Control of emerging foodborne viruses using cranberry extracts and chemical sanitizers

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Control of emerging foodborne viruses using cranberry extracts and chemical sanitizers

A Thesis Presented for the

Master of Science

The University of Tennessee, Knoxville

Snigdha Nitin Sewlikar

December 2014
Dedication

To the loving memory of my grandparents, Meena Sewlikar and Govindrao Sewlikar
Acknowledgments

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Abstract

Human norovirus (HNoV), hepatitis A virus (HAV) and Aichi virus (AiV) outbreaks that cause severe foodborne disease are on the rise globally. Strategies to prevent and mitigate the spread of these viral infections need to be investigated. HNoV, HAV and AiV are known to be resistant to most of the conventionally used chemical inactivation methods. Cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) have demonstrated antimicrobial and anti-inflammatory properties. CJ and C-PAC have been shown to exhibit antiviral activities against HNoV surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) at room temperature. Hence, the objectives of this research were (1) To evaluate dose and time-dependence effects of CJ and C-PAC against HAV and MNV-1; (2) To determine reduction of AiV by CJ and C-PAC (3) To study the survival of HAV and AiV in cranberry-based juices at refrigeration (4°C) and (4) To compare the activity of commercially available chemical compounds (an alkaline wash and a 0.5% levulinic acid-0.5% SDS wash) against reduction of HAV, MNV-1 and AiV titers. CJ and C-PAC show promise as natural antivirals against HAV, MNV-1 and AiV at 37°C. Damage to viral capsids by CJ was seen in TEM studies. HAV and AiV were found to survive for 21 days in cranberry juices at refrigeration temperature. The commercially available chemical washes were found to be effective in reducing titers of HAV, MNV-1 and AiV to some extent. This study thus helps in evaluating natural as well as chemical control measures for prevention of foodborne viral illnesses.
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Introduction

Human noroviruses (HNoVs), hepatitis A virus (HAV) and Aichi virus (AiV) are increasingly associated with foodborne illness outbreaks worldwide. HNoV infections continue to be on the rise with more than 900 reports of laboratory-confirmed outbreaks between 1993 and 2011 (Matthews et al., 2012). Currently vaccines are unavailable for the prevention of HNoV or AiV infections. While vaccines to prevent HAV infection are available, that resulted in a 90% decrease in HAV cases in the US over 20 years, an estimated 2,700 new cases of HAV illness are still reported annually in the US (CDC, 2014). Hence effective measures/strategies to prevent spread of these viruses or to treat these infections need to be researched.

The demand for natural plant-based products to prevent and treat infections is on the rise. Cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) have demonstrated antiviral activity against human norovirus surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) at room temperature. However, studies to evaluate the efficacy of CJ and C-PAC at 37°C are needed if they are to be consumed as preventive or therapeutic measures against HNoV infections. In addition, the effects of CJ and C-PAC against HAV and AiV have not been reported. Furthermore, it is also essential to understand the mechanism of antiviral action of CJ and C-PAC.

HAV and AiV are known to exhibit resistance to low pH with the ability to survive adverse environmental conditions. This resistance and stability leads to their ease of transmission and outbreaks. Temperature plays an important role in viral survival. Survival of these enteric viruses in cranberry-based juices at refrigeration is needed so as to understand the risk of transmission associated with the consumption of contaminated juices.

Given the fecal-oral transmission route for HNoVs, HAV and AiV, there remains a growing need to develop potent and cost-effective strategies to prevent the spread of these viruses.
Conventionally used chemical disinfectants or sanitizers do not appear to be very effective as antiviral agents. Also, cost and consumer safety are important factors that need to be considered when evaluating new chemicals. Hence, antiviral activity of generally recognized as safe substances need to be explored.

The objectives of this research are as follows:

Objective 1: Determine the dose and time-dependence effects of commercial CJ and C-PAC (0.06, 0.5, 1, and 2 mg/ml) over 24 h at 37°C on HAV, MNV-1, and AiV titer reduction.

Objective 2: Determine the mechanism of HAV, MNV-1, and AiV inactivation by CJ and C-PAC using viral adsorption and replication blocking assays and transmission electron microscopy.

Objective 3: Determine the survival of HAV and AiV in CJ over 21 days at refrigeration to determine transmission risk.

Objective 4: Compare the effects of commercially available chemicals (an alkaline wash and a 0.5% levulinic acid-0.5% sodium dodecyl sulfate wash) on HAV, MNV-1 and AiV titer reduction.
Chapter I

Literature Review
1. Foodborne Illnesses

Foodborne illnesses are a major threat to human health around the globe. According to Scallan et al. (2011), 9.4 million cases of foodborne illnesses occur every year in the United States. These illnesses are attributed to 31 major pathogens of which 5.5 million (59%) are caused by human noroviruses (HNoVs), the leading cause of foodborne illnesses (non-bacterial gastroenteritis) that affect all age groups (Hall et al., 2013). In addition to HNoVs, most of the viral foodborne and waterborne outbreaks have been attributed to hepatitis A virus (HAV), hepatitis E virus, sapoviruses, parvoviruses, rotaviruses, astroviruses, other small round viruses and human enteroviruses including polioviruses, coxsackieviruses, and echoviruses (Sair et al., 2002).

2. Foodborne Viruses

2.1. Human noroviruses

Human noroviruses (HNoVs) are non-enveloped, small (27-35 nm in diameter), single-stranded positive-sense RNA viruses with icosahedral symmetry that belong to the Caliciviridae family with very low infectious doses (10-100 viral particles) (Turcios et al., 2006; Siebenga et al., 2009; Widdowson et al., 2004). The size of the norovirus genome is ~7.5 kb with three open reading frames (ORF1, ORF2, and ORF3) encoding for structural as well as non-structural genes (Donaldson et al., 2008). ORF1 (5 kb in size) encodes for a polyprotein (~200 kDa) which is processed to yield a non-structural protein that is vital for replication of the virus while ORF2 (1.8 kb) and ORF3 (0.6 kb) encode for the structural capsid protein (VP1) and basic structural protein, respectively (Donaldson et al., 2008).
On the basis of similarities in the genome sequence, the currently reported 40 strains belonging to the Norovirus genus have been classified into five distinct genogroups (Green et al., 1995; Fankhauser et al., 2002; Karst et al., 2003; Oliver et al., 2003; Donaldson et al., 2008). These include Genogroup I (GI), GII, GIII, GIV and GV. GI, GII and GIV cause infections in humans while GIII and GV are bovine and murine pathogens, respectively (Zheng et al., 2006; D’Souza et al., 2007). Genogroups, GI, GII, GIV, are further divided into eight, seventeen and two genoclusters, on the basis of phylogenetic analysis and genome similarity (Donaldson et al., 2008), while GIII and GV each have only one genocluster (Zheng et al., 2006). HNoV GI and GII are the common causative agents in most of the norovirus-related outbreaks in humans, with GII being more frequent (Zheng et al., 2006; Donaldson et al., 2008).

HNoVs with a short incubation period of 24 to 72 h, cause self-limiting gastrointestinal infection with symptoms like nausea, fever, vomiting and diarrhea (Grove et al., 2006; Cliver et al., 2002; Grohmann et al., 1981). Severe symptoms include non-bloody diarrhea and vomiting which generally resolve within 2-3 days (Patel et al., 2009). HNoV infections caused by the newly emergent virulent viral strains are known to be life threatening particularly to the elderly and immuno-compromised individuals (Siebenga et al., 2009; Su et al., 2010a). The binding site for HNoVs, post ingestion, are the histoblood group antigens (HBGA) in epithelial cells of the gut (Huang et al., 2005) and hence the blood group of an individual is believed to be a key factor in determining the risk of infection with HNoV.

HNoVs are reported to cause an average of ~570–800 deaths, 56,000–71,000 hospitalizations, 400,000 emergency department visits, 1.7–1.9 million outpatient visits, and 19–21 million illnesses per year in the US (Hall et al., 2013). Between the year 1993 and 2011, more than 900 reports of laboratory-confirmed HNoV outbreaks have been published (Matthews et al.,
Some of the recent outbreaks associated with HNoVs are listed in Table 1.1. These outbreaks are common in closed settings such as cruise ships, healthcare facilities, restaurants, hotels and schools (CDC, 2014c). Owing to their high stability, resistance to degradation in the environment and a majority of chemical (for e.g. low pH) inactivation methods, the spread of these viruses are difficult to control (Cheesbrough et al., 2000; Kuusi et al., 2002; Widdowson et al., 2004). No vaccine is yet available to prevent HNoV infection (CDC, 2014c).

2.1.1. Human Norovirus Surrogates

Human noroviruses currently remain uncultivable in the laboratory. However, as human norovirus infections are on the rise, it is important to evaluate and develop control strategies. Human feeding studies to evaluate the efficacy of various control strategies are effective but an expensive option (Richards, 2012). This issue, hence, demands the use of surrogate organisms that mimic the human norovirus in terms of disease symptoms, genetics, binding and transmission characteristics and resistance to various environmental factors (Kniel, 2014). A surrogate also must also demonstrate characteristics including non-pathogenicity and ease of cultivability in the laboratory (Busta et al., 2003). Cultivable surrogates such as feline calicivirus (FCV-F9), San Miguel sea lion virus, rotavirus, murine norovirus (MNV-1), Tulane virus (TV), porcine saprovirus, as well as virus-like particles and bacteriophage have been used to determine viral persistence and efficacy of control measures (Kniel, 2014; Richards, 2012; Li et al., 2012; D’Souza et al., 2006, D’Souza et al., 2009: Su et al., 2011; Herneisen et al., 2013; Hirneisen et al., 2010; Fino and Kniel, 2008). Table 1.2 summarizes the characteristics of various HNoV surrogates. A drawback with use of surrogates is that they fail to exhibit variation in structure that is usually demonstrated by HNoVs (Donaldson et al., 2010; Kniel, 2014). MNV-1 and TV are being
increasingly used in research to determine survival and inactivation by processing conditions as they display similarity to human norovirus and are easy to use (Kniel, 2014).

