 180 copies of the VP1 major structural protein which are packed as an icosahedron and VP2 minor capsid protein, which has potential stability contribution, formed the capsid of HuNoV (Bertolotti-Ciarlet et al., 2002; Prasad et al., 2001). The VP1 major structural protein consists of five domains: one small N-terminal domain,

one S domain, which forms the inner shell of the capsid, two P1 domains and one P2 domain, which is the hypervariable region of VP1 protein (Hardy, 2005). The P2 domain possesses histoblood group antigen receptor sites and carbohydrate-receptor binding regions (Cao et al., 2007; Donaldson et al., 2008; Lundborg et al., 2013; Tan et al., 2004; Tan et al., 2003).

There are three open reading frames (ORF) in the HuNoV genome (ORF1, ORF2, ORF3) (Jiang et al., 1993). ORF1 is the longest, which encodes a polyprotein that is cleaved by the viral protease into at least six nonstructural proteins including a protease and RNA-dependent RNA polymerase (Sosnovtsev et al., 2006). ORF2 encodes the VP1 major structural protein while ORF3 encodes the VP2 minor capsid protein (Clarke and Lambden, 2000).

Recently, based on RNA-dependent RNA polymerase gene regions of the genome and the VP1 major structural protein, a standardized nomenclature was proposed to classify noroviruses into 29 genetic clusters which fall into five genotypes (GI, GII, GIII, GIV and GV) (Zheng et al., 2006). Genotypes I and II consist of most human disease related HuNoV strains while genotype III is associated with bovine, genotype IV is associated with human and canine and genotype V is associated with murine (Patel et al., 2009).

2.1.2 Clinical and Epidemiologic Features

HuNoV is considered the leading cause of acute gastroenteritis. Atmar and Estes (2006) reported that HuNoV caused 47 – 96% of outbreaks of acute gastroenteritis 5 – 36% of sporadic cases of acute gastroenteritis reported around the world. In the United States, norovirus is the most common cause of acute gastroenteritis, leading to 19-21 million illnesses, 56,000-71,000 hospitalizations and

570-800 deaths each year (Hall et al., 2013). Furthermore, norovirus is also the most common cause of foodborne-disease outbreaks in the United States (Centers for Disease control and prevention, 2014).

The symptoms of HuNoV includes acute onset of nausea, vomiting, abdominal cramps, myalgia, low-grade fever and non-bloody diarrhea (Patel et al., 2009; Pegues and Woernle, 1993). Symptoms of HuNoV infection usually show up 24 – 48 hours after infection and the symptoms will resolve in 2 -3 days (Lopman et al., 2004; Teunis et al., 2008). Deaths were reported in nursing home during HuNoV outbreaks due to severe dehydration (Chadwick et al., 2000; Dedman et al., 1998). HuNoV infection could also cause necrotizing enterocolitis (Turcios-Ruiz et al., 2008). Longer illness was observed among children younger than 11 years old during hospitalization (Lopman et al., 2004; Rockx et al., 2002). The elderly are experiencing higher risk for severe symptoms and death considering the compromised immune system (Donaldson et al., 2008). For most people, only mild symptoms will show up for HuNoV infection and no treatment is required. For patients experiencing severely volume depleted, intravenous fluid and electrolyte replacement may be required. No specific antiviral therapy is currently available (Patel et al., 2009).

Though HuNoV outbreaks happen throughout the year, the peak of HuNoV outbreaks is in colder months in temperate climate (Mounts et al., 2000). Research in England also demonstrated that spring and summer could also be the peaks of HuNoV outbreaks (Lopman et al., 2003). HuNoV can infect persons of all ages (Rockx et al., 2002). Children younger than 5 years old tend to be more susceptible to HuNoV infection (Boga et al., 2004).

2.1.3 Transmission and Outbreaks

The most important mode of transmission of HuNoV is fecal-oral spread (Patel et al., 2009). HuNoV has some significant characteristics that contribute to the spread of HuNoV: first, low dose of HuNoV is needed for infection (< 10 viral particles) (Teunis et al., 2008); second, viral shedding period could be long (one year was recorded) (Simon et al., 2006); third, HuNoV is stable in the environment (resistance to high temperatures up to 60 °C, frozen storage and high concentration of chlorine) (Duizer et al., 2004a); fourth, HuNoV infection could be repeated due to the lack of complete cross-protection against the diverse NoV strains and inadequate long-term immunity (Patel et al., 2009). Transmission of HuNoV could be through infectious vomit, either by mechanical transmission from environmental surfaces (i.e., through hand/mouth contact) or by aerosolization. This might account for the rapid and extensive spread of disease outbreaks in closed settings, such as hospitals, hotels, cruise ships, and day-care centers (Widdowson et al., 2005b). Contaminated environmental surfaces have been recorded as a reservoir for HuNoV during an outbreak (Centers for Disease Control and Prevention, 2008). Restrooms in different facilities and vehicles also served as a source of HuNoV (Ho et al., 1989; Widdowson et al., 2005a).

HuNoV GII strains are the leading cause of most human norovirus infection (Ramani et al., 2014). HuNoV GI strains are usually involved with water-borne outbreaks while GII strains are often linked to food-borne outbreaks or person-to-person transmission (Matthews et al., 2012). Of all HuNoV strains, GII.4 strains turned out to be the predominant strains of HuNoV infection, resulting in approximately 55–85% of the gastroenteritis cases worldwide (Ramani et al., 2014). From the 1990s to early 2013, seven different GII.4 variants were identified for global

epidemics of gastroenteritis (Ramani et al., 2014). In late 2012, Sydney 2012, a new HuNoV GII.4 strain emerged, causing several HuNoV outbreaks in Australia, the United States, Belgium and Denmark (van Beek J, 2013). It should be noted that dominant HuNoV GII.4 strain was largely replaced by a new emerged GII.4 variant every 2 – 3 years (Belliot et al., 2014).

As is mentioned, norovirus is also the most common cause of foodborne-disease outbreaks in the United States (Centers for Disease control and prevention, 2014). Most foodborne outbreaks of HuNoV infection are caused by contaminated food or water by a food handler immediately before its consumption (Acheson et al., 2002). Cold foods and ready-to-eat foods, such as sandwiches, salads and bakery products, are classically involved in outbreaks of HuNoV infection (Parashar et al., 2001). Shellfishes are also commonly linked with HuNoV outbreaks all around the world (Mattison, 2011). In the 1990s, three large outbreaks of HuNoV associated with the consumption of raw oysters were reported from Louisiana, leading to more than 300 illness in total (Niu et al., 1992).

HuNoV has also been identified as a main cause of foodborne pathogen outbreaks in fresh produce (i.e., fruits and vegetables) (Markland et al., 2014), including berries and berry products. Frozen strawberries were implicated in a massive human norovirus gastroenteritis outbreak which affected about 11,000 people (Mäde et al., 2013). In 2002, strawberries on wedding cake was linked to a HuNoV outbreak which 332 illness (Friedman et al., 2005). Raspberries have also been associated with some HuNoV outbreaks (Korsager et al., 2005; Le Guyader et al., 2004).

2.1.4 Pathogenesis and Immunology

Understanding of the norovirus life cycle in human body is hindering due to the lack of proper cell culture system or suitable small animal model (Karst et al., 2015). Previous human challenge experiment showed that infected volunteers developed immunity to HuNoV after challenge (Johnson et al., 1990; Parrino et al., 1977; Wyatt et al., 1974). In two human challenge studies, around 13–40% of volunteers never became infected and only 50% developed HuNoV infection symptoms (Parrino et al., 1977; Wright and Morris, 1991). This could be explained by that persons without antibodies to a particular NoV strain may be immune to be infected as the presence of specific human histo-blood group antigen (HBGA) receptors in the gut of susceptible hosts are key to HuNoV infection (Lindesmith et al., 2003; Patel et al., 2009).

Based on the clinical features of infection, it was indicated that the primary site of human norovirus infection and replication could be small intestine (Karst et al., 2014). However, HuNoV particles were not observed in intestinal biopsies from human norovirus-infected volunteers via electron microscopic visualization (AGUS et al., 1973; Dolin et al., 1975). Several researchers tried to use epithelial cells to cultivate HuNoV *in vitro* but the majority were unsuccessful, including the use of three-dimensional cell cultures to simulate human condition (Duizer et al., 2004b; Herbst-Kralovetz et al., 2013; Papafragkou et al., 2013; Straub et al., 2007; Takanashi et al., 2014; Wobus et al., 2004). Recent research showed that HuNoV could infect human B cells *in vitro* at a low level and this infection process was enhanced by histo-blood group antigens (HBGA) expressed by enteric bacteria (Jones et al., 2014; Miura et al., 2013).

It has been demonstrated that histo-blood group antigens in the human intestinal tract are the receptors of HuNoV (Marionneau et al., 2002). Further, porcine gastric mucin (PGM), which is antigenically-similar to HBGAs, can bind to HuNoV and norovirus-like particles (Tian et al., 2008; Tian et al., 2007; Tian et al., 2010). Increasing HBGAs which can bind to HuNoV GII.4 were identified which might provide an explanation why GII.4 is the predominant strain in the world (Bull and White, 2011).

2.2 Intervention for HuNoV Contamination

2.2.1 Antimicrobial Agents

Antimicrobial agents are substances or mixtures of substances that are used in the food industry to destroy or suppress the growth of harmful microorganisms on foods, fomites, and other surfaces (Hirneisen et al., 2010). Antimicrobials approved for washing of fruits and vegetable includes chlorine, peroxy acids, ClO_2, ozone, acidified sodium chlorite, and organic acids (Baert et al., 2009a).

Chlorine is the most commonly used antimicrobial agent which usually existes as chlorine gas (Cl_2), calcium hypochlorite ($CaClO_2$), and sodium hypochlorite ($NaOCl_2$) (Fonseca and Matthews, 2006). Calicivirus are usually resistant to chlorine treatment. 200 ppm of NaOCl treatment for 5 min could only reduce MNV-1 by 1.0 log on lettuce (Baert et al., 2009b). 1.6 log reduction of FCV was achieved with a 200 ppm of NaOCl treatment for 0.5 min on strawberries (Butot et al., 2008). Less than a 2 log reduction of FCV in medium was achieved with a 300 ppm of NaOCl treatment for 10 min (Duizer et al., 2004a). The unsubstantial chlorine inactivation effect of norovirus makes it difficult for application.

Chlorine dioxide is an oxidizing agent which is extremely soluble in water while due to its instability, it must be generated on site for use (Hirneisen et al., 2010). Thurston-Enriquez et al. (2005a) reported that 0.9 mg/L chlorine dioxide treatment at 5 °C could reduce FCV by 3.6 in 45 s at pH 8 while 20 min would be needed to achieve same inactivation effect at pH 6.

Ozone is considered as a broad-spectrum oxidizing agent which can be applied to inactivate bacteria, fungi, and their spores, viruses, and protozoa (Hirneisen et al., 2010). FCV could be reduced by 4.28 log with a 15s ozone treatment at 1.0 mg/L and 1.85 log at 0.06 mg/L (Thurston-Enriquez et al., 2005b).

Peroxyacetic acid (PAA) has also been examined in inactivation of viral pathogens. Eighty ppm of PAA treatment for 5 min could reduce MNV-1 by 0.8 log on lettuce (Baert et al., 2009a). Three hundred ppm of PAA treatment for 10 min could reduce FCV by 3 log on strawberries while 150 ppm treatment could only achieve 1 log reduction of FCV (Gulati et al., 2001).

2.2.2 Thermal Processing

Thermal processing is a traditional way to prevent microbial contamination which could be divided into pasteurization and sterilization. Dolin et al. (1972) proved that human norovirus remained infectious after a 60 °C heating treatment for 30 min via a human challenge study, indicating that HuNoV had moderate heating resistance. While for MNV-1, a 80 °C heat exposure could achieve ~ 7.8 log reduction in 150 s Baert et al. (2008c). However, in order to maintain fresh berry flavor and nutritional contents as increasingly demanded by consumers, the pasteurization processes are sometimes mitigated and even omitted (Huang et al., 2014). Unfortunately, those mitigated thermal pasteurization process are sometimes not sufficient to eliminate

pathogens (Baert et al., 2008a). In that study, nearly no reduction of MNV-1 was observed with mild heat treatments of 30 s at 65 °C and 15 s at 75 °C in raspberry puree.

Low temperature storage also showed little inactivation effect of norovirus. Richards et al. (2012) showed that no significant reduction of HuNoV GII.4 in water after 14 freezing/thawing cycles (− 80 °C/+ 22 °C) or 120-day frozen storage at − 80 °C. Baert et al. (2008b) showed that no reduction of MNV-1 was observed on frozen onions and spinach during 6-month storage at − 21 °C. Horm and D'Souza (2011) observed a < 1-log reduction of MNV-1 in orange juice stored at 4 °C for 21 days.