Various studies indicate the persistence of HNoVs under various environmental conditions. HNoVs can survive under varying temperature conditions (freezing temperatures and heating up to 60°C for 30 min); at room temperature, HNoVs can survive on food-contact surfaces including formica, stainless steel, and ceramic coupons for 7 days and can persist in stool for 3 h at pH as low as 2.7; treatment of water with chlorine at 3.75-6.25 mg/L is ineffective for the inactivation of HNoVs (D’Souza et al., 2006; D’Souza et al., 2007; Patel et al., 2009; Teunis et al., 2008; Butot et al., 2008). However, boiling contaminated water (rolling boil- explain) for 1 min is known to be effective (>4 log reduction) in inactivating enteroviruses including HNoVs and HAV (CDC, 2009; Zuber et al., 2013).

2.1.1.1. Feline Calicivirus

Feline calicivirus (FCV-F9), a single-stranded RNA virus, is a member of the Vesivirus genus in the Caliciviridae family (the same family as HNoV) that causes widespread upper respiratory infections in cats with symptoms like oral ulcerations, ocular and nasal discharge and limping syndrome (Thiel and Konig, 1999). The route for transmission is oral, nasal or conjunctival (Radford et al., 2007) as opposed to the fecal-oral route for HNoVs. Cats continue to shed the virus for almost 30 days post infection (Radford et al., 2007). FCV-F9 can be cultivated in the laboratory and so was initially used as a surrogate for HNoV. At pH of 2, 3 and 4, FCV-F9 titers were reduced 4.4, 3.7, and 2.3 log, respectively in 30 min at 37°C (Cannon et al., 2006). FCV-F9 titers were reduced by 5.1 log at pH 10, with reductions of ~2 log between pH 5 and 9 (Cannon et al., 2006). FCV-F9 is known to be sensitive to inactivation by ethanol (70 and 90%) and isopropanol (40 to 60%) (Malik et al., 2006), sodium hypochlorite, chlorine dioxide, iodine,
or glutaraldehyde (Doultree et al., 1999; Duizer et al., 2004; Eleraky et al., 2002; Thurston-Enriquez et al., 2005; Urakami et al., 2007; Belliot et al., 2008). Even in the presence of organic load, FCV-F9 was reduced by 1 to 2 log TCID$_{50}$ by alcohol-based hand rubs (Belliot et al., 2008; Kampf et al., 2005). Cannon et al. (2006) reported D-values of 6.715, 0.406, and 0.118 min at 56, 63, and 72°C, respectively for FCV-F9. The disadvantage in using FCV-F9 as a surrogate is its greater susceptibility than HNoVs towards inactivation at low pH, by chemicals and environmental stress factors (Slomka and Appleton, 1998; Doultree et al., 1999; D'Souza and Su, 2010).

2.1.1.2. Murine Norovirus

Murine norovirus (MNV-1), being a member of the *Norovirus* genus of the *Caliciviridae* family, is closer to HNoVs in terms of genetic similarity than FCV-F9. It is also transmitted via the fecal-oral route as HNoVs. MNV-1 can replicate in cell culture as well as in mice, that makes it a convenient surrogate for HNoV and helps to derive a comparison of mechanism of pathogenicity in mice (its natural host) to that of infection and replication in cell culture (Wobus et al., 2006).

MNV-1 is stable at low pH with only 0.6, 0.6, and 0.5 log reductions obtained at pH 2, 3, and 4, respectively when incubated for 30 min at 37°C (Cannon et al., 2006). Between pH 5 to 9, reduction of less than 1 log were obtained for MNV-1, whereas at pH 10, reduction of 1.8 log was obtained (Cannon et al., 2006). Cannon et al. (2006) reported no significant difference in the reductions at the tested pH values when incubation time was increased to 2 h. Dolin et al. (1972) reported that Norwalk virus, after a 3 h incubation at pH 2.7, caused infection when used in feeding studies. Resistance to low pH is an important characteristic that needs to be demonstrated by a surrogate for HNoVs as they need to survive low pH of stomach before reaching the site of infection (small intestine) (Cannon et al., 2006). When subjected to extraction by organic solvents
like chloroform, trichlorofluoromethane, and Vertrel, no reduction was obtained for MNV-1 (Cannon et al., 2006). The D-values for MNV-1, using the capillary tube method, were 3.473, 0.435, and 0.166 min at 56, 63, and 72°C, respectively (Cannon et al., 2006). When incubated in suspension (wet) and on a stainless steel coupon (dry) with artificial feces at RT, MNV-1 was reduced below the detection limit in 5 days when subjected to dry conditions, with higher stability under wet conditions (reduction of <2 log PFU/mL over 7 days) (Cannon et al., 2006). Reduction for MNV-1 under both wet and dry conditions was lower (< 2 log over 7 days) at 4°C (Cannon et al., 2006).

2.1.1.3. Tulane virus

Tulane virus (TV), a member of the genus *Recoivirus* of the *Caliciviridae* family, was isolated at the Tulane National Primate Research Center from *Macaca mulatta* (rhesus macaques) (Farkas et al., 2008). TV demonstrates similarity to GII noroviruses and binds to type A and B HBGAs as is the case with HNoVs (Farkas et al., 2010), making it good HNoV surrogate.

TV was observed to demonstrate greater resistance to pressure in acidic condition than at neutral pH (Li et al., 2013) and no significant reduction was reported after 2 min treatment of TV on dry blueberries at 600 MPa at 4, 21 and 35 °C. However, treatment of TV on blueberries immersed in phosphate-buffered saline at 300 MPa caused reduction to below the detection limit after 2 min at 4 °C. The inactivation of TV on blueberries and oysters when subjected to pressure treatment was observed to be affected by temperature (increased with decrease in temperature of sample) and TV was found to display greater sensitivity to pressure than MNV-1 in culture media, oysters and blueberries (Li et al., 2013). Tian et al. (2013) reported that TV has D-values of 11.8, 2.6, and 4.3 min at 56, 63, and 72°C, respectively which are similar to those of MNV-1 and FCV-F9. 5D reduction in TCID₅₀ was obtained for TV at 63°C for 5 min and at 56°C for 30 min while
4D reduction was obtained when TV was exposed to UV (60 mJ/cm$^2$) or to chlorine (300 ppm for 10 min) (Tian et al., 2013). TV demonstrated stability to pH in the range pH 3.0 to 8.0 while reductions were obtained at pH 2.5 and 9.0, with complete inactivation (4 D) at pH 10.0 (Tian et al., 2013). TV exhibited resistance to inactivation by low concentrations of ethanol ($\leq$40%) with complete inactivation (5 D) on incubation with 50 to 70% ethanol in 20 s (Tian et al., 2013). Hirneisen and Kniel (2013) compared the resistance of MNV-1 and TV to inactivation treatments including chlorine, heat, pH, and studied their survival in tap water at 4°C and 20°C over 30 days. MNV-1 demonstrated greater stability in tap water at 4°C as well as at the tested pH (2.0 to 10.0) than TV. At 2 ppm chlorine, MNV-1 was more resistant than TV. Hirneisen and Kniel (2013) thus concluded that MNV-1 is a better surrogate than TV given its persistence under various environmental conditions.

2.1.1.4. Bacteriophages

MS2, a bacteriophage (virus that can only infect a bacterial cells) found in sewage with Escherichia coli as its natural host, has also been used as surrogate for human enteric viruses (Dawson et al., 2005). MS2 is a single-stranded RNA virus (26 nm in diameter with icosahedral symmetry) belonging to group 1 of RNA coliphages in the Leviviridae family (Dawson et al., 2005; Calender, 1998). MS2 exhibits resistance to inactivation by pressure (reduction of less than 1 log at 500 MPa in 5 min at 20°C) (Black et al., 2010). Dawson et al. (2010) observed that MS2 can survive for up to 50 days 4 and 8°C (reduction of less than 1 log PFU/mL) and for up to 9 days (>1 log PFU/mL reduction) at 22°C on fresh produce (iceberg lettuce, cabbage, baton carrot, tomato, curly leaf parsley, spring onion, capsicum pepper, cucumber, strawberries, and raspberries). Trisodium phosphate (TSP) was reported to be effective in inactivation of MS2 with
high initial titers (reductions of ≥ 6 and 4.5 log PFU/mL with 5% and 1% TSP, respectively in 30 seconds) (D’Souza and Su, 2010).

2.1.1.5. Porcine sapovirus

Porcine sapovirus (PS) belongs to the Sapovirus (includes human sapovirus responsible for gastroenteritis in human) genus within the Caliciviridae family (Wang et al. 2005, Wang et al. 2007). Although human sapovirus is also not cultivable, PS Cowden strain can be grown in cell-culture (Flynn and Saif 1988). PS is an enteric pathogen that replicates in intestinal cells of pigs leading to gastroenteritis and is genetically similar to HNoV (Flynn et al. 1988, Guo et al. 2001; Li et al., 2012). Wang et al. (2012) observed that PC can survive between pH 4 to 8 at RT for 1 h (<1 log reduction in TCID_{50} at pH 3) and exhibits similar resistance to heat and chlorine as HNoVs.

2.2. Hepatitis A Virus

The other epidemiologically significant foodborne virus, HAV belongs to the Hepatovirus genus classified under Picornaviridae family. It is a non-enveloped virus, 27-30 nm in diameter with an icosahedral capsid and a positive-sense single stranded RNA which is 7.5 kb long (Bidawid et al, 2000). HAV is usually transmitted via the fecal-oral route (Lemon, 1997). The average incubation period for HAV illness is 30 days (may range from 15 to 50 days) that makes the detection of source and infection challenging (Koff, 1998; CDC, 2012).

HAV strains are classified into seven genotypes (GI to GVII). GI, GII, GIII, and GVII are human pathogens while GIV, GV, and GVI are simian pathogens (D’Souza et al., 2007, Arauz-Ruiz et al., 2001; Robertson et al., 1992). GI comprises of a major portion (80%) of the strains that infect humans (Kokkinos et al., 2010). HAV sub-genotypes IA and IB are of greater concern in Europe while sub-genotype IA is more prevalent in America (Kokkinos et al., 2010).
HAV is important because of severity of the disease with typical symptoms that include fever, malaise, anorexia, nausea, abdominal discomfort, dark urine and jaundice. The illness does not usually last longer than 2 months, but some (~10%–15%) patients may demonstrate prolonged or relapsing symptoms for up to 6 months. Hepatitis caused by HAV, mainly, follows four clinical phases (Hollinger et al., 1996; Koff, 1998; Lemon, 1994; Lemon, 1997; Stapleton et al., 1994). The first is the incubation (also called preclinical phase) phase, during which the patient shows no symptoms in spite of viral replication. This phase spans over 10 to 50 days and the risk of transmission is high. In the next stage, called preicteric or prodromal phase that could span over a week, symptoms including fatigue, loss of appetite, nausea, abdominal pain, fever, diarrhea, vomiting, dark urine and pale stools being to appear. The icteric phase then follows with development of jaundice within ~10 days of appearance of the initial symptoms accompanied by liver enlargement. As the jaundice progresses, viremia reduces while virus continues to be shed in the feces continues for about 1 to 2 weeks. The symptoms fade over the final phase with the liver function going back to normal (Hollinger et al., 1996; Koff, 1998; Lemon, 1994; Lemon, 1997; Stapleton et al., 1994).

HAV is very sturdy, can persist for long durations in fresh as well as salt water and soil, and temperatures higher than 85°C are required for inactivation (Sattar et al., 2000; Sobsey et al., 1988; Mbithi et al., 1992). It also exhibits resistance to inactivation methods that include use of detergents, freezing and low pH (pH 2) (Brundage and Fitzpatrick, 2006; Grove et al, 2006). HAV does not possess a lipid envelope and is stable in the gastrointestinal tract after it is excreted from the liver of an infected person into the bile (Lemon, 1997; WHO, 2000). HAV (adapted for cell culture) retains infectivity post incubation for 5 h at pH 1 (Pallansch and Roos, 2001). HAV is resistant to inactivation by low pH ( retains infectivity at pH 1 for 2 h at RT), denaturation by heat
(survives for 10 min at 70°C), as well as to chemicals including chloroform, dichlorodifluoromethane, 20% ether, trichlorotrifluoroethane, perchloracetic acid (300 mg/l for 15 min at 20°C), sodium dodecyl sulfate (SDS, 1%) (Hollinger et al., 1996; Lemon, 1994; Stapleton, 1995; Stapleton and Lemon, 1994). HAV, however, is susceptible to inactivation by autoclaving (121°C for 20 min), heating to 85°C for 1 min, ultraviolet radiation (1.1 W at a depth of 0.9 cm for 1 min), β-propiolactone (0.03% for 72 h at 4°C), formalin (8% for 1 min at 25°C), iodine (3 mg/l for 5 min), potassium permanganate (30 mg/l for 5 min), chlorine (2.0 to 2.5 mg/l free chlorine for 15 min), sodium hypochlorite (3 to 10 mg/l at 20°C for 5 to 15 min) (Hollinger et al., 1996; Lemon, 1994; Stapleton, 1995; Stapleton and Lemon, 1994).

Given the insignificant diversity among the strains recovered from individuals around the globe, only one serotype of HAV exists in spite of the difference in nucleotide composition in the genome (Lemon, 1997). While a 90% decrease in HAV cases (mostly attributed to vaccination of children and people at risk for Hepatitis A) has been observed in the US over the last 20 years, there are still an estimated 2,700 new cases of HAV illness per year (CDC, 2014c). Recent outbreaks of HAV around the world are summarized in Table 1.3. A multistate (10 states) outbreak of HAV (first in ~10 years) that occurred in the United States in May 2013 resulted in 165 illnesses, 69 hospitalizations, 2 cases of fulminant hepatitis and 1 case in which liver transplant was needed and was traced back to consumption frozen pomegranate arils (imported from Turkey) (Collier et al., 2014). Collier et al. (2014) mentioned that decreased immunity in some adults coupled with import of foods is an important factor for such outbreaks. It is hence essential to evaluate preventive and control measures for HAV infections.
2.3. Aichi virus

Another emerging foodborne virus, Aichi virus (AiV) belongs to the *Kobuvirus* genus in the same *Picornaviridae* family as HAV (Reuter et al., 2011). It was first isolated in March 1989 at Aichi Prefecture in Japan from stool specimens of patients who were suffering from nonbacterial gastroenteritis after consuming raw oysters (Yamashita et al., 1991). Studies conducted in Japan, Germany, France and Spain suggest that around 80-95% of the population between 30–40 years demonstrate presence of antibodies against AiV which is indicative of infection with AiV at some point in their life (Yamashita et al., 1993; Oh et al., 2006; Goyer et al., 2010; Ribes et al., 2010). AiV is shed in feces and transmitted via the fecal-oral route (Reuter et al., 2011). Hence water and shellfish harvested from water bodies contaminated with sewage are the primary contamination sources (Yamashita et al., 2000 and Ambert-Balay et al., 2008; Le Guyader et al., 2008). Gastroenteritis caused by AiV is characterized by diarrhea, abdominal pain, nausea, vomiting and fever (Yamashita et al., 1991; Yamashita et al., 2001). The incubation period for AiV infection is 3-7 days and the illness can last for 3-10 days (Švraka-Latifovic, 2011). AiV has been detected all over the world, in human stool samples in Japan, Germany, Brazil, Bangladesh, Thailand, Vietnam, France, Tunisia, Hungary, China and Finland (Yamashita et al., 1991 and 1993; Oh et al., 2006; Pham et al, 2007.; Ambert-Balay et al., 2008; Sdiri-Loulizi et al., 2008; Reuter et al., 2009b; Yang et al., 2009; Kaikkonen et al., 2010). AiV is an emerging human pathogen and not much is known about the control measures to prevent or treat AiV infections. It is hence essential to evaluate the potential of natural plant products and chemical sanitizers as antivirals against AiV.

AiV, *in vitro*, exhibits stability in acidic conditions as low as pH 2. It is resistant to conventional methods of inactivation including heat, alcohols, chlorine, high hydrostatic pressure, chloroform, ether and non-ionic detergents (Yamashita et al., 1998; Cromeans et al. 2014). A
recent study conducted by Cromeans et al. (2014) reported AiV to be more resistant than feline calicivirus (FCV), murine norovirus (MNV), porcine enteric calicivirus (PEC) and Tulane virus (TV) to generally used disinfection and inactivation treatments like pH, heat, alcohols, chlorine and high hydrostatic pressure (HHP).

3. Viral Transmission and Persistence

HNoV, HAV and AiV illnesses are frequently spread by indirect transmission of viral particles i.e. through contact with contaminated surfaces or consumption of contaminated water and food (Kotwal and Cannon, 2014; Reuter et al., 2011). Typically, non-enveloped viruses exhibit longer persistence in the environment than enveloped viruses (Kotwal and Cannon, 2014). Fruits (blueberries) and juices have been vehicles for outbreaks of HNoV and HAV (Calder et al., 2003; Fiore, 2004; Frank et al., 2007). Consumption of contaminated orange juice in Egypt in 2004 led to an HAV outbreak affecting 351 people from nine European countries (Frank et al., 2007). Horm and D’Souza (2011) studied the survival of HNoV surrogates in orange and pomegranate juices, milk and juice blends (1:1 v/v orange juice: pomegranate juice and 1:1 v/v orange juice: pomegranate polyphenols (4 mg/ml)) at 4°C over 21 days. No reduction for MNV-1 in milk and orange juice was observed even after 21 days cold refrigerated storage, while reduction of 1.4 log PFU/mL in pomegranate juice was obtained only after 21 days. Complete reduction was obtained for MNV-1 after 7 days in a juice blend containing orange and pomegranate juice. Complete reduction was obtained for FCV-F9 (initial titer being 6 log PFU/mL) in pomegranate and orange juices after 14 days while reduction of 3 log PFU/mL was obtained in milk after 21 days. This study demonstrated that HNoV surrogates can persist in the juices tested at refrigeration temperature over time and hence contaminated juices can become a potential source for foodborne
illness. It is hence essential to study the survival of these viruses in juices over time to determine risk of transmission.

4. Control Methods/Strategies for inactivation of viruses

Various conventional as well as non-conventional methods have been tested, used and some are being researched for their efficacy against viruses on food and food-contact surfaces. Some of these methods are discussed below.

4.1. Washing

Washing of fresh produce is generally used to get rid of any dirt from the external surfaces. Croci et al. (2002) observed that when cut lettuce, carrot and fennel were washed using potable water for 5 min minimal reductions in the range of 0.1 and 1 log were obtained for HAV. Fraisse et al. (2011) reported reductions of 0.7 log for FCV and HAV and that of 1 log for MNV-1 when lettuce was washed with tap water. Butot et al. (2008) studied viral reductions on herbs and berries (inoculated with FCV and HAV) by washing (by stirring) for 30 s in chlorinated water (200 ppm free chlorine) followed rinsing with tap water. FCV was found to be reduced by >3.5, >2.7, and <1 log in berries, and parsley, respectively while 1.4 and <1 log reduction was obtained for HAV in parsley and raspberries, respectively. Depuration, a commonly employed technique for decontamination of shellfish, for 44 h resulted in less than 2 log reduction of HAV in oysters at 25°C (Love et al., 2010).

4.2. Chemical disinfectants, sanitizers and washes

Considering the prevalence of infections and fecal-oral transmission route for HNoVs, HAV and AiV, it is crucial to develop control strategies to prevent the spread of these viruses. Use of chemical sanitizers/disinfectants for disinfection of surfaces (fomites, produce, and ready-to-eat
foods) is one such important strategy/method. Chemical sanitizers/disinfectants as washes are cost-effective approaches for the control of foodborne pathogens on contaminated surfaces or fresh produce. Ozone, chlorine, organic acids (tannic acid, lactic acid, peroxycetic acid), alcohols, benzalkonium chloride, glutaraldehyde, hydrogen peroxide, trisodium phosphate, potassium monosulfate and sodium metasilicate have been evaluated for their antibacterial and antiviral properties (Allwood et al., 2004; Gehrke et al., 2004; Su and D’Souza, 2011a and 2012; Doltree et al., 1999; Moadab et al., 2001; Fraisse et al., 2011; Kim et al., 1999; Li et al., 2011; Lindsay, 1985; Anipsitakis et al., 2008; USDA FSIS, 2011). The target for inactivation of enteric viruses include the viral genome and the viral capsid (Kingsley et al., 2014). The factors that influence the choice of a disinfectant are the target microorganisms, type and amount of organic material, time of exposure, concentration, pH of the system, temperature, and topography of the food product in in consideration (Warf et al., 2007).