2.2.3 Nonthermal Processing

2.2.3.1 Ultraviolet Light

Ultraviolet (UV) light is electromagnetic radiation with wavelengths shorter than visible light that can induce damage in a variety of organisms (Hirneisen et al., 2010).Based on wavelength, UV light can be divided into different ranges including UVA (400 to 320 nm), UVB (320 to 280 nm), and UVC (280 to 100 nm). UVC is commonly used for microbial inactivation due to the mutation inducing ability at this wavelength range (Kowalski et al., 2000). The predominant mechanism underlying UV inactivation of virus is causing mutation of viral nucleic acid while high enough UV light dose (> 1000 mJ /cm^2) could also affect virus capsid (Nuanualsuwan and Cliver, 2003; Sena and Jarvis, 1981; Smirnov et al., 1983).

Lee et al. (2008) demonstrated that 25 mJ /cm^2 UV treatment could inactivate MNV-1 by 3.3 log in PBS. As for FCV, 36 mJ/cm^2 UV treatment achieve 4 log reduction in buffered demand-free water while in wastewater, 19.4 mJ/cm^2 UV

treatment achieve 4 log reduction (Thurston-Enriquez et al., 2002; Tree et al., 2005). Nuanualsuwan et al. (2002) demonstrated that 125 mJ /cm^2 UV treatment could ~ 2.9 log reduction of FCV in cell culture medium. A lot of factors contribute to the efficacy of UV inactivation effect of norovirus, like viral strain, virus aggregation, produce topography and experimental conditions (Fino and Kniel, 2008).

2.2.3.2 Irradiation

Ionizing radiation targets at both RNA and DNA. There are different types of ionizing radiation including X-rays, gamma-rays and beta-rays (Hirneisen et al., 2010; Josephson, 1983). Usually the smaller the pathogens are, the more resistant to ionizing irradiation. Due to their small size and genome, norovirus is more resistant to ionizing irradiation inactivation compared to bacteria (Farkas, 1998; Patterson, 1993). Gamma irradiation at 0.5 and 0.3 kGy achieved 3-log decreases in FCV and canine norovirus titers, respectively, in low protein solutions, but high amounts of protein appreciably reduced the effectiveness of ionizing irradiation (de Roda Husman et al., 2004).

2.2.3.3 Pulsed Electric Field

Pulsed electric field (PEF) processing uses short bursts of electricity for microbial inactivation while minimizing side effects on food quality (Hirneisen et al., 2010). Few studies have focused on the effects of PEF inactivation on norovirus. (Khadre and Yousef, 2002) observed that rotavirus of varying concentrations was resistant to PEF treatment of 20 to 29 kV/cm for 145.6 μs.

2.3 High Hydrostatic Pressure

High hydrostatic pressure (HHP), also known as high pressure processing (HPP) has been applied to numerous kinds of food, both liquid and solid foods (Farkas

and Hoover, 2000). During HHP, foods are usually subjected to pressures between 100 MPa and 800 MPa with or without additional heat to achieve desirable microbial inactivation effect while maintaining certain food qualities (Ramaswamy et al., 2004). Comparing to common used thermal pasteurization, HPP could provide consumers with better appearance, texture, taste and nutrition, especially for heat-sensitive foods (Ramaswamy et al., 2004). The very first commercial HPP-applied product was a line of jams, jellies and sauces by Meidi-ya in Japan (Thakur and Nelson, 1998). Currently, HPP has been used to process a wide range of foods, like shellfish, cooked or cured ham, guacamole, fruit jellies, juices and ready-to-eat (RTE) products (Rastogi et al., 2007). Currently, commercial size batch type HPP equipment has been made by Avure Technologies (Kent, WA), reaching up to 215 liter capacity. Besides inhibiting undesirable enzymes and microorganisms, HPP has also been used in seafood shucking, like lobsters and oysters (Hirneisen et al., 2010). Furthermore, other technologies, like traditional thermal processing, irradiation, alternating current, ultrasound, carbon dioxide and antimicrobial peptides have been combined with HPP to achieve better effect of food processing.

2.3.1 Theory of HHP

According to Le Chatelier's principle, biochemical and physicochemical phenomena in equilibrium are accompanied by changes in volume and are thus influenced by pressure. Pressure affects reaction systems in two possible ways: reduction in the available molecular space and enhancement of inter-chain reactions (Hoover et al., 1989). During HHP treatment, covalent bond is not affected with exceptions of sulphydryl groups and thiol-disulphide interchange reactions (Funtenberger et al., 1997). Without disruption of covalent bond, flavor of food is well

preserved. At relative low pressure level hydrogen bonds appear to be strengthened while at high pressure levels (> 600 MPa), hydrogen bonds will be disrupted (Hendrickx et al., 1998; Knorr, 1995). Hydrogen bonds stabilize the secondary structure of protein. Thus, extremely high pressure level processing will lead to denaturation of proteins (Knorr, 1995). Between 400 and 600 MPa, proteins are readily denatured (Jay, 1978). With the decrease of volume during HHP treatment, ionic and possible hydrophobic bonds will be disrupted, which will lead to changes of tertiary structure of proteins (Hendrickx et al., 1998).

The high hydrostatic pressure will be applied to all parts of a food, regardless of its size or shape which enables food to be treated evenly throughout. This overcomes problems that might be found in other processing techniques, like thermal conduction. Compared to thermal processing, there is no heating and cooling period for HHP treatment, making the treatment cycles shorter and more likely to be combined with other techniques (Fellows, 2009). Energy is only consumed during pressure building up period, leading to less energy consuming than other techniques (Farr, 1990).

HHP will produce adiabatic compression heating: when a medium is pressurized, the temperature goes up due to the compressive work against intermolecular forces (Fellows, 2009). Adiabatic temperature increase rate of water is 3 °C per 100 MPa at initial temperature of 30 °C while for oils, it is ~ 9 °C per 100 MPa (Rasanayagam et al., 2003). It should be noted that under adiabatic conditions, the higher the initial temperature, the greater the temperature increase rate (°C/MPa) is. Food composition, temperature and the geometry of the processing equipment will

affect the actual adiabatic compression heating (Hartmann and Delgado, 2002; Otero et al., 2007).

2.3.2 HHP Equipment

Currently, nearly all commercial HHP equipment is batch HHP equipment. Similar to the processing of batch-type thermal processing retort system, batch HHP processing consists of loading food products into pressure vessel, closing and sealing the vessel, bringing up the pressure level in the vessel, venting the vessel and removing the foods out. A batch HHP equipment usually consists of: (1) a pressure vessel, usually cylindrical design, (2) two end closures, (3) a means for restraining the end closures (for example, yoke, threads, pin), (4) a low pressure pump, (5) an intensifier which uses liquid to generate a higher pressure level from the low pressure pump, (6) a controlling system. The pressure vessel is a key part. It needs to be able to hold high pressure. For running under 400 MPa, single cylinder of high tensile strength steel should be enough. For higher pressure demand, usually two or more concentric cylinders would be needed. The outer cylinder may also be wire wound or immersed in liquid-filled, permanent pressurized outer cylinder to ensure routine procedures. The intensifier amplifies the pressure based on the well-known principle of hydraulic pressure amplification using the area ratio of two faces of the main piston. Usually the two faces are exposed to different liquid, generally oil (Farkas and Hoover, 2000).

In the present, commercial HHP equipment can reach up to a volume of 687 liters with a limit of 300 MPa or a volume of 525 liters with a limit of 500 MPa (Avure Technologies, Kent WA). Other than batch-type HHP equipment, semi-continuous HPP equipment used a low pressure food pump to fill the pressure vessel

and then introduced high pressure water to compress the food. Furthermore, some ideas about continuous HHP equipment were proposed, but no commercial continuous HPP equipment showed up (Farkas and Hoover, 2000; Torres and Velazquez, 2005).

2.3.3 HHP Inactivation of Virus

2.3.3.1 Mechanism of HHP Inactivation of Virus

Different virus showed various resistances to HHP treatment, indicating that mechanism underlying HHP inactivation could vary from one virus to another. For instance, to achieve 7 log reduction, HAV requires a HHP treatment at 450 MPa and 22 °C for 5 min while FCV only need a HHP treatment at 275 MPa and 22 °C for 5 min (Kingsley et al., 2002).

One hypothetical mechanism of HHP inactivation of virus is that HHP could dissociate and/or denature the capsid for viruses or impair the envelop of the enveloped viruses (Hirneisen et al., 2010). Silva et al. (1992) found that a bulge showed up on the surface of pressurized vesicular stomatitis virus, indicating capsid subunit might be misplaced under the envelop. Lou et al. (2011) also found that 350, 500 and 600 MPa HHP treatments disrupted the structure of MNV-1 caspid but primary and secondary structures of the VP1 protein, a major capsid structural protein, were not disrupted. Pressurized herpes simplex virus type 1 and human cytomegalovirus had damaged virus envelop via electron microscopic examination (Nakagami et al., 1992). Other than the capsid and envelop disruption, attachment protein could be damaged during HHP treatment, resulting in loss of infectious ability (Kingsley et al., 2002; Pontes et al., 2001). Another theory is that HHP destroyed the virus structure, releasing the genome of the virus, which could prevent the virus from

replication (Hirneisen et al., 2010). 500 MPa HHP was treated to HAV, which showed no release of RNA genome after treatment, even the capsid remained intact (Kingsley et al., 2002).

2.3.3.2 Factors Influencing HHP Inactivation of Virus

Numerous factors could make an impact on HHP inactivation of virus, like treatment time, pressure level, sample temperature, pH, pressure oscillation, target virus and food matrix properties (Hirneisen et al., 2010).

Generally HHP inactivation effect of virus would be enhanced with increasing treatment time. Chen et al. (2005) investigated pressure inactivation curves of FCV strain KCD in Dulbecco's modified Eagle medium with 10% fetal bovine serum at 200 and 250 MPa as a function of time at room temperature. Tailing effect was observed in both inactivation curves, indicating that pressure inactivation rate was higher at the beginning phase of the HHP treatment. However, HHP inactivation effect of MNV-1 showed a linear relationship with treatment time at 350 MPa (Huang et al., 2014).

Better HHP inactivation effect of virus is usually obtained at higher pressure level. It should be noted that there seemed to be certain threshold of pressure of pressure inactivation of virus. Significant different viral inactivation rates were observed at different pressure level during HHP inactivation of HuNoV GI.1 and GII.4 strain (Li et al., 2013a). In that study, when pressure level increased from 450 to 500 MPa, the log reduction of GI.1 strain increased from 0 to 0.5 while when pressure level increased from 500 to 550 MPa, log reduction increased from 0.5 to 2.2. Similar results were obtained at different temperature and in HuNoV GII.4 strain. However, pressure inactivation of MNV-1 showed a linear relationship with pressure level (Kingsley et al., 2007).

Sample temperature played an important role in pressure inactivation of virus. Lower sample temperature tends to have better pressure inactivation effect of virus (Hirneisen et al., 2010). Li et al. (2013a) demonstrated that lower sample temperature could enhance pressure inactivation of HuNoV GI.1 and GII.4 strains. At 21 °C, HuNoV GI.1 strain wasn't inactivated at all with a 2 min HHP treatment at 450 MPa while at 1 °C, > 3.0 log reduction of GI.1 strain was achieved. Similar results were also observed in HuNoV GII.4 strain, MNV-1, FCV and TV (Chen et al., 2005; Huang et al., 2014; Li et al., 2013a; Li et al., 2013b; Ye et al., 2014).

Enhanced pressure inactivation of HuNoV was obtained at neutral pH compared to lower pH. When being pressure-treated at 550 MPa for 2min, HuNoV GI.1 strain was inactivated by 3 log at pH 7 while only 1.5 log reduction was achieved at pH 4. MNV-1 and TV also showed more resistant to HHP treatment in an acidic environment compared to a pH neutral environment (Li and Chen, 2014). Kingsley and Chen (2008) also proved that FCV could be more easily inactivated by pressure treatment at neutral pH than lower pH.

Pressure oscillation was few investigated in viral inactivation with variant outcomes. Only slightly increase MNV-1 inactivation was observed in higher frequent pressure cycling than no cycling (Huang et al., 2014). Kingsley et al. (2006) also found that pressure cycling with 2, 4, 6 and 8 cycles did not significantly enhance HAV inactivation compared with continuous HHP treatment when HAV in DMEM were treated at 400 MPa and 20 °C or 400 MPa and 50 °C. On the other hand, Bradley et al. (2000) found that the inactivation of phage λ, a bacterial virus, was improved by pressure cycling. For an equal holding time of 7.5 min at 275 MPa, phage λ was

inactivated by an additional 2 log PFU/ml as the number of cycles increased from 1 to 5.