4.2.1. Oxidizers

Commonly used oxidizers comprise of household bleach (sodium hypochlorite), chlorine, chlorine dioxide, iodine-based disinfectants, ozone, hydrogen peroxide and peracetic acid (Nims and Plasvic, 2013).

4.2.1.1. Chlorine

Chlorine is one of the conventional disinfectants, with approval for use in three forms including chlorine gas, sodium hypochlorite, and calcium hypochlorite (Fonseca 2006). Disinfectant efficacy of chlorine depends on the quantity of free chlorine and organic matter, temperature and pH (Hirneisen et al., 2010). The concentration of chlorine generally used is 50–200 mg/L, at a pH of 6-7.5, with a contact time of 1-2 min (Hirneisen et al., 2010). Noroviruses
are found to be quite resistant to inactivation by chlorine, leading to outbreaks caused due to insufficiency of the use of common disinfection methods (Hirneisen et al., 2010). Keswick et al. (1985) found that when water inoculated with norovirus was treated with chlorine at a level of 10 mg/L and was consumed by eight volunteers, illness did not occur. However, when chlorine at 3.75 to 6.25 mg/L (concentrations generally used for water treatment/disinfection) was used for water treatment, five volunteers out of eight were found ill (Keswick et al., 1985). Li et al. (2002) reported that complete inactivation of HAV was obtained in 30 min using chlorine at 10 or 20 mg/L. Reductions of 1.7 log were obtained for HAV when tomatoes, strawberries and lettuce were treated with 20 ppm chlorine (Casteel et al., 2008). Chlorine is typically used at levels of 50-200 ppm in washes for fresh produce (Herdt and Feng, 2009). Treatment of HNoV with free chlorine at 33 and 189 ppm for 1 min resulted in reductions in binding of virus by 1.48 and 4.14 log, respectively as analyzed using the porcine gastric mucin binding magnetic bead (PGMB) assay (Kingsley et al., 2014). Sodium hypochlorite at 200 mg/L was found to reduce high (~7 log PFU/mL) and low titers (5 log PFU/mL) of FCV-F9 on lettuce and jalapeno peppers in 15 s by <1.4 log PFU/mL and to undetectable levels, respectively (Su and D’Souza, 2011). For high and low titers of MNV-1, Su and D’Souza (2011) observed reductions of ~2-3 log PFU/mL and <1.3 log PFU/mL, respectively in 15 s with 200 mg/ml of sodium hypochlorite. HAV was reduced by 5 log using 3000 ppm of sodium hypochlorite at 22°C for 5 min (Jean et al., 2003). When viruses dried on stainless steel discs were exposed to 200 or 1,000 ppm chlorine for 5 min, reductions of 0.9±0.2 log and 1.3±0.9 log were obtained, respectively for AiV (Cromeans et al., 2014).

The mechanism of action of chlorine and chlorine-releasing compounds against viruses is not clearly established yet. O’Brien and Newman (1979) observed that inactivation of poliovirus with chlorine involved release of RNA as a second step while the first step of
inactivation remained unclear. When used in presence of organic matter, chlorine may form carcinogenic by-products like trihalomethanes (Di Cristo et al., 2013), thus making it necessary to search for alternative chemicals that are safe.

4.2.1.2. Chlorine dioxide

Chlorine dioxide (ClO₂), an oxidizing agent, is gaining popularity as an antimicrobial (Khadre et al., 2001) as it does not form harmful trihalomethanes by-products as is the case with chlorine. It is approved to be used as an antimicrobial agent for treatment of water at levels not to exceed residuals of 3 ppm ClO₂ (Kingsley et al., 2014). It is highly water soluble (five times higher solubility than chlorine), is unaffected by presence of organic matter or ammonia (White, 1978; Schnoor et al., 1979) and shows relatively higher pH stability than chlorine (Aieta and Berg, 1986). However, ClO₂ can be explosive at high concentrations and it needs to be generated at the site of use making it difficult to work with (Beuchat, 1998).

Alvarez and O’Brien (1982) suggested that chlorine dioxide caused inactivation of poliovirus by hydrolyzing the viral RNA causing problems in viral replication. Reductions of 1.7 log were obtained for HAV when tomatoes, strawberries and lettuce were treated with 20 ppm chlorine (Casteel et al., 2008). Chlorine dioxide at 7.5 mg/L caused complete reduction (~6 log) of HAV in 10 min, with damage to the 5’NTR region of the genome as well as reaction with the capsid proteins (causing loss of antigenicity) being suggested as the mechanism of action of ClO₂ (Li et al., 2004). Kingsley et al. (2014) observed that treatment of HNoVs with 350 ppm of ClO₂ dissolved in water for 30 min resulted in no reduction while treatment for 60 min caused reduction of 2.8 log.
The difference in mechanism of action of chlorine and ClO$_2$ is thought to be caused by the tendency of chlorine to damage capsid proteins by affecting its polypeptide backbone while that of ClO$_2$ to target the amino acid side chains (Kingsley et al., 2014; Sigstam et al., 2013).

4.2.1.3. **Hydrogen peroxide**

Hydrogen peroxide (H$_2$O$_2$), a strong oxidizing agent, is an antimicrobial that can oxidize capsid proteins (thiol groups) and nucleic acids leading to damage to viral RNA (Finnegan et al., 2010). For use as produce wash, H$_2$O$_2$ is used at concentrations of 1-4% (Herdt and Feng, 2009). Liquid H$_2$O$_2$ (2.5%) caused ~1 log reduction of MNV-1 in 5 min, similar to that obtained with water when washing lettuce (Li et al., 2011). For MS2, reduction of 3.2 log was obtained in 60 s when 2% H$_2$O$_2$ was used as a spray on lettuce (Xie et al., 2008). Spray of 2% H$_2$O$_2$ for 10 s followed by exposure to UV (0.632 mW/cm²) for 30 s increased the reduction of MS2 to 4 log; this increase in reduction could be attributed to the ability of free radicals (formed on exposure to UV) to enter the viral capsid and cause inactivation. When studied using the PGMB assay, Kingsley et al. (2014) observed that treatment of HNoV with 4% H$_2$O$_2$ for 60 min did not cause significant reduction (~0.1 log) in viral binding. H$_2$O$_2$ retains its activity in the presence of organic matter and does not form toxic by-products (Herdt and Feng, 2009; De Luca et al., 2008).

4.2.1.4. **Potassium peroxymonosulfate**

Potassium peroxymonosulfate (PPMS) is an oxidizer used to disinfect water in swimming pools against *Escherichia coli* (Anipsitakis et al., 2008). Applications of PPMS (4%) also include being used for inactivation (>6 log in 30 min) of *S. aureus* and *S. Typhimurium* in veterinary hospitals (Patterson et al., 2005). PPMS has also been studied for its effect against viruses and was found to inactivate FCV (animal isolate and not the surrogate for HNoV) within 10 min (Eleraky
et al., 2002). When used in an embryo model, Virkon S (containing PPMS) was observed to inactivate the astrovirus related to poultry enteric mortality syndrome (Schultz-Cherry et al., 2001). FCV-F9 was found to be reduced to undetectable levels (initial titer: 4-5 log PFU/mL) within 10 min of treatment with 1% Virkon (5 mg/mL PPMS) (Solomon et al., 2009). High (~7 log PFU/mL) and low (~5 log PFU/mL) titers of FCV-F9 and low titers of MNV-1 were shown to be reduced to undetectable levels by PPMS (5, 10, and 20 mg/mL) (Su and D’Souza, 2012). Reductions of 0.92 and 3.44 log PFU/mL were obtained for MNV-1 in 2 h on treatment with 5 and 10 mg/mL of PPMS, respectively at RT (Su and D’Souza, 2012).