Pressure inactivation of virus also relies on the intrinsic characteristic of target virus and food matrix properties. When treated at HHP condition, HuNoV GI.1 tended to be the most resistant virus followed by MNV-1, HuNoV GII.4 and FCV (Chen et al., 2005; Huang et al., 2014; Kingsley and Chen, 2008; Li et al., 2013a). It should be noted that even within the same HuNoV genotype, different variants might still reacts in various ways to HHP treatment (Ramani et al., 2014). Sometimes, food matrix might provide protective effect for virus. Kingsley et al. (2009) demonstrated that HAV was more resistant to HHP treatment when blended into oyster homogenate compared to in NaCl solutions.

2.3.3.3 HHP Inactivation of Norovirus

Due to the lack of available *in vitro* cell culture system or a small animal model for HuNoV, research about pressure inactivation of norovirus relied on using HuNoV surrogates and molecular biotechnology. As is shown in Table 2.1, human norovirus and its surrogate showed different resistance to HHP treatment in cell culture medium or on berries. Kingsley et al. (2002) demonstrated that a HHP treatment at 275 MPa and 21 °C for 2 min could completely inactivate FCV (> 7 log reduction) in DMEM supplemented with 10% FBS. In 2005, a HHP treatment at lower pressure level and lower temperature (200 MPa and – 10 °C) for 2 min could reduce FCV by 5 log (Chen et al., 2005). Deeper and more comprehensive behavior of FCV towards HHP was investigated by Buckow et al. (2008) in both DMEM and mineral water. MNV-1 could be pressure inactivated by 6.85 log at 450 MPa and 20 °C for 5 min (Kingsley et al., 2007). Furthermore, HHP was applied to inactivate MNV-1 in

food, such as strawberries, blueberries and strawberry puree (Kovač et al., 2012; Li et al., 2013b; Lou et al., 2011). Kovač et al. (2012) also proved that under similar HHP treatment condition, MNV-1 would be more easily inactivated in water than in strawberry puree. A HHP treatment at 350 MPa and 4 °C for 2 min could reduce titers of TV by > 4.0 log in DMEM (Li et al., 2013b). HHP treatment was tested for inactivation of HuNoV via PGM-MB binding assay, which offered good discrimination of infectious HuNoV particles, and qRT-PCR. Li et al. (2013a) found that a HHP treatment at 450 MPa and 1 °C for 2 min could reduce titers of HuNoV GI.1 strain by > 3.0 log in PBS; a HHP treatment at 350 MPa and 21 °C for 2 min could reduce titers of HuNoV GII.4 strain by > 4.0 log in PBS. As for HHP inactivation of HuNoV GI.1 strain on blueberry with water surrounding the berries, more intense HHP treatment condition (500 MPa/1 °C /2 min or 600 MPa/21 °C /2 min) was needed compared to in PBS (Li et al., 2013a).

Table 2.1 Pressure inactivation of HuNoV and its surrogates

Virus Name	Substrate	HHP condition			Log reduction	Reference
		Pressure (MPa)	Temp. (°C)	Time (min)		
HuNoV GI.1	PBS	450	1	2	> 3.0	(Li et al., 2013a)
HuNoV GII.4	PBS	350	21	2	>4.0	(Li et al., 2013a)
HuNoV GI.1	Blueberry with water	500	1	2	2.7	(Li et al., 2013a)
HuNoV GI.1	Blueberry with water	600	21	2	> 3.0	(Li et al., 2013a)
MNV-1	Strawberry puree	400	4	5	> 3.33	(Kovač et al., 2012)
MNV-1	Water	400	4	4	> 5.13	(Kovač et al., 2012)
MNV-1	Blueberry with water	400	4	2	> 5.7	(Li et al., 2013b)
MNV-1	DMEM with 10% FBS	500	25	15	2.23	(Sánchez et al., 2011)
MNV-1	Strawberry puree	400	4	2	5.45	(Lou et al., 2011)
MNV-1	Strawberry quarter	350	4	2	2.2	(Lou et al., 2011)
MNV-1	DMEM with 10% FBS	450	20	5	6.85	(Kingsley et al., 2007)
TV	Blueberry with water	350	4	2	> 4.0	(Li et al., 2013b)
FCV	DMEM with 10% FBS	200	- 10	4	5.0	(Chen et al., 2005)
FCV	DMEM with 10% FBS	275	21	5	> 7.0	(Kingsley et al., 2002)
FCV	DMEM or mineral water	450	15	1	> 7.0	(Buckow et al., 2008)

2.3.4 HHP Impact on Quality of Berries and Berry Products

Beyond the increasing concern of safety of berries and berry products, more attention is drawn to the quality issue, including sensory attributes and nutritional compounds. The effect of HHP on color, texture and flavor has been comprehensively investigated in fruits and vegetables. Researches of HHP impact on nutritional compound focus on two parts: micronutrients (vitamins and polyphenols) and macronutrients (proteins and lipids).

According to Pilar Cano et al. (2005), texture of fruit and vegetable mainly relies on the structure of cell wall and middle lamella. HHP has been confirmed to have a softening impact on fruits and loss of tissue firmness was observed during HHP treatment (Belie et al., 2002). Slight softening was found in strawberries, blueberries and raspberries after a 350 MPa pressure treatment at 4 °C for 2 min; HHP treated strawberry quarters were described as partially lost the opacity of the inner white tissue, appearing more translucent (Lou et al., 2011). Huang et al. (2013) reported that increasing viscosity of strawberry puree was recorded after 2 min HHP treatment at either 250 or 300 MPa and 21 °C (from 5.1 Pa S to 5.8 and 6.1 Pa S, respectively). Nearly no significant texture change of strawberry puree was observed after 600 MPa pressure treatment (Lou et al., 2011).

Ludikhuyze and Hendrickx (2001) stated that once thresholds of temperature and/or pH were determined, color of most fruit could be easily preserved during high pressure processing. A maximum increase of 8.8% in L∗a∗/b∗ parameter was found for strawberry juice samples (pH 5) during a combination of thermal (100–140 °C, 0–120 min) and high pressure thermal (300–700 MPa, 60 min, 65 °C) treatments (Rodrigo et al., 2007). Huang et al. (2013) demonstrated that all three parameters

would experience an increase for pressure treated (250 or 300 MPa/21 °C /2 min) strawberry puree. 96% color was retained in strawberry puree after a pressure treatment at 600 MPa for 15 min (Patras et al., 2009).

Compared to thermal processing, nearly no loss or change of flavor would be observed in high pressure processing (Gamlath and Wakeling, 2011). Lambadarios and Zabetakis (2002) found that little impact was done by high pressure processing to strawberry flavor compounds while the best flavor retention pressure level would be 400 MPa. An interesting result was that amount of total phenols in strawberry samples was increasing as the pressure increases in high pressure processing, whereas the amount of total phenols was lower in thermally processed samples than the amount in unprocessed samples (Patras et al., 2009).

Ascorbic acid (known as Vitamin C) is the focus of researches of HHP effect on vitamins in fruits (Gamlath and Wakeling, 2011). No significant loss (> 90% retained) of vitamin C was found in strawberry puree after a HHP treatment at 400, 500 and 600 MPa and 10 – 30 °C for 15 min (Patras et al., 2009). Oey et al. (2008) have reported that the anthocyanin content of various fruits had minimally affected from HHP treatment at room temperature.

Sugar content, protein and lipid are usually considered in macronutrients research works. Butz et al. (2003) reported that a HHP treatment at 600 MPa and 25 °C for 15 min could retain more than 95% of sucrose in strawberry and raspberry. Soluble solids content in strawberry puree wasn't affected during a HHP treatment (250 or 300 MPa/21 °C /2 min) (Huang et al., 2013).

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Chapter 3

STRATEGIES TO ENHANCE HIGH PRESSURE INACTIVATION OF MURINE NOROVIRUS IN STRAWBERRY PUREE AND ON STRAWBERRIES

3.1 Abstract

Due to the increasing concern of viral infection related to berries, this study investigated strategies to enhance high hydrostatic pressure (HHP) inactivation of MNV-1, a human norovirus surrogate, on strawberries and in strawberry puree. Strawberry puree was inoculated with ~10^6 PFU/g of MNV-1 and treated at 350MPa for 2 min at initial sample temperatures of 0, 5, 10 and 20 °C. MNV-1 became more sensitive to HHP as initial sample temperature decreased from 20 to 0 °C. To determine the effect of pressure cycling on MNV-1 inactivation, inoculated puree samples were treated at 300 MPa and 0 °C with 1, 2 and 4 cycles. Pressure cycling offered no distinct advantage over continuous HHP treatment. To determine the effect of presence of water during HHP on MNV-1 inactivation, strawberries inoculated with ~4×10^5 PFU/g of MNV-1 were either pressure-treated directly (dry state) or immersed in water during pressure treatment. MNV-1was very resistant to pressure under the dry state condition, but became sensitive to pressure under the wet state condition. The inactivation curves of MNV-1 in strawberry puree and on strawberries were obtained at 300 and 350 MPa and initial sample temperature of 0 °C. Except for the curve of strawberries treated at 350 MPa which had a concave downward shape, the other three curves were almost linear with R^2 value of 0.99. The fate of MNV-1 in

the untreated and pressure-treated strawberries and strawberry puree during frozen storage was determined. The virus was relatively stable and only reduced by 1.2 log during the 28-day frozen storage. In all, this study provides practical insights of designing strategies using HHP to inactivate HuNoV on strawberries and in strawberry puree assuming that HuNoV behaved similarly to MNV-1 when treated by HHP.

3.2 Introduction

Strawberry is a healthy fruit which is rich in vitamin C, folic acid, anthocyanins as well as other polyphenols with a potential of anti-cancer and heart disease attributes (Beattie et al., 2005). In the USA, the production of strawberries increased from 606 thousand tons in 1992 to 1,294 thousand tons in 2010 (Food and Agricultre Organization of United Nations, 2013). However, recent foodborne illness outbreaks associated with strawberries have raised concerns about the microbial safety of these small fruits. Both bacterial and viral pathogens have caused outbreaks in fresh strawberries and strawberry products. *Escherichia coli* O157:H7 was reported to be involved in an outbreak due to consumption of fresh strawberries (Laidler et al., 2013; Stone and Oregon Health Authority, 2011). Strawberries, raspberries and blueberries have also been frequently associated with foodborne viral outbreaks (Calder et al., 2003; Cotterelle et al., 2005; Friedman et al., 2005; Hutin et al., 1999; Korsager et al., 2005; Le Guyader et al., 2004; Mäde et al., 2013; Niu et al., 1992). In 1990 and 1997, hepatitis A virus (HAV) caused two multistate outbreaks associated with frozen strawberries (Niu et al., 1992). In 2012, frozen strawberries were implicated in a massive HuNoV gastroenteritis outbreak which affected about 11,000 people (Mäde et al., 2013). Indeed, HuNoV is the leading cause of foodborne illnesses in the USA. It is estimated that 19-21 million cases of acute gastroenteritis were caused by HuNoV

every year, leading to 56,000-71,000 hospitalizations including 570 - 800 deaths (Hall et al., 2013).

Strawberries are usually sold as fresh or processed into frozen berries, puree or other products. Strawberries destined for fresh market are usually picked by hands and not washed before sale for the sake of fruit quality and shelf-life. In the production of frozen strawberries and berry puree, fresh berries are washed with a mild sanitizer such as chlorine before being subjected to further processing such as freezing (for frozen berries) and thermal pasteurization (for puree). The reported outbreaks associated with frozen strawberries demonstrate that the sanitizer wash probably reduces, but does not eliminate, the risk of viral infection. Therefore, interventions are urgently needed for frozen strawberries to prevent outbreaks. Berry puree is usually pasteurized to extend shelf life and enhance food safety. However, in order to maintain fresh berry flavor and nutritional contents as increasingly demanded by consumers, the pasteurization processes are sometimes mitigated and even omitted (Deuel & Plotto, 2004). Unfortunately, those mitigated thermal pasteurization processes are sometimes not sufficient to eliminate pathogens (Baert et al., 2008).

In the last two decades, high hydrostatic pressure (HHP) has been successfully used to process a variety of foods such as oysters, guacamole, salsa, ready-to-eat meats and juices since it has less detrimental effects on nutrients and can better retain the fresh-like characteristics and flavors of foods compared with thermal processing. Recently, Huang et al. (2013) found that *E. coli* O157: H7 and *Salmonella* in strawberry puree could be effectively inactivated by a pressure treatment of 300 MPa for 2 min at 21 °C with a subsequent 4-day frozen storage at -18 °C. Lou et al. (2011) also found that MNV-1, a common surrogate for HuNoV, in strawberries and puree

could be inactivated by HHP at levels ≤ 450 MPa. In their study, strawberries and puree were treated at 350-450 MPa for 2 min at two initial sample temperatures of 4 or 20 °C. They reported that the 4 °C pressure treatments had better pressure inactivation of MNV-1 than the 20 °C treatments.

Since the capital costs of HHP equipment increase exponentially with operating pressure level and the processing and maintenance costs are also positively correlated to it, it is economically beneficial to use lower pressure levels to achieve the target pathogen reduction. The overall goal of this study was, therefore, to explore ways to enhance pressure inactivation of MNV-1 in strawberries and puree so that HHP processing could be conducted at a lower pressure level. First, the pressure inactivation of MNV-1 was compared at four initial sample temperatures of 0, 5, 10 and 20 °C to identify the best temperature. Second, the effect of pressure cycling (cycling between high pressure and atmospheric pressure) was investigated since some studies have shown that it could increase the pressure inactivation of microorganisms (Bradley et al., 2000; Hayakawa et al., 1994; Palou et al., 1998). Third, the influence of presence of water during HHP was studied since Li et al. (2013b) showed that MNV-1 on wet blueberries was more effectively inactivated than that on dry blueberries. Fourth, the pressure inactivation curves were obtained to help identify optimum processing conditions. Finally the fate of MNV-1 during frozen storage was determined.

3.3 Materials and Methods

3.3.1 Virus and Cell lines

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 Dulbecco's modified eagle medium (DMEM) (Gibco, Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37 °C under a 5% CO_2 atmosphere. To prepare MNV-1stock, confluent RAW264.1 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h incubation at 37 °C under a 5% CO_2 atmosphere, 25 ml of DMEM supplemented with 2% FBS was added. MNV-1 was harvested 2 days after post-inoculation by three freeze-thawing cycles and centrifugation. Virus was stored at −80 °C until use.

3.3.2 Inoculation of Strawberries and Puree

Fresh strawberries were purchased from a local store the day before each experiment and stored at 4 °C until use. To prepare strawberry puree, strawberries were washed by tap water and dried in a strainer. Calyxes of the strawberries were removed with a flame-sterilized stainless knife and strawberries were blended in a food blender at high speed for 1 min until no chunk of strawberries was visible. Strawberry puree (0.2, 1 and 2 g) was inoculated with the MNV-1 stock (20, 100 and 200 µl, respectively) to a final level of ~ 10^6 PFU/g and bagged in individual sterile stomacher bag, sealed and double-bagged. To prepare strawberries for HPP treatment, they were cleaned and their calyxes were removed. The whole strawberries were then cut into quarters (~5 g/quarter) from the top to the bottom with a flame-sterile knife since the pressure chamber could not hold a whole strawberry. Strawberry quarters were spot-inoculated with the MNV-1 stock (200 µl) to a level of ~ 4×10^5 PFU/g on

the surface and dried for 2 h in a biosafety cabinet without UV light for virus attachment. Each strawberry quarter was either placed into an empty sterile stomacher bag (dry state) or a bag containing 5 ml of sterile double distilled water (ddH_2O) (wet state). The bags were sealed and double-bagged.

3.3.3 Effect of Initial Sample Temperature and Pressure Cycling on Pressure Inactivation of MNV-1 in Strawberry Puree

All the pressure treatments were conducted using a pressure unit using water as a hydrostatic medium with temperature control (model Avure PT-1; Avure Technologies, Kent, WA). The pressure come-up rate was approximately 22 MPa/s. The pressure-release was almost immediate (< 4 s). Pressurization time reported in this study did not include the pressure come-up or release times. A thermal couple was inserted in a strawberry quarter or strawberry puree to measure the sample temperature during pressure treatment. Temperature increases during pressure treatment due to adiabatic heating were 2.1, 2.2, 2.4, and 2.7 °C/100 MPa at initial sample temperatures of 0, 5, 10, and 20 °C, respectively. To determine the effect of temperature on pressure inactivation of MNV-1 in strawberry puree, puree samples were treated at 350 MPa for 2 min at initial sample temperatures of 0, 5, 10 and 20 °C. To determine whether pressure cycling would enhance virus inactivation, puree samples were treated at 300 MPa and initial sample temperature of 0 °C with 1 cycle of 2 min, 2 cycles of 1 min/cycle and 4 cycles of 30 s/cycle. Control samples (without pressure treatments) were prepared in the same way as the pressure-treated samples. To determine the pressure inactivation effect of MNV-1 in strawberry puree during pressure come-up and release periods, puree samples were treated at 300 and 350 MPa at 0 and 20 °C without the pressure holding time period. After treatments, surviving virus in the

samples was extracted and quantified by a viral plaque assay as described in Section 3.3.6 and 3.3.7.

3.3.4 Effect of Presence of Water during HHP on Pressure Inactivation of MNV-1 on Strawberries

Dry-state (without water in the bags) and wet-state (with 5 ml of water in the bag) strawberry quarter samples were treated at 300 MPa for 2 min at initial sample temperatures of 0 and 20 °C. After treatments, surviving virus in the treated and un-treated samples was extracted and quantified by the viral plaque assay. For the wet-state samples, the 5 ml of water were also quantified for MNV-1 by the viral plaque assay.

3.3.5 Pressure Inactivation Curves of MNV-1 and the Effects of Post-HHP Frozen Storage on Virus Survival on Strawberries and in Puree

To obtain pressure inactivation curves of MNV-1, strawberry puree and wet-state quarter samples were treated at 300 for 1-5 min and 350 MPa for 1-3 min all at initial sample temperature of 0 °C. To determine the fate of MNV-1 during post-HHP frozen storage, strawberry puree and wet-state quarter samples were treated at 300 and 350 MPa for 1 min at initial sample temperature of 0 °C and immediately stored at -20 °C for 0, 7, 14, 21 and 28 days. After treatments, surviving virus in the treated and un-treated samples was extracted and quantified by the viral plaque assay.

3.3.6 Extraction of MNV-1 from Strawberry Puree and Strawberry

MNV-1 was extracted from the strawberry puree and quarter samples using the method described by (Kingsley et al., 2002) with minor modifications. Individual strawberry puree sample was transferred into a sterile stomacher bag and individual strawberry quarter sample into a sterile stomacher bag with filter (Whirl-Pak, Nasco,

USA). Nine volumes of vegetable extraction buffer (100 mM Tris (Thermo Fisher Scientific Inc.), 50 mM glycine (Promega Corporation), 3% (m/v) beef extract (Becton Dickinson Company), 50 mM $MgCl_2$ (Thermo Fisher Scientific Inc.), pH 9.5) was added to the bags and the samples were homogenized by a stomacher (Seward 400, Seward, London, U.K.) at 220 RPM for 1 min. The homogenate was incubated at 37 °C for 30 min. Puree was pelleted at 10,000 × g for 15 min at 4 °C. One volume of 16% (m/v) PEG8000 (Thermo Fisher Scientific Inc.) and 0.525M NaCl (AMRESCO LLC) were added to the supernatant, followed by incubation on ice for 1 h. The mixture was then centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was resuspended in 500 µl phosphate buffered saline.

3.3.7 Viral Plaque Assay

The MNV-1 was quantified using the procedure 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 ~2×10^6 cells per well. After 24 h of incubation, cell monolayers were infected with 400 µl 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, 10mM HEPES (pH 7.7), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2mM L-glutamine (Life Technologies Corporation) and 0.5% (w/v) low-melting agarose (SeaPlaque, Lonza Group Ltd.). Plates were incubated at 37°C in 5% CO_2 for 48 h, fixed in 3.7% formaldehyde for 30 min and plaques were then visualized by staining with 0.05% (w/v in 10% ethanol) crystal violet.

3.3.8 Statistical Analysis

Three independent trials were conducted for all the experiments. Virus counts were converted into log PFU/g and expressed as mean ± standard deviation. Statistical analyses were performed using JMP (SAS Cary, NC, USA). A P value < 0.05 was considered significantly different among treatments using one-way analysis of variance and Tukey's multiple comparisons.

3.4 Results

3.4.1 Effect of Initial Sample Temperature on Pressure Inactivation of MNV-1 in Strawberry Puree

The extraction rate of MNV-1 from strawberry puree was approximately 15%. As shown in Fig. 3.1, initial sample temperature had a significant impact on pressure inactivation of MNV-1 in strawberry puree. Within the temperature range tested, MNV-1 became more sensitive to HHP as treatment temperature decreased from 20 to 0 °C. A 2-min treatment at 350 MPa and 0 °C reduced the titer of MNV-1 by 4.4 log; while at 20 °C the same treatment only reduced MNV-1 by 0.5 log. During the pressure come-up and release periods, the 350-MPa treatment at 20 and 0 °C reduced MNV-1 in the samples by 0.1 and 0.3 log, respectively.

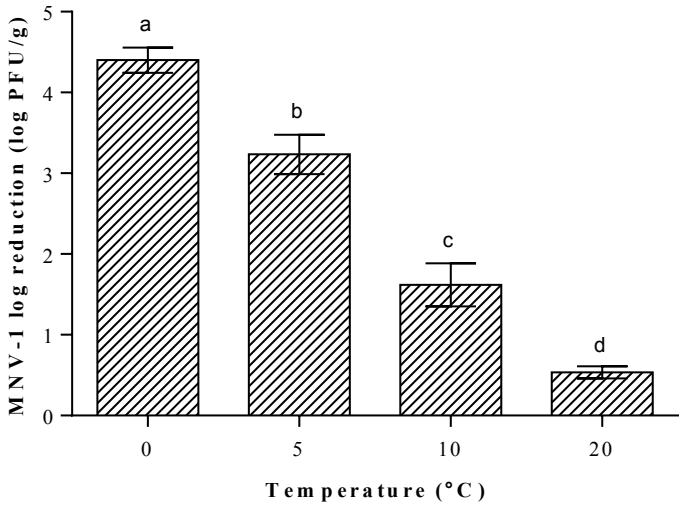

Figure 3.1: Effect of initial sample temperature on HHP inactivation of MNV-1 in strawberry puree. Puree samples with initial MNV-1 inoculation level of ~10^6 PFU/g were treated at 350 MPa for 2 min at initial sample temperatures of 0, 5, 10, and 20 °C, respectively. Error bars represent one standard deviation. Bars with same lowercase letter are not significantly different ($p > 0.05$).

3.4.2 Effect of Pressure Cycling on Pressure Inactivation of MNV-1 in Strawberry Puree

Pressure cycling was investigated to determine whether it could be used to enhance pressure inactivation of MNV-1. Puree samples were treated at 300 MPa and initial sample temperature of 0 °C with 1, 2 and 4 cycles. Pressure cycling offered no distinct advantage over continuous HHP treatment, as only slightly greater inactivation (<0.8 log) was observed for the 2-cycle and 4-cycle HHP treatments (Fig. 3.2). In comparison with continuous HHP treatment, the 2-cycle and 4-cycle HHP treatments had additional 1 and 3 come-up time periods and additional 1 and 3 depressurization time periods (additional 0.2 and 0.7 min in treatment time, respectively). During the pressure come-up and release periods, the 300-MPa treatment at 0 °C reduced MNV-1 in the samples by 0.2 log. Therefore the greater inactivation of MNV-1 for the 2-cycle and 4-cycle HHP treatment was probably due to these extra processing times instead of the cycling itself.

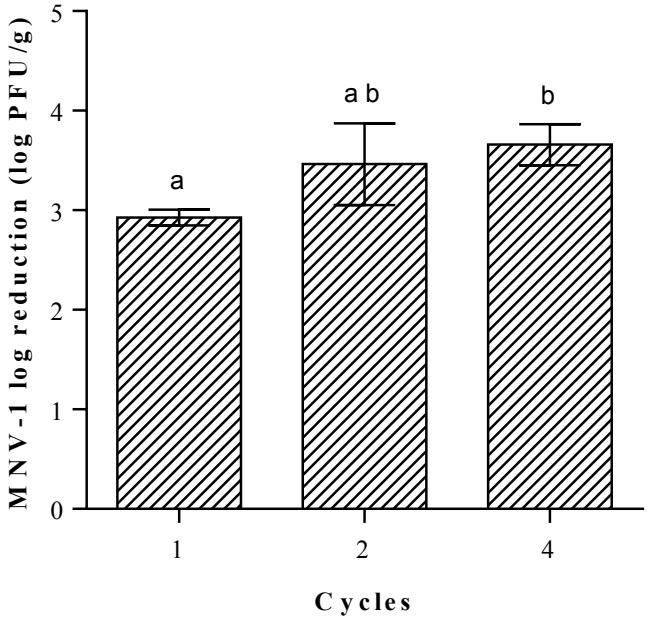

Figure 3.2: Effect of pressure cycling on the inactivation of MNV-1 in strawberry puree. Puree samples with initial MNV-1 inoculation level of ~10^6 PFU/g were treated at 300 MPa and initial sample temperature of 0 °C with 1 cycle of 2 min, 2 cycles of 1 min/cycle and 4 cycles of 30 s/cycle. Error bars represent one standard deviation. Bars with same lowercase letter are not significantly different ($p > 0.05$).