4.3. Acid sanitizers: Organic acids

Commonly used organic acids for viral inactivation (HNoV and human enteroviruses) include lactic acid, peroxyacetic acid and tannic acid. The mechanism of action of organic acids against these viruses are not completely clear yet with the inactivation thought of being the result of damage to the viral capsid as well as nucleic acids; it is not understood, however, if the agent for this damage is the low pH or the acid structure (Cliver, 2009; Salo and Cliver, 1976). In case of non-enveloped viruses, decrease in pH is thought to be the agent for protein denaturation in viral capsids (Rodger et al., 1977). The antimicrobial effect exerted by organic acids is generally attributed to lowering of pH of the environment (In et al., 2013). Other factors that alter this antimicrobial activity include the percentage of undissociated acid, degree of branching and chain length (Doores, 1993). Citric acid, in combination with sodium lauryl sulfate and malic acid demonstrated antiviral activity against rhinovirus (Hayden et al., 1985). Studies show that citric acid application on periodontally diseased root surfaces resulted in reduction of both aerobic and anaerobic cultures (Daly, 1982). A citric acid-based bathroom disinfectant (2.5% citric acid, pH 2.0) was shown to cause reduction of >3.17 log PFU/mL for FCV-F9 in 1 min (Whitehead and
McCue, 2010). Use of 0.3% and 0.4 % D, L-lactic acid with pH 3.4-3.5 and 3.2-3.3, respectively in suspension tests resulted in significant reduction in the titer of FCV-F9 at 20°C after 7 days of exposure (Straube et al., 2011). Studies show that peroxyacetic acid (PAA) is not very effective in reduction of HNoV, as <1 log reduction in viral binding was obtained for HNoV on treatment with 195 ppm of PAA (Kingsley et al., 2014). Use of a peroxy acetic acid-based biocide (100 ppm) for washing lettuce did not result in significant reduction of HAV while reductions of 3.2 log and 2.3 log were obtained in 2 min for FCV-F9 and MNV-1, respectively (Fraisse et al., 2011). At 250 mg/L, PAA was found to reduce MNV-1 by 1 log on shredded lettuce, with the activity being unaffected by organic matter (Baert et al., 2009). Venno Vet 1 Super, a disinfectant containing a mixture of formic acid (55-60%) and glyoxylic acid (7%), at 0.5% concentration was found to reduce FCV-F9 by 3 log in 15 min and 60 min as analyzed using cell culture and RT-PCR, respectively; greater concentration (4%) was required when 40% fetal bovine serum or 25% feces were used in the suspension test (Poschetto et al., 2007). Malic acid, found in apple juice, on combination with sodium lauryl sulfate and citric acid demonstrated potential antiviral effect against rhinovirus (Hayden et al., 1985). Gallic acid from black raspberry juice was shown to have no effect on reduction of FCV-F9 or MNV-1 while the juice itself did demonstrate antiviral activity (Oh et al., 2012). Su and D’Souza (2012) studied the effect of tannic acid and gallic acid against high (~7 log PFU/mL) and low (~5 log PFU/mL) of FCV-F9. Tannic acid (0.2 mg/mL) at RT was shown to reduce high and low titers of FCV-F9 by <1 and 1.95 log, respectively while gallic acid, at 0.1, 0.2, and 0.4 mg/mL reduced low titers of FCV-F9 by 2.50, 2.36, and 0.86 log PFU/mL, respectively in 2 h (Su and D’Souza, 2012). Acetic acid, an effective antibacterial against Salmonella Typhimurium, Enterobacter sakazakii and Listeria monocytogenes (Alvarez-Ordonez et al., 2009; Back et al., 2009; Vasseur et al., 1999), when used to dissolve chitosan caused
reductions of 1.19-1.29 log PFU/mL for φX174, 1.88-5.37 log PFU/mL for MS2, 0.09-0.28 log PFU/mL for MNV-1 and 2.27-2.94 log PFU/mL for FCV-F9 after 3 h at 37 °C (Davis et al., 2012).

4.4. Alkaline sanitizers

4.4.1. Trisodium Phosphate

Studies show that HNoV capsid can be unstable under alkaline conditions leading to a possibility of viral inactivation (Ausar et al., 2006). Trisodium phosphate (TSP), in 1 % solution, is alkaline (pH~12). It is a generally recognized as safe (GRAS) substance as per the Food and Drug Administration and is known to be effective in inactivation of several pathogens like Salmonella spp., E. coli O157: H7, poliovirus 1 and bacteriophages (Jerzy et al., 2003). However, when used as a wash, it increases the phosphate content of wastewater posing risk of eutrophication in the water environment (Kingsley et al., 2014). D’Souza and Su (2011) studied the effect of TSP as a wash for 15 s on lettuce and jalapeno peppers contaminated with FCV-F9 and MNV-1. They found that low initial titers (~5 log PFU/mL) of FCV-F9 were reduced to undetectable levels by 2 % and 5 % TSP while high initial titers (~7 log PFU/mL) were reduced by >5 log PFU/mL and to undetectable levels with 2% and 5 % TSP, respectively. For MNV-1, with low initial titers, reduction by~2-3 log PFU/mL and to undetectable levels was obtained with 2% % and 5 % TSP, respectively; high titers of MNV-1 were reduced by ~2-3.4 log PFU/mL and to undetectable levels with 2% % and 5 % TSP, respectively. Hence, D’Souza and Su (2011) suggested that TSP at 5% could be used as a produce was for reduction of HNoV on fresh produce. Treatment of HNoV with 5% TSP for 5 min reduced viral binding by 1.6 log, as analyzed by the PGMB assay indicating that TSP is effective in reduction of HNoVs.


4.4.2. Sodium Metasilicate

Sodium metasilicate (NaMS), an alkaline compound (1% solution has pH 12.5-13.0), has USDA FSIS approval for use as a processing aid. As an antimicrobial, NaMS may be used in meat and poultry marinades (at 2% of weight of the marinade) and on raw beef carcasses, trimmings, and subprimals (as a spray or rinse at concentration of 6% in solution (USDA FSIS, 2012). NaMS (at concentrations of 0.6 to 4%) has been reported to inhibit \textit{E. coli} O157: H7 as well as six non-O157 Shiga toxin-producing \textit{E. coli}, \textit{Campylobacter jejuni} and \textit{Salmonella} Typhimurium in beef and chicken products (Carlson et al., 2008; Geornaras et al., 2012; Pohlman et al., 2009; Weber et al., 2004; Sharma et al., 2012a, b; Adler et al., 2011). NaMS has also been tested for effectiveness against viruses. NaMS, at 0.5 to 5% concentration, was found to enhance the antiviral activity of quaternary ammonium-based disinfectants against human rotaviruses (Springthorpe et al., 1986). Whereas, NaMS (5%, pH 12.1) did not exhibit any antiviral activity against vesicular stomatitis virus in 10 min (Wright, 1970).

4.4.3. Alkaline Wash

One of the chemical-based antibacterial washes and sanitizers available commercially and being used in the food processing industry is Alkaline Wash (ALKW), an alkaline wash (pH 14) consisting of a mixture of calcium hydroxide, sodium lactate, ethanol, lactic acid and water has shown to be a potent disinfectant against bacteria. Calcium hydroxide (1%) has shown to inactivate low pathogenic avian influenza virus on a metal surface effectively after 10 min (Alphin et al., 2009). Calcium hydroxide (pH 9.5 to 10.5) treatment of wastewater resulted in a 2 log PFU/mL reduction of poliovirus (type I, Sabin) (Sattar and Ramia, 1978). Calcium hydroxide (pH 11.2) was also reported to reduce bacteria, enteric viruses, and coliphages in wastewater with the pH being regarded as the key factor for the antimicrobial activity (Grabow et al., 1978). The disinfection
caused by alkalis is attributed to their ability to denature proteins, even in the presence of organic matter (Agriculture and Resource Management Council of Australia and New Zealand, 2000). When dipped in 2.5% (w/v) aqueous solution of sodium lactate the growth of *Pseudomonas* spp., H2S-producing bacteria, lactic acid bacteria, and *Enterobacteriaceae* on fresh salmon slices was inhibited (Sallam, 2007). Sodium lactate also inhibited the growth of lactic acid bacteria and aerobic psychrotrophic bacteria on refrigerated sliced poultry sausage when stored under refrigeration (Cegielska-Radziejewska and Pikul, 2004).

4.5. Alcohols

Ethanol (70%) is a conventionally used disinfectant and sanitizer which is effective in inactivation of bacteria. Ethanol acts against viruses by targeting the capsid, causing denaturation of the proteins, and in case of enveloped viruses, it dissolves the lipids in the envelope (McDonnell and Russell, 1999). For non-enveloped viruses like the HNoV, results for efficacy of ethanol in viral reduction are contradictory. Various studies report that ~70% ethanol is effective in reducing titers of FCV-F9 with reductions ranging between 2 to 5 log PFU/mL obtained within 30 s to 10 min (Di Martino et al., 2010; Malik et al., 2006; Gehrke et al., 2004; Macinga et al., 2008) and that of MNV-1 by ~4 log within 5 min of exposure (Belliot et al., 2008; Magulski et al., 2009; Park et al., 2010). However, some studies report that ethanol is not effective in reduction of viral titers of HNoV surrogates; with reductions ranging from 0 to 3 log in 30 s to 30 min for FCV-F9 with 70% ethanol (D’Souza and Su, 2010; Park et al., 2010; Duizer et al., 2004; Doultree et al., 1999; Whitehead and McCue, 2010; Lages et al., 2008).

In addition to ethanol, some other alcohols have also been tested for their effects on HNoV surrogates. Majority of the studies conducted to evaluate the efficacy of isopropanol for its antiviral activity suggest that it is highly effective in reduction of FCV-F9 and MNV-1 being
more resistant to inactivation (Belliot et al., 2008; Gehrke et al., 2004; Malik et al., 2006; Park et al., 2010). However, Park et al. (2010) reported that no reduction in viral titers were obtained for FCV-F9 on treatment with 50%, 70% or 90% isopropanol for 1 or 5 min. MNV-1 was reported to be reduced by 2.6-3.8 log on exposure to 60-70% isopropanol (Park et al., 2010; Belliot et al., 2008; Magulski et al., 2009). Reductions of 4 log PFU/mL were obtained for FCV-F9 on treatment with 50 to 70% 1-propanol, in suspension, for 30 s (Gehrke et al., 2004). When used on surface, reductions of ≥4 log was obtained for MNV-1 on treatment with 30% 1-propanol for 5 min (Magulski et al., 2009). Treatment with 70% isopropanol, 90% isopropanol, 70% ethanol or 90% ethanol resulted in less than 0.5 log reduction in AiV titer in 1 and 5 min indicating its resistance to alcohols that are generally used as sanitizers (Cromeans et al., 2014).

4.6. Quaternary ammonium compounds

Quaternary ammonium compounds are surface-acting agents typically used in hand sanitizers (Moadab et al., 2001) and surface disinfectants (Gradel et al., 2004) as they demonstrate antimicrobial activity against a variety of bacteria, fungi, and viruses (Bélec et al., 2000; Jira et al., 1982; Bastiani et al., 1974). At lower concentrations, they are not very effective in reduction of HNoV or its surrogates MNV-1 and FCV-F9 (reduction of ~1 log) with higher concentrations exhibiting increased reductions of 2-3 log with increased time of treatment. Benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride) is one of the quaternary ammonium compounds that is used as a disinfectant. The antimicrobial activity of benzalkonium chloride (BAC) is thought to be due to the changes it causes at or adjacent to the surface of the virus leading to its inability in attaching to the host cell receptor (Wood and Payne 1998). BAC has been reported to exhibit antiviral activity against canine respiratory syncytial virus, coronavirus, herpes simplex virus, adenovirus, cytomegalovirus and hominis type 2 virus, human immunodeficiency virus type 1 and
human coxsackie virus (Belec et al., 2000; Pratelli, 2007; Wood and Payne, 1998). In suspension tests, low titers (\(~5 \log 10 \text{ PFU/mL}\)) of FCV-F9 and MNV-1 were reduced to undetectable levels when treated with BAC at concentrations of 0.2, 0.5, and 1 mg/mL for 2 h at room temperature. High titers (\(~7 \log \text{ PFU/mL}\)) of MNV-1 were reduced by 1.55, 2.32, and 2.75 log PFU/mL and that of FCV-F9 were reduced by 2.87, 3.08, and 3.25 PFU/mL, by 0.2, 0.5, and 1 mg/mL of BAC respectively (Su and D’Souza, 2012).