3.4.3 Effect of Presence of Water during HHP on Pressure Inactivation of MNV-1 on Strawberries

The extraction rate of MNV-1 from strawberry samples was approximately 18.75%. Under the dry-state condition (dry strawberries without water around during HHP treatment), MNV-1 was resistant to pressure with < 1-log reduction at both initial sample temperatures of 0 and 20 °C (Fig. 3.3). When strawberries were surrounded by water during pressure treatment (wet state), MNV-1 became sensitive to pressure; a treatment of 300 MPa at initial sample temperature of 0 °C achieved 2.9-log reduction of MNV-1. Since a considerable amount of virus particles probably were released from the strawberry surface into the 5 ml water, the water sample was also analyzed for MNV-1. No virus was detected in the water surrounding the strawberry quarter (detection limit was 6.25 PFU/sample). The results also demonstrated that decreasing the initial sample temperature from 20 to 0 °C significantly ($P < 0.05$) enhanced the pressure inactivation of MNV-1, which agreed well with the results shown in Fig. 3.1.

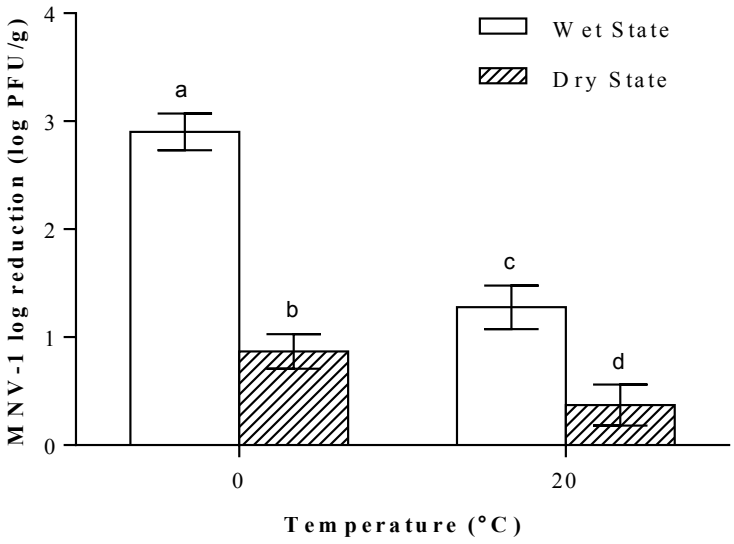

Figure 3.3: Effect of water presence on HHP inactivation of MNV-1 on strawberries. Strawberry quarters with initial MNV-1 inoculation level of ~10^6 PFU/g were either placed into bags (dry state) or bags containing water (wet state). They were then treated at 300 MPa for 2 min at initial sample temperatures of 0 and 20 °C. Error bars represent one standard deviation. Within the same temperature, bars with same lowercase letter are not significantly different ($p > 0.05$).

3.4.4 Pressure Inactivation Curves of MNV-1 in Puree and on Strawberries

The inactivation curves of MNV-1 in strawberry puree and on strawberries were obtained at two pressure levels, 300 and 350 MPa, and initial sample temperature of 0 °C (Fig. 3.4). The short treatment times were used since commercially processing times are usually ≤ 5 min. The degree of MNV-1 inactivation depended on the pressure level and hold time at a specific pressure. As pressure and/or treatment time increased, the degree of virus inactivation generally increased. For example, a treatment time of 2 min was needed to achieve a 4.3 log reduction of MNV-1 in puree at 350 MPa while a treatment time of 4 min was needed to obtain the same level of kill at 300 MPa. For the 300 MPa treatment, the degree of inactivation at each treatment time was very similar for both puree and strawberries. In addition, both curves were almost linear with R^2 values of 0.99. The calculated averaged D value at 300 MPa for both curves was 0.86 min.

For the 350 MPa treatment, the two inactivation curves were different with an almost linear curve for the strawberry puree ($R^2 = 0.99$) and a concave downward curve for the strawberries. The calculated D value at 350 MPa for the puree curve was 0.46 min. The strawberries curve at 350 MPa had a rapid drop in titer at the first 1 min of pressure treatment and a slower and almost linear inactivation rate at 1-4 min.

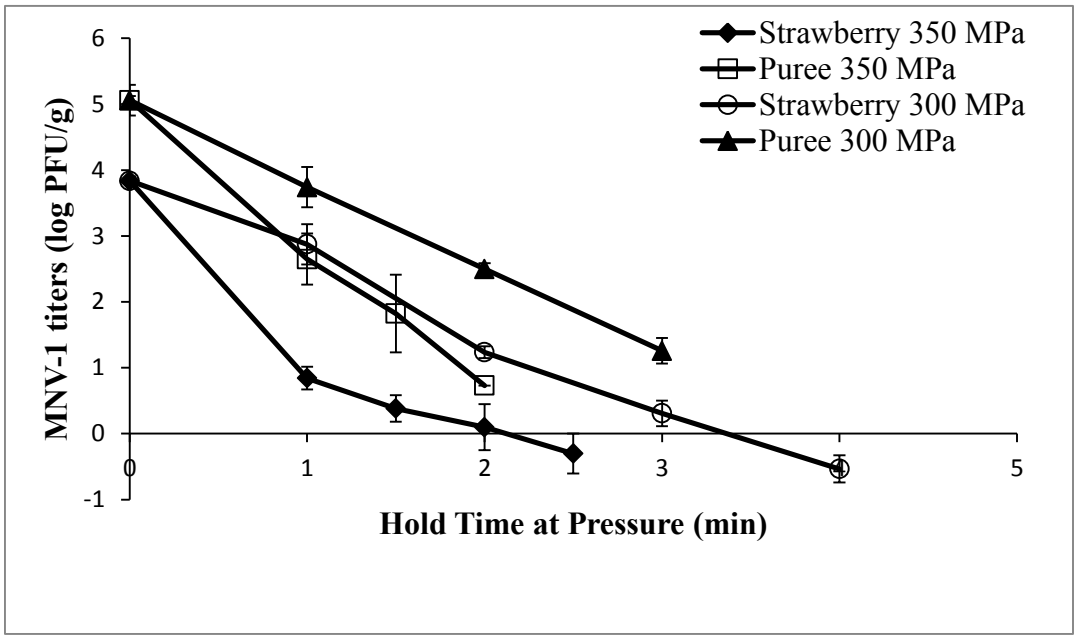

Figure 3.4: Pressure inactivation curves of MNV-1 on strawberries and in puree. Strawberries and puree samples with initial MNV-1 inoculation level of ~4×10^5 PFU/g and ~10^6 PFU/g, respectively, were treated at 300 and 350 MPa for different time intervals at initial sample temperature of 0 °C. Error bars represent one standard deviation. Four data points, puree treated at 300 MPa for 2.5 and 3 min and treated at 350 MPa for 5 min and strawberries treated at 350 MPa for 5 min, are not shown in the figure since the data of three replicates were either near the detection limit or below the detection limit.

3.4.5 Post-HHP Frozen Storage on MNV-1 Survival in Puree and on Strawberries

MNV-1 in the un-treated and HHP-treated samples decreased slightly during the 28-day frozen storage at -20°C (Fig. 3.5). For strawberry puree, additional 0.9 and 0.8 log reductions of MNV-1 were achieved after the 28-day frozen storage for the 300 and 350 MPa treatments, respectively, while the un-treated control showed 1.0 log reduction after the 28-day frozen storage. Similar trend was observed for the strawberry samples. Additional 0.4 and 0.6 log reductions of MNV-1 were achieved after the 28-day frozen storage for the 300 and 350 MPa treatments, respectively, while the un-treated control showed 1.2 log reductions after the 28-day frozen storage.

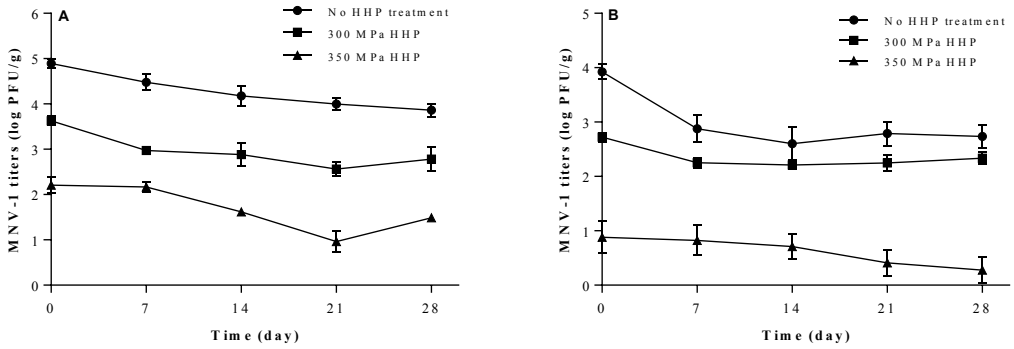

Figure 3.5: Survival curves of MNV-1 in strawberry puree (A) and on strawberries (B) during frozen storage. Strawberries and puree samples with initial MNV-1 inoculation level of ~4×10^5 PFU/g and ~10^6 PFU/g, respectively, were treated at 300 and 350 MPa for 1 min at initial sample temperature of 0 °C. The control (without HHP treatment) and HHP-treated samples were then stored at -20 °C for 28 days. Error bars represent one standard deviation.

3.5 Discussion

Since there is no available *in vitro* cell culture systems or a small animal models for HuNoV, current studies of HuNoV rely on surrogate viruses or molecular biology techniques. Currently, MNV-1 is widely used as a surrogate of HuNoV since it not only belongs to genus norovirus and has similar biochemical features compared with HuNoV, but also has similar genome size and gene organization as HuNoV (Cannon et al., 2006; Karst et al., 2003; Li et al., 2012; Wobus et al., 2006). Recently, Li et al. (2013a) utilized the binding ability of porcine gastric mucin (PGM), which is chemically and antigenically similar to human histo-blood group antigens, to HuNoV to extract potentially infectious viral particles and then quantified them using qRT-PCR. Using this approach, they showed that MNV-1 responded to pH, treatment temperature, and presence of water under HHP in ways similar to HuNoV. Based on the pressure sensitivity data, they concluded that MNV-1 was probably a good surrogate for HuNoV GII.4, which is the leading cause of norovirus gastroenteritis outbreaks (Glass et al., 2009; Zheng et al., 2010).

Results from HHP treatments at different temperatures showed that the efficacy of HHP inactivation of MNV-1 increased with decreasing initial sample temperatures, These results are in agreement with those reported by Lou et al. (2011) who found that MNV-1 was more sensitive to HHP at 4 °C than at 20 °C in DMEM, strawberry puree, strawberries and lettuce. Our results are also consistent with reported results for feline calicivirus (FCV) (Chen et al., 2005), Tulane virus (TV) (Li et al., 2013b) and HuNoV GI.1 and GII.4 (Li et al., 2013a), which showed these viruses were substantially more sensitive to HHP at lower temperatures. Using the PGM binding assay and qRT-PCR to quantify potential infectious HuNoV, Li et al. (2013a) showed that both GI.1 and GII.4 became more sensitive to HHP as treatment

temperature decreased from 35 to 1 °C. For example, a 450 MPa treatments at 21 and 35 °C caused no reduction of the GI.1 strain, while the same pressure treatments at 1 °C resulted in > 3-log reduction. A recent human challenge study conducted by Leon et al. (2011) also suggested that cold temperature probably enhanced pressure inactivation of HuNoV GI.1. In that study, whole oysters were injected with HuNoV GI.1 and then treated at 400 MPa for 5 min at either 6 or 25 °C, followed by feeding to human subjects. For the 25 °C treatments, a 60% infection rate was observed, while at 6°C, a 21% infection rate was observed.

Pressure cycling only slightly increase MNV-1 inactivation (<0.8 log). Kingsley et al. (2006) also found that pressure cycling with 2, 4, 6 and 8 cycles did not significantly enhance HAV inactivation compared with continuous HHP treatment when HAV in DMEM were treated at 400 MPa and 20°C or 400 MPa and 50°C. On the other hand, Bradley et al. (2000) found that inactivation of phage λ, a bacterial virus, was improved by pressure cycling. For an equal holding time of 7.5 min at 275 MPa, phage λ was inactivated by an additional 2 log PFU/ml as the number of cycles increased from 1 to 5. Pressure cycling has been found to enhance the inactivation of *Zygosaccharomyces bailii* yeast (Palou et al., 1998), *Bacillus stearothermophilus* spore (Hayakawa et al., 1994), but it did not increase the inactivation of *Salmonella* (D'Souza et al., 2012).

Our results indicated that berries should be wet when treated by HHP since MNV-1 was very resistant to pressure at the dry state condition. The enhanced pressure inactivation of viruses in the presence of water was also recently reported by (Li et al., 2013a; Li et al., 2013b). They showed that the degrees of inactivation of TV, MNV-1, and HuNoV GI.1 were significantly higher when blueberries were immersed

in water than when they were pressurized under the dry state conditions. Using the PGM binding assay and qRT-PCR to quantify potential infectious HuNoV, Li et al. (2013a) demonstrated that a treatment of 500 MPa for 2 min at 1 °C resulted in a 2.7-log reduction of GI.1 under the wet state condition, while a treatment of 600 MPa for 2 min at 1 °C achieved a < 1-log reduction under the dry state condition. The authors in those two studies speculated that under the wet state condition, water was forced by pressure into the solvation cage surrounding the viral capsid by HHP, disrupting hydrophobic interactions and facilitating the unfolding, or misfolding of capsid proteins. The conformation changes of key viral protein domains likely resulted in the inactivation of viruses. This water enhancing pressure inactivation effect was also observed for bacteria. Neetoo et al. (2008) showed that pressure inactivation of *E. coli* O157:H7 on alfalfa seeds was significantly higher when seeds were immersed in water than when they were pressurized in the dry state.

Except for the inactivation curve of strawberry samples treated at 350 MPa, the other three curves were almost linear. The reason for this difference in shape is not clear. Supposedly MNV-1 should have behaved similarly under HHP in both strawberries and puree since both are similar substrates. The strawberry curve at 350 MPa was characterized by rapid reduction of titers at the beginning of pressurization followed by tailing. These tailing effect was also observed for FCV (Chen et al., 2005), HAV (Kingsley et al., 2006), and MNV-1 in a DMEM culture medium (Kingsley et al., 2007).

The titers of MNV-1 in un-treated (control) and HHP-treated strawberry puree and in strawberries decreased slightly during the 28-day frozen storage. The results were not surprising since viruses are known to be stable at frozen temperatures and

frozen storage is a widely used laboratory preservation technique for viruses. Richards et al. (2012) showed that no significant reduction of HuNoV GII.4 in water after 14 freezing/thawing cycles (-80 °C/+22 °C) or 120-day frozen storage at -80 °C. Baert et al. (2008) showed that no reduction of MNV-1 was observed on frozen onions and spinach during 6-month storage at -21°C. The pH of the strawberry used in this study was ~3.4 and this low pH probably did not cause substantial inactivation of MNV-1 during frozen storage since it is extremely stable under acidic condition. Li et al. (2013b) observed no reduction of MNV-1 in a culture medium adjusted to pH 2, 3 and 4 after 1-h exposure at ~ 25 °C. Horm and D'Souza (2011) observed a <1-log reduction of MNV-1 in orange juice stored at 4 °C for 21 d. Enteric viruses, including HuNoV, TV and MNV-1, are expected to be acid tolerant because they must survive the low pH environment of a human stomach to reach their target cells in the small intestine (Cannon et al., 2006).

It was recently shown in our laboratory that HHP did not adversely affect the physical properties and visual appearance of strawberry puree (Huang et al., 2013), demonstrating that HPP could potentially be used to process it. For strawberries, although the pressure-treated berries retained their shape and raw characteristics, they became softer and showed visual signs of damage. Therefore, HHP could not be used to process berries destined for the fresh market. Its potential application probably will be for frozen berries since freezing and frozen storage cause similar and more severe texture damage.

3.6 Conclusion

The overall goal of our study was to identify and develop effective HHP processing techniques and strategies to inactivate HuNoV in berries. MNV-1 was used

as a surrogate for HuNoV in this study. It should be noted that our conclusions below are based on the assumption that MNV-1 is a good surrogate for HuNoV. The results obtained here confirm that lower initial sample temperatures dramatically enhance pressure inactivation of MNV-1 and advocate the use of cold temperatures for HHP processing. In this study, 0 °C was chosen as the lowest sample temperature since it could be easily achieved by mixing ice with water to maintain the pressure chamber temperature at 0 °C prior to HHP. Processing lower than 0 °C would involve the use of non-water hydrostatic medium such as mixture of water and propylene glycol, which is commercially undesirable. The results also advocate the use of wet state for HHP processing of strawberries since the presence of water during pressure treatment substantially enhanced inactivation of MNV-1 on strawberries. The use of pressure cycling is not supported since it only enhanced pressure inactivation of MNV-1 slightly. In addition, pressure cycling increases energy consumption, increases maintenance cost, and shortens the life of a pressure unit. The HHP inactivation curves demonstrate that increasing pressure level and/or extending treatment time could increase pressure inactivation of MNV-1. Since MNV-1 was relatively stable during frozen storage, strawberries and strawberry puree need interventions to prevent HuNoV infection. It is also advisable to use an adequate pressure treatment to achieve complete inactivation of HuNoV.

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Chapter 4

VALIDATION OF HIGH HYDROSTATIC PRESSURE INACTIVATION OF HUMAN NOROVIRUS ON STRAWBERRY, BLUEBERRY, RASPBERRY AND IN THEIR PUREE

4.1 Abstract

Human norovirus has been an increasing concern of foodborne illness related to fresh and frozen berries. In this study, high hydrostatic pressure (HHP) inactivation of HuNoV on strawberries, blueberries, and raspberries and in purees was determined. Porcine gastric mucin (PGM)-conjugated magnetic beads (PGM-MBs) and real-time reverse transcriptional polymerase chain reaction (qRT-PCR) was utilized for infectious HuNoV discrimination and quantification. Strawberry puree inoculated with HuNoV genogroup I.1 (GI.1) strain was HHP-treated at 450, 500 and 550 MPa for 2 min at initial sample temperatures of 0, 4 and 20 °C. HuNoV GI.1 strain became more sensitive to HHP treatment as the temperature decreased from 20 to 0 °C. HuNoV GI.1 or genogroup II.4 (GII.4) strains were inoculated into three berry purees or onto berry surface and treated at 250 to 650 MPa for 2 min at initial sample temperatures of 0 °C. For the purees, the HHP condition needs to be ≥ 550 MPa for 2 min at 0 °C to achieve > 2.9 log reduction of HuNoV GI.1 strain and > 4.0 log reduction of HuNoV GII.4 strain. HHP treatment showed more inactivation effect of HuNoV on blueberries than on strawberry quarters and raspberries. HuNoV GI.1 strain showed more resistance to HHP treatment than HuNoV GII.4 strain under different temperatures and environment. In all, HHP treatment was proved to be a suitable nonthermal

intervention for HuNoV in strawberry puree, blueberry puree, raspberry puree and on blueberries.

4.2 Introduction

Human norovirus, a member of family *Caliciviridae*, has been the leading cause of foodborne outbreaks and most frequent cause of acute nonbacterial gastrointestinal disease in the United States (Hall et al., 2013; Scallan et al., 2011). Fresh and frozen berries and berry products have been frequently associated with foodborne illness outbreaks which led to increasing concern of microbial safety for these products. Foodborne viral outbreaks have been linked to strawberries, blueberries and raspberries (Calder et al., 2003; Cotterelle et al., 2005; Falkenhorst et al., 2005; Friedman et al., 2005; Hutin et al., 1999; Korsager et al., 2005; Le Guyader et al., 2004; Mäde et al., 2013; Niu et al., 1992). In 2012, frozen strawberries were involved in a massive HuNoV outbreak in Germany, affecting about 11,000 people (Mäde et al., 2013). In Denmark, HuNoV was reported to be assosciated with an outbreak in frozen raspberries, involving about 180 cases (Korsager et al., 2005). Raw blueberries was linked to be responsible for a outbreak of HAV in New Zealand (Calder et al., 2003). In 2013, an outbreak of HAV happened in Austria which imported frozen berry mix might be the source (Wenzel et al., 2014). Berry products may get contaminated by virus from many sources, such as irrigation water, fertilizer, pesticide and food handler (Carter, 2005; Potera, 2013; Rodríguez‐Lázaro et al., 2012). Thus an intervention is needed for berry products to prevent and control HuNoV contamination.

Berries and berry products lack proper interventions to control viral contamination. Fresh berries are usually sold without washing step, while other berry

products such as frozen berries and berry puree include a washing step using a sanitizer such as chlorine prior to further processing. Thermal processing is not usually used in berry products as it negatively impacts sensory quality. As a nonthermal processing technique, HHP has been successfully used in many food products, such as oysters, guacamole, fruit jams, ready-to-eat meats, salsa, and orange juice. Researches also showed that HHP could be applied to inactivate viruses. Kingsley et al. (2002) demonstrated that a HHP treatment at 450 MPa and 22 °C for 5 min could reduce HAV by 7 log in DMEM with 10% FBS. HuNoV GI.1 strain could be inactivated by more than 3 log on blueberry with water surrounding via a HHP treatment at 600 MPa and 21 °C for 2 min (Li et al., 2013a). In oyster and clam homogenates, a HHP treatment at 450 MPa and 1 °C for 5 min could achieve a 4 log reduction of HuNoV GI.1 strain. Recent study in our laboratory has shown that physical properties and visual appearance of strawberry puree can be well preserved during HHP treatment (Huang et al., 2013).

The main issue hindering research work on norovirus is the lack of a proper *in vitro* cell culture system or small animal model. Surrogates, like MNV-1, FCV and TV are commonly used (Cannon et al., 2006; Chen et al., 2005; Li et al., 2013b). However, different behaviors under processing techniques of these surrogates and various HuNoV strains make it difficult to predict actual inactivation of HuNoV (Li et al., 2013a). Other than surrogates, molecular biotechnologies were used to quantify the RNA content to estimate the inactivation of HuNoV (Ye et al., 2014). However, methods are still needed for discrimination of infectious virus particles from those impaired virus which still have detectable RNA genome. It has been demonstrated that histo-blood group antigens (HBGAs) in the human intestinal tract were the receptors

of HuNoV (Marionneau et al., 2002). Porcine gastric mucin (PGM), which is antigenically-similar to HBGAs, can bind to HuNoV and norovirus-like particles (Tian et al., 2008; Tian et al., 2007; Tian et al., 2010). Dancho et al. (2012) showed that HHP treatment could abolish the ability of HuNoV to bind to PGM-MBs. In Li et al. (2013a), PGM-MB binding assay showed a good capability to discriminate infectious HuNoV and reflect HHP inactivation of HuNoV GI.1 and GII.4 strains which is the same as used in this study. Furthermore, comparison between result of PGM-MB binding assay and result of HuNoV surrogate, human challenge study and original PGM-MB binding assay study indicated that PGM-MBs could offer reliable discrimination of infectious HuNoV(Chen et al., 2005; Dancho et al., 2012; Leon et al., 2011; Li et al., 2013b).

The overall goal of this part of study is to verify HHP inactivation effect of HuNoV GI.1 and GII.4 on strawberries, blueberries, raspberries and in their purees and identify suitable HHP treatment condition for HuNoV intervention and commercial use. First, initial sample temperature effect on HHP inactivation of HuNoV GI.1 was investigated in strawberry puree. Then both HuNoV GI.1 and GII.4 strains were tested at 250 – 650 MPa for 2 min at an initial sample of 0 °C on strawberry quarters, blueberries, and raspberries and in their purees.

4.3 Materials and Methods

4.3.1 HuNoV Stock Preparation

A HuNoV GII.4 strain in fecal suspensions was provided by Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. The information about the 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.