4.7. Glutaraldehyde

Glutaraldehyde is an active ingredient in commercially available sanitizers. Greater than 3 log reduction (TCID50 assays were used) was obtained for HAV when treated with 0.10% glutaraldehyde for 30 min and in 3 min when treated with 0.5% glutaraldehyde (Passagot et al., 1987). HAV titers were reduced by 99.9% on stainless steel discs using 2% glutaraldehyde after 1 min (Mbithi et al., 1990)). Reduction of 7 log for HAV in 5 min was reported when treated with a disinfectant solution consisting of 5% glutaraldehyde and 10% quaternary ammonium at the final concentration of 3,000 ppm but only 3 log reduction when treated on surfaces (Jean et al., 2003). Alternative GRAS sanitizers are being researched for improved efficacy.

4.8. Combinations of chemicals

Using synergistic effect of multiple chemicals is an effective method to enhance microbial inactivation and hence several combinations of chemicals have been evaluated for their antiviral activity. Some of the combinations are discussed below.

4.8.1. Levulinic acid and sodium dodecyl sulfate

Levulinic acid and sodium dodecyl sulfate are generally recognized as safe (GRAS) substances that could be used for addition to food as a flavoring substance or adjunct (21 CFR
172.515) and multipurpose additive (21 CFR 172.822) respectively. Cannon et al. (2012) reported that a combination of 0.5% levulinic acid and 0.5% SDS (pH 2.8) when combined with MNV-1 or FCV-F9 at 10:1 ratio resulted in reductions of 3 to 4.21 log PFU/mL within 1 min. When inoculated on stainless steel, MNV-1 reduction of >1.50 log PFU/mL after 1 min and >3.3 log PFU/mL after 5 min of exposure to a liquid or foaming solution of 5% levulinic acid plus 2% SDS was obtained. The effect of this combination was found to be unaffected by the presence of up to 10% of organic matter. However, the mechanism of action of this sanitizer is not known. Predmore and Li (2011) observed that the reduction of MNV-1 on produce was enhanced when a combination of surfactants (including SDS) was used with chlorine at 200 ppm. They suggested that SDS helped detach the virus from the surface of the produce and hence made the viral particles available for the action of the antiviral compound. However, Cannon et al. (2012) observed no difference in reduction when stainless steel discs contaminated with HNoV were subjected to static treatment with water or water containing 2% SDS. The results suggest that the antiviral action is dependent on the pH of the sanitizer because lower reduction was obtained for MNV-1 when pH of the sanitizer was increased to 4.0 from 2.8. But when MNV-1 was treated at pH 2.0 only minimal reduction was obtained even after exposure for 2 h. The authors hence suggested that the sanitizer targets the capsid of the viral particles. The application of LA and SDS wash against HAV or AiV has not yet been reported in literature, and this research would help determine the spectrum of antiviral activity of LA-SDS combinations.

4.9. Storage at low temperature

Storing food at low temperatures is a conventional method used to preserve food and to prevent growth of spoilage organisms or pathogens (Zuber et al., 2013). HAV, in mineral water packed in plastic bottles (viruses may adhere to the wall), was found to survive better for 9 months
at 4°C (0.5 log reduction) than at RT (4.1 log reduction) (Biziagos et al., 1988; Butot et al., 2007). Storage of carrots and fennel at 4 °C for 7 days caused >2.5 log reduction for HAV while 2 log reduction was observed in lettuce under the same conditions (Croci et al., 2002). When stored at 5 °C for 15 days, and at -17.5 °C (freezing) for 12 weeks, poliovirus (more sensitive to environmental conditions than other enteric viruses) on oysters was reduced by just 1 log (Di Girolamo et al., 1970; Abad et al., 1994). Hence, low temperature storage might not reduce viral loads to a great extent (Zuber et al., 2013).

4.10. Use of Heat

Heat is a conventionally used food processing method that can be used for inactivation of pathogens and spoilage organisms. Boiling water to a rolling boil for a minimum of 1 min is highly (>4 log reduction) effective in inactivation of HNoVs and HAV (Zuber et al., 2013; CDC, 2009). Heating water to 72 °C for 1 min resulted in reduction of HAV and MNV by >3.5 log with similar reductions obtained in milk (Hewitt et al., 2009). When heated to 63 °C for 1 min, MNV-1 and HAV in water were reduced only by 1.1 and 1.3 log, respectively and that in milk were reduced by 1.4 and 1.6 log, respectively; increasing the heating time to 5 and 10 min at 63 °C resulted in >3 log reductions for both MNV-1 and HAV (Hewitt et al., 2009). AiV was rapidly inactivated when subjected to heat treatment at 60°C and 63°C and that at 56°C, 4 log PFU reduction (maximum measurable reduction being 5 log10 PFU) in infectivity was obtained for AiV in 20 min (Cromeans et al., 2014).

However, for food, which is complex matrix, greater time would be needed to achieve inactivation of viruses (Zuber et al., 2013). Hewitt and Greening (2006) reported that when New Zealand greenshell mussels were immersed in boiling water for 3 min, reduction of 3.5 log was obtained for HAV. The Codex Guidelines on the application of general principles of food hygiene
to the control of viruses in food (Codex Alimentarius Commission, 2012) and those of the UK Ministry of Fisheries directive (Waterman, 2001) require mussels to reach and maintain an internal temperature of 90 °C for 90 s (Zuber et al., 2013). Hewitt and Greening (2006) observed that the mussels would need to be heated in boiling water for about 170 s just to reach 90 °C. When heated in boiling water for 1 min, FCV in cockles was found to be reduced to undetectable levels with 2 log reduction obtained in 30 s (Hewitt and Greening, 2006). Hewitt et al. (2006) also observed that reduction of less than 2 log was obtained for HAV when mussels are steamed for about 3 min (cooking time in general practice).

Steam blanching of basil for 2.5 min at 95 °C inactivated HAV and FCV by >3 and >4 log respectively (Butot et al., 2009; Zuber et al., 2013). Wet heat demonstrates higher efficiency in inactivation of viruses than dry heat as heating at 100 °C for 20 min was required to inactivate HAV (2 log reduction) in freeze-dried strawberries, raspberries, and blackberries (Zuber et al., 2013; Butot et al., 2009). MNV-1 in spinach was reduced by 2.4 log by blanching at 80 °C for 1 min (Baert et al., 2008b). MNV-1 in raspberry puree was reported to be reduced by 1.9 and 2.8 log when pasteurized at 75 °C for 15 s and 65 °C for 30 s, respectively (Baert et al., 2008a). D-value for HAV in strawberry mash at 52 °Brix (sucrose) was found to be ~8 times the D-value at 28 °Brix (Deboosere et al., 2004) suggesting that high sugar content could be enhancing the resistance of HAV to thermal treatments (Zuber et al., 2013). When heated for 20 h at 56.4 and 65.9 °C (dehydration process), HAV on green onions was reduced by 1.6 and >3.9 log, respectively (Laird et al. 2011).

4.11. Acidification

As discussed earlier, MNV-1 is more resistant to inactivation by low pH than FCV (can be attributed to the transmission route for FCV) (Zuber et al., 2013). Hewitt and Greening (2004)
evaluated the effect of a marination process (treating with boiling water and steam for 3 min with subsequent marination in an acetic-acid marinade (pH 3.75). In mussels, FCV was found to be reduced by >4 log in 24 h without being subjected to heat treatment whereas HAV was found to survive for 4 weeks of storage at 4°C. When subjected to both heat and marination, HAV was not completely reduced (3 out of 9 were positive for HAV) in mussels. AiV exhibited resistance to extreme pH with no significant reduction when subjected to pH 2, 3, 9 and 10 for 30 min at 37°C (Cromeans et al., 2014).

4.12. High hydrostatic pressure

In this processing method, the food is subjected to pressures ranging from 100 to 600 MPa. High hydrostatic pressure (HHP) is an alternative method for inactivation of pathogens that can be used for foods in which other treatments (like heat) would lead to a change in sensory quality of the end product (as in case of shellfish which is preferred to be eaten raw). The mode of antiviral action of HHP is considered to be due to disruption of capsids of viral particles with probable change in tertiary and quaternary structures of capsid proteins while no significant effect is seen on primary and secondary structures of proteins (Lou et al., 2011).

HHP was found to inactivate FCV-F9 and MNV-1 completely at ≥300 mega Pascal (MPa), TV at 600 MPa while AiV demonstrated complete resistance to pressures up to 800 MPa (Cromeans et al., 2014). MNV-1 on clams was observed to be reduced by <1 log on HHP treatment to 400 MPa (Arcangeli et al., 2012) while complete inactivation was reported by Kingsley et al. (2007) for MNV-1 using 400 MPa after 5 min and 500 MPa in 1 min at 5 °C (Zuber et al., 2013). This difference in reductions could be attributed to either the difference in the shellfish tested, the temperature of treatment or the equipment used (Zuber et al., 2013). For HAV, reduction of 3 log was obtained when oysters were treated at 400 MPa after 1 min (Calci et al., 2005) and 4 log
reduction was obtained when green onions and strawberries were treated at 375 MPa (Kingsley et al., 2005). High pressure processing treatment of AiV at 600 MPa in minimum essential growth medium with 10% fetal bovine serum for 5 min was found to be ineffective in reducing AiV titers (Kingsley et al., 2004).