4.3.2 Inoculation of Strawberries, Blueberries, Raspberries and Purees

Fresh strawberries, blueberries and raspberries were purchased from a local store the day before each experiment and stored at 4 °C until use. To prepare strawberry puree, strawberries were washed with tap water and dried in a strainer. Calyxes of the strawberries were removed with a flame-sterilized stainless knife and strawberries were blended in a food blender at high speed for 1 min until no chunks of strawberries were visible. Blueberry puree and raspberry puree were prepared in a similar way. 1 g strawberry puree, blueberry puree or raspberry puree was inoculated with 10 μl of the HuNoV GI.1 stock or GII.4 stock and bagged in individual sterile stomacher bag, sealed and double-bagged. To prepare berries for HHP treatment, they were cleaned and calyxes of strawberries were removed. The whole strawberries were then cut into quarters (~5 g/quarter) from the top to the bottom with a flame-sterile knife since the pressure chamber could not hold a whole strawberry. One strawberry quarter (~5 g/quarter), three blueberries (~5 g in total) or one whole raspberry (~4 g) was spot-inoculated with 10 μl of the HuNoV GI.1 stock or GII.4 stock on the surface and dried for 1 h in a biosafety cabinet without UV light for virus attachment. Each sample was placed into a sterile stomacher bag containing 5 ml of sterile double distilled water (ddH_2O). The bags were sealed and double-bagged.

4.3.3 Effect of Initial Sample Temperature on Pressure Inactivation of HuNoV GI.1 in Strawberry Puree

All the pressure treatments were conducted using a pressure unit using water as a hydrostatic medium with temperature control (model Avure PT-1; Avure

Technologies, Kent, WA). The pressure come-up rate was approximately 22 MPa/s. The pressure-release was almost immediate (<4 s). Pressurization time reported in this study did not include the pressure come-up or release times. To determine the effect of initial temperature on pressure inactivation of HuNoV GI.1 in strawberry puree, strawberry puree samples were treated at 450, 500 and 550 MPa for 2 min at an initial temperature of 0, 4 and 20 °C. And then HuNoV were extracted from the samples and quantified by qRT-PCR as described in Sections 4.3.5, 4.3.6 and 4.3.7.

4.3.4 HHP Treatment of HuNoV GI.1 and GII.4 on Strawberries, Blueberries, Raspberries and in Purees

To determine HHP inactivation of HuNoV GI.1 and GII.4 strains, berries and their puree samples were prepared as described in 4.3.2 and treated at 200 – 650 MPa for 2 min at an initial sample temperature of 0 °C. After HHP treatments, HuNoV were extracted from the samples and quantified by qRT-PCR. pH of the surrounding water were tested after the HHP treatment for strawberry quarters, blueberries and raspberries.

4.3.5 Extraction of HuNoV from Strawberries, Blueberries, Raspberries and Purees

HuNoV GI.1 and GII.4 were extracted from strawberries, blueberries and raspberries following the protocol described by Li et al. (2013a) with minor modifications. Briefly, each berry sample along with 5 ml of water in the bag was placed into a 50 ml sterile centrifuge tube and additional 5 ml of water was added to the tube. To release the virus from the berry surface, each tube containing the berry sample was vortex for 5 times and each pulse lasted for 10 s on a Vortex-Genie 2 vortexer (Scientific industries, Bohemia, NY) with speed set at “4”-“5”. The

suspension was then transferred to a 15 ml sterile centrifuge tube. 10 × PBS (KeraFAST) was added to the tube to buffer the suspension to 1 × PBS.

HuNoV GI.1 and GII.4 were extracted from the three berry purees following the protocol described by Kingsley et al. (2002) with minor modifications. Each puree sample was transferred to a 50 ml sterile centrifuge tube and 9 volumes of vegetable extraction buffer (100 mM Tris (Thermo Fisher Scientific Inc.), 50 mM glycine (Promega Corporation), 3% (m/v) beef extract (Becton Dickinson Company), 50 mM $MgCl_2$ (Thermo Fisher Scientific Inc.), pH 9.5) was added to the tube. The sample was then homogenized by a vortexer (Scientific industries, Bohemia, NY) and incubated at 37 °C for 30 min. The homogenate was centrifuged at 10,000 × g for 15 min at 4 °C. One volume of 16% (m/v) PEG8000 (Thermo Fisher Scientific Inc.) and 0.525MNaCl (AMRESCO LLC) were added to the supernatant, followed by incubation on ice for 1 h. The mixture was then centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was resuspended in 5 ml PBS.

4.3.6 Preparation of PGM-MBs and PGM-MB Binding Assay

PGM-MBs were prepared as described by Li et al. (2013a). One ml of MagnaBind carboxyl-derivatized beads (Thermo Scientific, Rockford, IL) was washed 3 times with 1 ml PBS for each wash and separated from the liquid using a bead attractor (EMD Millipore). One ml of 10 mg/ml type III mucin from porcine stomach (Sigma, St. Louis, MO) and 0.1 ml of 10 mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), both in conjugation buffer (0.1 M MES (2-(N-morpholino) ethanesulfonic acid), 0.9% NaCl, pH 4.7), were added to the beads. The mixture was rotated for 30 min on a Labquake shaker rotisserie (Thermo Scientific,

Waltham, MA). The beads were then washed 3 times using 1 ml PBS for each wash, suspended in 1 ml PBS containing 0.05% sodium azide, and stored at 4 °C

Before PGM-MB binding assay, each sample was treated with 100 µg of RNase A at 37 °C for 30 min. The PGM-MB binding assay was conducted as described by Li et al. (2013a) with slight modifications. One hundred and 200 µl of PGM-MBs were added to samples inoculated with HuNoV GI.1 or GII.4, respectively. The samples were then incubated at room temperature 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 PBS for each wash, suspended in 140 µl ddH_2O and stored on ice.

4.3.7 RNA Extraction and qRT-PCR Detection

HuNoV RNA was extracted using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol with slight modifications. For each sample, 560 µl buffer AVL containing 5.6 µl 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 µl 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 × g for 1 min. The column containing the RNA was then washed by 500 µl buffer AW1 and 500 µl buffer AW2. Finally, the RNA was eluted in 2 × 40 µl AVE elution buffer.

The qRT-PCR quantification, including the primers, the TaqMan probes and their concentration, followed Dr. Li's protocol(Li et al., 2013a). For HuNoV GI.1, NIF4 (+) CGCTGGATGCGNTTCCAT (500 nM), NV1LCR (−)CCTTAGACGCCATCATCATTTAC (900 nM), and NVGG1p 6′ -FAM-

TGGACAGGAGAYCGCRATCT-BHQ (250 nM) were used. For HuNoV GII.4, QNIF2 (+) ATGTTCAGRTGGATGAGRTTCTCWGA (500 nM), COG2R (−) TCGACGCCATCTTCATTCACA (900 nM) and QNIFS 6′ -FAM-AGCACGTGGGAGGGCGATCG-BHQ (250 nM) were used. All primers were synthesized by Life technologies and all probes were synthesized by Integrated DNA Technologies. qRT-PCR was performed using the Fast Virus 1-Step Master Mix (Life Technologies). For each sample, 10 µl qRT-PCR system was adopted, containing 2.5 µl master mix, 0.5 µl forward primer, 0.5 reverse primer, 0.5 µl probe and 6 µl 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. HuNoV GI.1 or GII.4 virus stock (280 µl) was extracted for RNA standard template and eluted in 200 µl AVE buffer. The RNA standard template was aliquoted and store at – 80 °C until use. The RNA standard template was 10-fold diluted for qRT-PCR. Serial 10-fold dilutions of GI.1 RNA and GII.4 RNA were used as standard for qRT-PCR. 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 qRT-PCR was below the most diluted RNA standard, the result was considered as below detection limit. The maximum reduction that could be detected was ~ 3.0 log (GI.1) or ~ 4.0 log (GII.4).

4.3.8 Statistical Analysis

At least three independent trials were conducted for all the experiments. Virus log reductions were calculated via the difference between untreated samples and

treated samples. Statistical analyses were performed using JMP (SAS Cary, NC, USA). A p value < 0.05 was considered significantly different among treatments using one-way analysis of variance and Tukey's multiple comparisons.

4.4 Results

4.4.1 Effect of Initial Sample Temperature on Pressure Inactivation of HuNoV GI.1 in Strawberry Puree

As shown in Fig. 4.1, the initial sample temperature had a significant impact on the HHP inactivation of HuNoV GI.1 in strawberry puree. Under 450, 500 and 550 MPa HHP treatments, HuNoV GI.1 became more sensitive to HHP treatment as the initial temperature decreased from 20 to 0 °C. HHP treatments at 550 MPa for 2 min at initial sample temperature of 0 and 4 °C could achieve > 3.0 log reductions of HuNoV GI.1. The HHP treatment of 450 MPa for 2 min at 0 °C could achieve HuNoV GI.1 inactivation effect similar to the treatment of 550 MPa for 2 min at 20 °C.

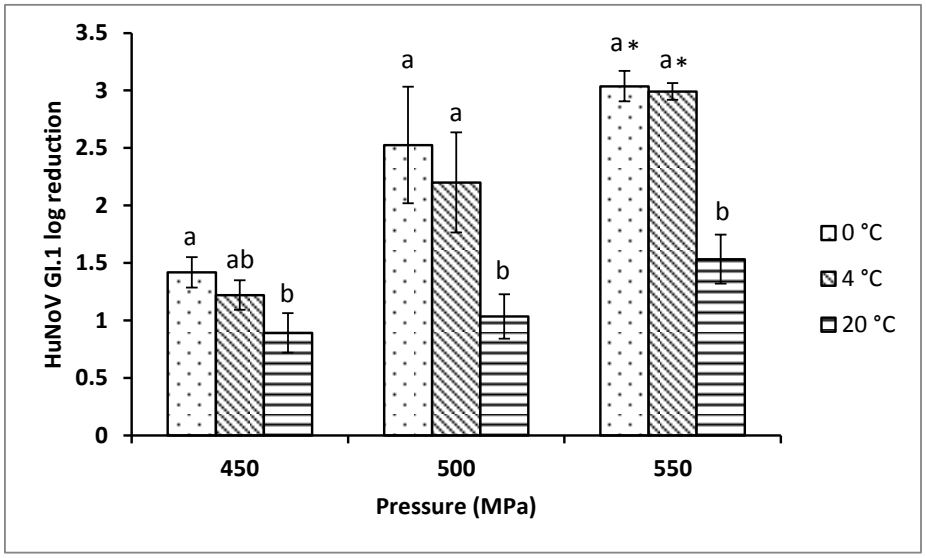

Figure 4.1: Effect of initial sample temperature on HHP inactivation of HuNoV GI.1 in strawberry puree. Puree samples were inoculated with HuNoV GI.1 virus stock and treated at 450 - 550 MPa for 2 min at initial sample temperatures of 0, 4 and 20 °C. Error bars represent one standard deviation. Bars with different letters under the same pressure are significantly different ($p < 0.05$). Treatments marked with asterisk were below detection limit. The maximum reduction could be detected was 3.0 log.

4.4.2 HHP Inactivation of HuNoV GI.1 and GII.4 on Strawberry, Blueberry, Raspberry and in Their Puree

As for HuNoV GI.1 strain, the extraction rates were ~ 11.2%, 16.8%, 13.4%, 11.2%, 10.8% and 10.8%, for strawberry quarter, blueberry, raspberry, strawberry puree, blueberry puree and raspberry puree, respectively. As for HuNoV GII.4 strain, the extraction rates were ~ 11.2%, 13.4%, 13.4%,16.8%, 18.75% and 16.8%, for strawberry quarter, blueberry, raspberry, strawberry puree, blueberry puree and raspberry puree, respectively. The detection limits for HuNoV GI.1 and GII.4 strains were around 10^{-3} and 10^{-4} dilution levels, respectively. All tests in this part were conducted at an initial sample temperature of 0 °C and HHP treatment time was 2 min.

As is shown in Table 4.1, HuNoV GI.1 strain was more sensitive to HHP treatment than GII.4 strain on strawberries, blueberries, raspberries and in purees. As for HuNoV GI.1 strain, HHP treatments of 550 MPa could achieve > 2.9 log reduction for three berry purees. A HHP treatment of 550 MPa could also reduce > 3.2 log of GI.1 strain on blueberries while HHP treatments of 650 MPa merely achieved 1.7 and 2.5 log reduction of GI.1 strain on strawberry quarters and raspberries, respectively. As for HuNoV GII.4 strain, a HHP treatment of 600 MPa was needed to achieve > 4.4 log reduction of GII.4 strain in blueberry puree while HHP treatments of 400 MPa and 350 MPa was needed to achieve > 4.2 log reduction of GII.4 strain in blueberry puree and raspberry puree, respectively. HHP treatment of 300 and 600 MPa could also reduce > 4.1 log of GII.4 strain on blueberries and raspberries, respectively while a HHP treatment of 650 MPa could reduce 3.1 log of GII.4 strain on strawberry quarters.

The pH of surrounding water for strawberry quarters, blueberries and raspberries after the HHP-treatment was tested. As is shown in Table 4.2, among all three berries, surrounding water pH of blueberry was the highest either in control sample or pressure-treated samples. Control samples had higher pH than treated samples while 400 and 600 MPa treatments had similar pH of surrounding water.

Table 4.1: HHP inactivation of HuNoV GI.1 and GII.4 on strawberry, blueberry, raspberry and in their puree

HuNoV Strain	Pressure (MPa)	Target					
		Strawberry Puree	Blueberry Puree	Raspberry Puree	Strawberry quarter	Blueberry	Raspberry
GI.1	450	1.4 ± 0.1^{Aab}	1.6 ± 0.5^{Aa}	2.7 ± 0.1^{Ac}	0.6 ± 0.3^{Ab}	2.7 ± 0.2^{Ac}	1.5 ± 0.3^{Aa}
	500	2.5 ± 0.5^{Bab}	2.4 ± 0.6^{ABab}	2.7 ± 0.4^{Aa}	0.9 ± 0.4^{Ac}	$> 3.2 \pm 0.1^{Ba}$	1.9 ± 0.4^{Ab}
	550	$> 3.0 \pm 0.1^{Ba}$	$> 2.9 \pm 0.2^{Ba}$	$> 2.9 \pm 0.4^{Aa}$	1.4 ± 0.4^{Bb}	$> 3.2 \pm 0.1^{Ba}$	2.1 ± 0.5^{Ac}
	600	ND	ND	ND	1.9 ± 0.5^{Ba}	ND	2.2 ± 0.4^{Aa}
	650	ND	ND	ND	1.7 ± 0.4^{Ba}	ND	2.5 ± 0.2^{Ab}
GII.4	250	0.1 ± 0.1^{Aa}	ND	ND	ND	3.2 ± 0.3^{Ab}	ND
	300	0.7 ± 0.1^{Ba}	1.1 ± 0.3^{Aa}	3.7 ± 0.2^{Ab}	ND	$> 4.1 \pm 0.2^{Ab}$	ND
	350	2.4 ± 0.1^{Ca}	1.6 ± 0.1^{ABb}	$> 4.2 \pm 0.3^{Bc}$	ND	ND	ND
	400	$> 4.2 \pm 0.2^{Da}$	2.1 ± 0.4^{BCb}	$> 4.2 \pm 0.2^{Ba}$	ND	ND	ND
	450	$> 4.2 \pm 0.1^{Da}$	2.6 ± 0.2^{Cb}	ND	ND	ND	ND
	500	$> 4.2 \pm 0.4^{Da}$	3.5 ± 0.2^{Da}	ND	ND	ND	3.5 ± 0.3^{Aa}
	550	ND	$> 4.4 \pm 0.1^{Ea}$	ND	2.8 ± 0.4^{Ab}	ND	$> 4.1 \pm 0.3^{Aa}$
	600	ND	$> 4.4 \pm 0.1^{Ea}$	ND	3.0 ± 0.5^{Ab}	ND	$> 4.1 \pm 0.3^{Aa}$
	650	ND	ND	ND	3.1 ± 0.3^{A}	ND	ND

Berry and puree sample were inoculated with 10 µl HuNoV GI.1 or GII.4 stock and HHP-treated at an initial sample temperature of 0 °C for 2 min. Data are the means of log reductions ± 1 standard deviation from at least 3 replicates. Data with different lowercase letters in the same row are significantly different ($p < 0.05$). Data with different uppercase letters in the same column for the same virus are significantly different ($p < 0.05$). ND: not done.

Table 4.2: pH of surrounding water for strawberry quarters, blueberries and raspberries after the HHP treatments

	Strawberry quarter	Blueberry	Raspberry
Control	3.92 ± 0.02	5.62 ± 0.15	4.24 ± 0.23
400 MPa	3.81 ± 0.01	4.82 ± 0.08	3.72 ± 0.14
600 MPa	3.79 ± 0.02	4.88 ± 0.10	3.92 ± 0.06

Strawberry quarters, blueberries and raspberries were HHP treated at 400 and 600 MPa for 2 min at initial sample temperature of 0 °C or untreated (control) without virus inoculation. Surrounding water was taken out right after HHP treatments and pH was tested. Data are the means of log reductions ± 1 standard deviation from at least 3 replicates.

4.5 Discussion

Since there is currently no suitable *in vitro* cell culture system or small animal model for HuNoV, surrogates or molecular biology techniques are usually used in HuNoV research. In this part of study, PGM-MB binding assay was used for discriminating potentially infectious HuNoV. Although no direct research proved that PGM-MB binding assay could reveal actual HHP inactivation effect of HuNoV with reproducible *in vitro* or *in vivo* data (Li et al., 2013a), the PGM-MB binding assay could probably provide a good estimate of HHP inactivation of HuNoV. A human challenge study showed that a HHP treatment of 600 MPa, 6 °C and 5 min could inactivate ≥ 4 log of HuNoV GI.1 while qRT-PCR detectable HuNoV GI.1 RNA was reduced by ≥ 4.7 log after a HHP treatment of 600 MPa, 5 °C and 5 min (Dancho et al., 2012; Leon et al., 2011). Similar result was also observed in HuNoV surrogate, MNV and FCV (Li et al., 2014). In that study, MNV and FCV were pressure treated at

250 – 450 MPa (MNV) and 50 – 300 MPa (FCV) at 4 or 21 °C for 2 min and quantification of MNV and FCV via plaque assay and PGM-MB binding/qRT-PCR assay showed almost same inactivation curve when reduction was below 2 log. The result of this part of study was based on an assumption that PGM-MB binding assay provided a good estimate of pressure inactivation effect of HuNoV.

Results from HHP treatments of HuNoV GI.1 strain at different initial sample temperatures showed that GI.1 strain became more sensitive to HHP treatment with the decrease of initial sample temperature. This result was consistent with those reported by Li et al. (2013a). Both HuNoV GI.1 and GII.4 strains became more sensitive to HHP treatment as the initial sample temperature decreased from 31 °C to 1 °C. Previously, Ye et al. (2014) showed that the HHP inactivation effect of HuNoV GI.1 and GII.4 strains in oysters and clams increased while the temperature decreased from 25 °C to 1°C. Similar result was also observed from a human challenge study conducted by Leon et al. (2011). A 21% infection rate was observed with human subjects fed with whole oyster injected with HuNoV GI.1 pressure treated at 400 MPa for 5 min at 6 °C while a 60% infection rate was recorded with 25 °C group. The temperature effect on HHP inactivation of HuNoV was also in agreement with studies of HuNoV surrogates. MNV-1, FCV and TV showed more sensitive to HHP treatment at lower temperature (Chen et al., 2005; Huang et al., 2014; Kingsley et al., 2007; Li et al., 2013b; Lou et al., 2011).

The result in Table 4.2 showed that HuNoV GII.4 strain was more sensitive to HHP treatment than GI.1 strain on three berry fruits and purees. This result was in agreement with those reported Li et al. (2013a). In that study, a HHP treatment of 350 MPa, 2 min and 21 °C could achieve > 4.0 log reduction of HuNoV GII.4 strain in

PBS while only 0.5 log reduction of HuNoV GI.1 strain in PBS was observed with a HHP treatment of 500 MPa, 2min and 21 °C. Ye et al. (2014) also proved that a 5 min HHP treatment at 400 MPa and 25 °C could reduce HuNoV GII.4 strain by 3.6 log but could only reduce HuNoV GI.1 by 1.0 log. The different sensitiveness of HuNoV strains to HHP treatment makes it important to select proper strain for validation studies in various situations. HuNoV GII strains were commonly associated with foodborne outbreaks while GI strains also showed similar prevalence as GII strains for waterborne outbreaks (Bitler et al., 2013; Vega et al., 2014). From 2009 to 2013 in the U.S.A., GI.6 was the most prevalent strain among GI strains while GII.4 was the most prevalent strain among GII strains (Vega et al., 2014). It should be noted that HuNoV GII.4 is considered the leading cause of HuNoV foodborne outbreaks with higher hospitalization and mortality rates compared with other HuNoV strains (Desai et al., 2012; Lysén et al., 2009; Widdowson et al., 2004).

One of the objectives in this study was to identify suitable HHP treatment conditions for processing berry fruits and purees to enhance their safety in terms of HuNoV inactivation. Previous studies showed that infectious dose of HuNoV was relatively low (10–100 virions) (Hutson et al., 2004). Butot et al. (2009) reported that titers of HuNoV in shellfish ranged from 10^2 to 10^4 genome copies per gram of digestive tissues. In raspberries and strawberries, concomitant level of HuNoV genomic copies ranged between 2 and 3.5 log/g (Baert et al., 2011). Based on these data, we thought > 3 log reduction would be adequate for inactivation of HuNoV in berries and berry purees. For strawberry puree, blueberry puree and raspberry puree, the HHP condition needs to be ≥ 550 MPa for 2 min at 0 °C. HHP treatment didn't show substantial inactivation effect for strawberry quarters and raspberries. But for

blueberries, HHP treatment of ≥ 550 MPa for 2 min at 0 °C would be enough. Whole berries were treated in this part of study with surrounding water as it has been proved that water presence could significantly enhance HHP inactivation effect of HuNoV GI.1 on blueberries (Li et al., 2013a). MNV-1 has also been demonstrated to be more sensitive to HHP treatment with water presence on strawberry quarter (Huang et al., 2014).

As is shown in Table 4.2, significant difference was observed in HHP inactivation effect of HuNoV between in blueberries, strawberry quarters and raspberries. Considering that the virus was inoculated on the berry surface and the difference of surrounding water pH (Table 4.1), pH would be the main influence of the HHP inactivation effect of HuNoV. HHP treatment tended to have better inactivation effect of HuNoV at neutral pH. This result was in consistent with those reported by Li et al. (2013) who found that both HuNoV GI.1 and GII.4 strains became more sensitive to HHP treatment at pH 7.0 than pH 4.0. Similar phenomenon was also observed for HuNoV surrogates, MNV-1 and TV, which tended to be more resistant to HHP treatment under acidic environment (Li et al., 2013b; Lou et al., 2011). Though there is no specific explanation for mechanism of the different responses to HHP treatment under different pH, it is known that enteric virus is usually acid-tolerant as they need to go through human stomach.

4.6 Conclusion

The overall goal of this study was to identify suitable and optimum HHP processing condition for HuNoV inactivation in three berry fruits and purees. It should be noted that conclusions made in this study were based on the assumption that the PGM-MB binding assay could provide a good estimate of HHP inactivation of

HuNoV. The temperature effect results advocate the use of cold temperature in HHP to enhance HuNoV inactivation. The results obtained also demonstrate that HHP treatment could serve as a good HuNoV inactivation method for the three berry purees. For strawberries and raspberries, HHP treatment seemed to be less effective for HuNoV inactivation while for blueberries, HHP treatment could still be a good inactivation method. It seems the mechanism underlying the differences in HHP inactivation effect among the three berries could be attributed to the substrate pH during HHP.

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Chapter 5

FUTURE RESEARCH

Current and previous research work showed that application of high hydrostatic pressure technique on human norovirus inactivation was promising in strawberry puree, blueberry puree and raspberry puree and on blueberry. As for strawberry and raspberry, pressure inactivation effect of human norovirus was not ideal. Other techniques, like antimicrobials, mild heating, UV and pulse light could be combined with HHP to achieve better human norovirus inactivation effect on strawberry and raspberry. Furthermore, with combination of other techniques, higher retention of sensory characteristics and nutrients might be achieved with a relatively mild HHP treatment. Though high pressure processing is known for less detrimental effect on quality compared with thermal processing, sensory test is still needed to be conducted to confirm that quality of pressurized berries and berry puree meet the anticipation of consumers. Besides, validation of pressure inactivation of human norovirus needs to be done in commercial size HHP equipment which might experience different pressure building up rate and pressure level limit. Additionally, pressure-time-temperature profile might vary a lot due to the different size and shape of the pressure chamber, which might lead to more loss of quality. Beyond berry puree, HHP also has a potential to be applied to more berry products and fruit products. Various juice products and jam could utilize HHP technique for human norovirus inactivation. HHP might also serve as an intervention method before freeze-drying for cereal or fruit chips products.

In the study, PGM-MB binding assay was used for discrimination of infectious human norovirus while no direct evidence could show that the infection ability of human norovirus could be unbiased revealed by PGM-MB binding assay. Human challenge experiment should be carried out in the future to confirm the validity of PGM-MB binding assay. Certain progress has been made in human norovirus tropism and *in vitro* cell culture system. The pressure inactivation effect of human norovirus could be confirmed in viral infection experiment in the future. PGM-MB binding assay might also be applied to quick detection and concentration of human norovirus which need to be further studied.