4.13. Ultrasound

Schultz et al. (2012) tested the effect of steam-ultrasound treatment (combination of steam under pressure and high power ultrasound) on inactivation of MS2 in raspberries and observed reduction of ~1 log in 1 min. Nevertheless, the steam-ultrasound treatment was reported to be more efficient on solid (plastic) surfaces causing reduction of >4 log for FCV and MS2 and 3.7 log reduction for MNV-1 (Schultz et al., 2012; Zuber et al., 2013). Su et al. (2010) evaluated the effect of high-intensity ultrasound (HIU) (known to damage cell wall, cell membrane as well as nucleic acids) against FCV, MS2 and MNV-1. They reported MNV-1 to be the most resistant of the three viruses with reductions of >3.5 log (low initial titer of ~4 log PFU/mL) obtained for MNV-1 in PBS when subjected to 20 kHz HIU treatment for 30 min while no reduction was obtained for MNV-1 with initial titers of 6 log PFU/mL. When tested in orange juice, reductions due to HIU treatment were found to decrease which was attributed to the protective effect of complex matrix of orange juice (Su et al., 2010c).

4.14. Other methods

Vacuum freeze drying, typically used to dehydrate berries (with minimal losses in flavor, colour and antioxidants) of raspberries over 18 h (heating at 115 °C for 3 h, cooling to 60 °C for 13 h and heating again to 115 °C in 2 h) caused HAV to reduce by 1.5 log (Butot et al., 2009; Zuber et al., 2013). Modified atmosphere packaging (MAP), a technique used to enhance shelf-
stability of food products, using 70% CO$_2$ and 30% N$_2$ was found to be ineffective in reduction of HAV on lettuce stored at 4°C for 12 days (Bidawid et al., 2001).

4.15. Natural plant extracts as antivirals

Given the pronounced effect that foodborne viruses have on the human population, it is essential to evaluate natural plant extracts to control their spread (Su et al. 2010a). Natural components are preferred by consumers as they are perceived to be safer than chemicals used in food processing and preservation, can be lower in cost, and are sustainable. Berries and fruits are gaining increasing popularity associated with health benefits that include high antioxidant capacity. Table 1.4 summarizes the use of natural extracts as antivirals from various plant sources. Oh et al. (2012) observed that black raspberry juice, at concentrations of 3% and 6%, demonstrated antiviral activity against MNV-1. However, quercetin and gallic acid (the principal phenolic components in black raspberry juice), did not exhibit any antiviral activity against MNV-1 when used individually for treatment (Yoon et al., 2003; Ju et al., 2009).

4.15.1. Pomegranate polyphenols

Pomegranate (*Punica granatum*) is shrub grown in the Mediterranean region, China, Russia, India, Japan, and US (Haidari et al., 2009). Pomegranate is a very good source of polyphenols that comprise of anthocyanins (flavonoids), ellagitannins and gallotannins (hydrolysable tannins), and proanthocyanidins (condensed tannins) and hence has been studied for its nutritional as well as medicinal properties (Jaiswal et al., 2010; Gil et al., 2000; Hernandez et al., 1999; Santagati et al., 1984). Pomegranate juice, with greater content of antioxidants than cranberry, grape, orange or grapefruit juice, has been reported to be three times more effective than green tea and red wine in scavenging free-radicals as well as chelating iron (Basu and
Pomegranate juice and extracts exhibit antibacterial activity against *Staphylococcus aureus* and *Bacillus cereus* which is thought to be due to the disruption of cell membranes by the phenolics (Kanatt et al., 2010). Pomegranate extract, juice and polyphenols were shown to be effective in reduction of influenza virus, FCV-F9, MNV-1 and MS2 (Su et al., 2010b; Haidari et al., 2009). Su et al. (2010b) have shown that high (~7 log PFU/mL) titers of FCV-F9, MNV-1, and MS2 were reduced by 1.20, 0.06, and 0.63 log PFU/mL respectively when treated with pomegranate juice for 1 h at room temperature. However, when these viruses were treated with 8, 16, and 32 mg/mL of pomegranate polyphenols for 1 h at room temperature, both high and low titers of FCV-F9 were reduced to undetectable levels, while MNV-1 (low initial titer of ~ 5 log PFU/mL) was reduced by 1.30, 2.11, and 3.61 log PFU/mL and MNV-1 (high initial titer) was reduced by 1.56, 1.48, and 1.54 log\textsubscript{10} PFU/mL respectively. Su et al. (2010b) suggested that the pomegranate juice and polyphenols could be preventing binding of the viral particles to the host cells by blocking the host cell receptors.

**4.15.2. Grape seed extract**

Grape seed extract (GSE) is a byproduct of the wine industry and is a low cost source of antioxidants. The bioactive components in GSE include flavonoids, ascorbic acid, citric acid, limonoids, sterols, and tocopherols (Bevilacqua et al., 2010; Armando et al., 1998). When treated with GSE at 0.25, 0.5, and 1.0 mg/ml at 37°C for 2 h, reductions of 1.49, 1.72, and 1.97 log PFU/mL for MNV-1 and 1.86, 2.26, and 2.89 log PFU/mL for HAV were respectively observed by Su et al. (2011). GSE has also been tested as a produce wash against FCV-F9, MNV-1 and HAV on lettuce and jalapeno peppers (Su and D’Souza, 2013). On lettuce FCV-F9 (initial titers of ~7 log\textsubscript{10} PFU/mL) was reduced by 2.33, 2.58, and 2.71 log\textsubscript{10} PFU/mL and on jalapeno peppers
it was reduced by 2.20, 2.74, and 3.05 log₁₀ PFU/mL in 1 min with 0.25, 0.50, and 1 mg/mL GSE, respectively. On lettuce and jalapeno peppers MNV-1 (initial titer of ∼5 log₁₀ PFU/mL) was reduced by 0.2-0.3 and 0.8 log₁₀ PFU/mL, respectively. HAV at initial titers of ∼7 and ∼5 log₁₀ PFU/mL was reduced by 0.7-1.1 and 1-1.3 log₁₀ PFU/mL with 0.25-1 mg/mL GSE, respectively in 1 min.

4.15.3. Blueberry polyphenols

Blueberries, native to North America, are a source of polyphenols (flavonoids, anthocyanins and proanthocyanidins) (Wang et al., 2010; Smith et al., 2000; Howell et al., 1998; Bomser et al., 1996). Blueberry proanthocyanidins exhibit antimicrobial activity against bacterial and hepatitis C viral pathogens (Joshi et al., 2014; Lacombe et al., 2012; Chatterjee et al., 2004; Ofek et al., 1996). Blueberry proanthocyanidins have been reported to exert antiviral activity against hepatitis C virus by inhibiting its expression and replication (Takeshita et al., 2009).

4.15.4. Cranberries as antivirals

In addition to these fruits described above, cranberries (Vaccinium macrocarpon) contain bioactive polyphenols with known antimicrobial and anti-inflammatory properties. Cranberry proanthocyanidins (C-PAC), the bioactive polyphenols in cranberries, exhibit unusual A-type linkages (Foo et. al. 2000a, 2000b), while other foods rich in tannins possess proanthocyanidins with B-type linkages. Cranberries have been used over centuries to prevent bacterial urinary tract infections (UTI).

Although known for antibacterial effects especially against E. coli causing urinary tract infections and enteric bacterial pathogens, CJ and C-PAC have also shown significant antiviral activity against bacteriophages T4 and T2, the simian rotavirus SA-11, bovine reovirus (Lipson et
al., 2007) and influenza virus (Weiss et al., 2005). CJ and pomegranate juice were shown to be effective in decreasing the specific binding ability of HNoV to saliva when P particles of HNoV GII.4 were used as a research surrogate (Li et al, 2012). Su et al. (2010a) reported that CJ and C-PAC at 0.15, 0.3 and 0.6 mg/ml were effective in reducing the infectivity of human enteric viral surrogates, MNV-1, FCV-F9, bacteriophage MS2 and bacteriophage φ-X174 after 1 h at RT. Transmission electron microscopy (TEM) analysis showed structural damage to FCV-F9, but TEM studies on MNV-1 treated with C-PAC are needed.

4.15.4.1. Bioactive compounds in Cranberries

American cranberry (Vaccinium macrocarpon Ait) contains many bioactive compounds/phytochemicals. These include flavonols, anthocyanins, flavan-3-ols, tannins (proanthocyanidins and ellagitannins), and phenolic acid derivatives (Côté et al., 2010). The polyphenols present in cranberries have demonstrated antioxidant, antiviral, antibacterial, antimutagenic, anti-inflammatory, anticarcinogenic, antiangiogenic, and antitumorigenic properties when tested in vitro (Côté et al., 2010; McKay et al., 2007; Del et al., 2013).

Flavonoids occur naturally and contain two six-carbon aromatic centers, namely the A and B rings, with a three-carbon bridge, called the C ring that forms a phenol bridge with oxygen (Robards and Antolovich, 1997; Haslam, 1998; Gee and Johnson, 2001). Flavan-3-ols in cranberries exist as monomers, oligomers, and polymers (Pappas et al., 2009). These oligomeric and polymeric pigments are also called non-hydrolyzable condensed tannins or proanthocyanidins (PACs). PACs are produced in plants when subjected to stress or infection (Scalbert, 1991; Dixon et al., 2005; Howell, 2007). PACs can bind to proteins (Hagerman and Butler, 1981) and contribute to the astringent taste of cranberries (Bate-Smith, 1973). The biological activity of PACs is affected by the structure (Howell, 2007). Flavan-3-ols have the same molecular structure as the
flavanols except the absence of the C4 carbonyl group (Iwashina, 2000; Escarpa and Gonzalez, 2001). (-)-Epicatechin is the primary unit in cranberry PACs, with (epi) gallatechin and (+)-catechin being present in small amounts. On an average, the PAC content of raw cranberries has been reported to be 410 mg/100 g fresh fruit weight (USDA, 2004).

The successive monomeric flavan-3-ol units in B-type PACs (Fig. 1.1) are linked by C-C interflavan bonds between the C4 of the upper unit and the C6 (4β →6) or C8 (4β →8) of the lower unit (Hammerstonne et al., 2000; Gu et al., 2004; Dixon et al., 2005). A less frequent variation that occurs in the structure of PACs is when the flavan-3-ol units are linked by a mixture of C-C bonds and interflavonoid bonds by C-O oxidative coupling between both C2 and C4 of the upper unit and the oxygen at C7 and positions C6 or C8, respectively, of the lower unit (4β →8 and 2β →O→7) as can be seen in Fig. 1.2 (Côté et al., 2010). These PACs are called A-type PACs. Fifty one to ninety one percent of the total PAC content in cranberries consists of at least one A-type PAC (Foo et al., 2000; Feliciano et al., 2012). A- and B-type PACs demonstrate difference in biological activity with A-type PAC showing greater anti-adhesion activity against the attachment of P-fimbriated *Escherichia coli* to uroepithelial cells (attributed to effect on the structure of bacterial cells (Liu et al., 2006) and/or decrease in the expression of P-fimbrial gene (Liu et al., 2006; Ahuja et al., 1998)) in development of urinary tract infections (Howell et al., 2005).

Cranberries contain high amount of PACs bound to the cell-wall (important because they are reported to be bioavailable in the large intestine) which causes resistance to extraction using the conventional methods and has led to underestimation of quantity of PACs (Saura-Calixto et al., 2012 and 2007; Pérez-Jiménez et al., 2007). Cranberries are usually processed (juice, sauce or dried) prior to consumption. The processing of cranberries into juice, which includes pressing (where skin and seeds of the fruit are removed), could lead to ~30-40% loss in PACs and flavanols
(Blumberg et al., 2013). Losses in these compounds also occur when cranberries are subjected to high heat during processing into dried powders (Blumberg et al., 2013).

Based on the information currently available on the widespread nature of foodborne viruses and the available natural and chemical control options, research continues to be focused on improved, cost-effective and natural approaches to control their spread and to prevent emergence of virulent forms. As consumer demand for natural based products increase together with the sustainable nature of plant-derived products, these options are being researched as antiviral agents as well as improved chemical sanitizers.
References:


associated with orange juice among tourists, Egypt, 2004. Emerging Infectious Diseases 13, 156-158.


Haidari, M., Ali, M., Casscells, S.W., Madjid, M., 2009. Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. Phytomedicine 16, 1127-1136.


Sharma, C., Williams, S., Schneider, K., Schmidt, R., Rodrick, G., 2012a. Sodium metasilicate affects growth of Campylobacter jejuni in fresh, boneless, uncooked chicken breast fillets stored at 4 degrees Celsius for 7 days1, 2. Poultry Science 91, 2324-2329.

Sharma, C., Williams, S., Schneider, K., Schmidt, R., Rodrick, G., 2012b. Sodium metasilicate affects growth of Salmonella Typhimurium in fresh, boneless, uncooked chicken breast fillets stored at 4° C for 7 days. Poultry Science 91, 719-723.


In, Y., Kim, J., Kim, H., Oh, S., 2013. Antimicrobial activities of acetic acid, citric acid and lactic acid against _Shigella_ species. Journal of Food Safety 33, 79–85.


## Appendix

### Tables:

Table 1.1. Recent outbreaks of human norovirus in the United States

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<tr>
<th>Year</th>
<th>Location</th>
<th>Number of cases</th>
<th>Food vehicle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Illinois</td>
<td>196</td>
<td>Coleslaw; green beans; vegetables</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2012</td>
<td>Ohio</td>
<td>42</td>
<td>Spanish Piquillo Peppers with goat cheese</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2012</td>
<td>New York</td>
<td>103</td>
<td>Antipasto, ice, pizza, salad, water</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2012</td>
<td>Ohio</td>
<td>29</td>
<td>Mesclun mix and spinach</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2011</td>
<td>Massachusetts</td>
<td>50</td>
<td>American cheese (pasteurized), lettuce, tomatoes</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2011</td>
<td>Pennsylvania</td>
<td>24</td>
<td>Garden salad</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td>2014</td>
<td>Cruise Ships</td>
<td>8 outbreaks</td>
<td>Not specified</td>
<td>CDC, 2014a</td>
</tr>
<tr>
<td>2013</td>
<td>Cruise Ships</td>
<td>8 outbreaks</td>
<td>Not specified</td>
<td>CDC, 2014a</td>
</tr>
<tr>
<td>2012</td>
<td>Cruise Ships</td>
<td>16 outbreaks</td>
<td>Not specified</td>
<td>CDC, 2014a</td>
</tr>
<tr>
<td>2011</td>
<td>Cruise Ships</td>
<td>10 outbreaks</td>
<td>Not specified</td>
<td>CDC, 2014a</td>
</tr>
</tbody>
</table>
Table 1.2 Comparison of human norovirus surrogates (Adapted from Hoelzer et al., 2013 and Kniel, 2014)

<table>
<thead>
<tr>
<th></th>
<th>Human norovirus (HNoV)</th>
<th>Feline calicivirus (FCV)</th>
<th>Murine norovirus (MNV-1)</th>
<th>Tulane virus (TV)</th>
<th>Coliphage (MS2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
<td>Caliciviridae</td>
<td>Caliciviridae</td>
<td>Caliciviridae</td>
<td>Caliciviridae</td>
<td>Leviviridae</td>
</tr>
<tr>
<td>Genus</td>
<td>Norovirus</td>
<td>Vesivirus</td>
<td>Norovirus</td>
<td>Recovirus</td>
<td>Levivirus</td>
</tr>
<tr>
<td>Enteric/fecal shedding</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No/Yes</td>
</tr>
<tr>
<td>Envelope</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Virion diameter</td>
<td>27–38 nm</td>
<td>35–39nm</td>
<td>35–39nm</td>
<td>35–37 nm</td>
<td>27 nm</td>
</tr>
<tr>
<td>Host receptor, coreceptors</td>
<td>Histo blood group antigens (HBGA), heparan sulfate</td>
<td>JAM-1, sialic acid</td>
<td>Sialic acid, glycoproteins</td>
<td>HBGA</td>
<td>F-pilus</td>
</tr>
<tr>
<td>Genome composition</td>
<td>(+) ss RNA</td>
<td>(+) ss RNA</td>
<td>(+) ss RNA</td>
<td>(+) ss RNA</td>
<td>(+) ss RNA</td>
</tr>
<tr>
<td>Genome Size and organization</td>
<td>7.5 kb, 3 ORF</td>
<td>7.5 kb, 3 ORF</td>
<td>7.5 kb, 3 ORF</td>
<td>6.7 kb, 3 ORF</td>
<td>3.5 kb</td>
</tr>
</tbody>
</table>
Table 1.3. Recent outbreaks of hepatitis A virus (HAV)

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Number of cases</th>
<th>Food vehicle</th>
<th>Method used in investigation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>United States (10 states)</td>
<td>165</td>
<td>Pomegranate arils imported from Turkey</td>
<td>Epidemiology, genetic sequencing</td>
<td>Collier et al., 2014</td>
</tr>
<tr>
<td>2013</td>
<td>Ireland</td>
<td>21</td>
<td>Frozen berries</td>
<td>Descriptive epidemiology, case–control study, Microbiological testing of human and food specimens, Molecular typing of positive specimens and food traceback, Nested reverse transcriptase (RT) polymerase chain reaction (PCR)</td>
<td>Fitzgerald et al., 2014</td>
</tr>
<tr>
<td></td>
<td>European Union (12 countries)</td>
<td>1,440 (331 confirmed)</td>
<td>Frozen berries (mainly Bulgarian blackberries and Polish redcurrants)</td>
<td>RT-PCR, genotyping and sequencing (double strand sequencing and the sequence was checked by basic local alignment search tool)</td>
<td>Montaño-Remacha et al., 2014; European Food Safety Authority, 2014</td>
</tr>
<tr>
<td>2011</td>
<td>England</td>
<td>7</td>
<td>Semi-dried tomatoes</td>
<td>Genotyping</td>
<td>Carvalho et al., 2012</td>
</tr>
<tr>
<td>2010</td>
<td>France</td>
<td>59 (49 confirmed)</td>
<td>Frozen semidried tomatoes from Turkey</td>
<td>Case-control study, genotyping and phylogenetic analysis (RT-PCR)</td>
<td>Gallot et al., 2011</td>
</tr>
<tr>
<td>2010</td>
<td>Netherlands</td>
<td>13</td>
<td>Semi-dried tomatoes in oil</td>
<td>Case-control study, Two step RT-PCR</td>
<td>Petrignani et al., 2010</td>
</tr>
<tr>
<td>2009</td>
<td>Australia</td>
<td>153</td>
<td>Semi-dried tomatoes</td>
<td>Case-control studies, product trace-back, food sampling and genotyping (quantitative RT-PCR)</td>
<td>Donnan et al., 2012</td>
</tr>
<tr>
<td>Source</td>
<td>Bioactive component</td>
<td>Antiviral activity against</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>American Cranberry</td>
<td>Anthocyanins (flavonoids) and Proanthocyanidins (condensed tannins)</td>
<td>FCV-F9, MNV-1, MS2, Reovirus, Bacteriophages T4 and T2, Simian rotavirus SA-11, Bovine reovirus, Influenza virus, Bacteriophage φ-X174, HNoV</td>
<td>Li et al., 2012, Su et al., 2010a, Weiss et al., 2005, Lipson et al., 2007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black raspberry</td>
<td>Gallic acid and quercitin</td>
<td>MNV-1, FCV-F9</td>
<td>Oh et al., 2012, Ju et al., 2009, Yoon et al., 2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pomegranate</td>
<td>Anthocyanins, ellagitannins and gallotannins (hydrolysable tannins), and proanthocyanidins</td>
<td>Influenza virus, FCV-F9, MNV-1, MS2, HNoV</td>
<td>Su et al., 2010b, Haidari et al., 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blueberry</td>
<td>Phenolic acids, catechins and proanthocyanidins</td>
<td>Hepatitis C virus</td>
<td>Wang et al., 2010, Takeshita et al., 2009, Smith et al., 2000, Howell et al., 1998, Bomser et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grape seed</td>
<td>Flavonoids, ascorbic acid, citric acid, limonoids, sterols, and tocopherols</td>
<td>HAV, FCV-F9, MNV-1</td>
<td>Su et al., 2011, Bevilacqua et al., 2010, Armando et al., 1998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oregano</td>
<td>Carvacrol, thymol, p-cymene, γ-terpinene</td>
<td>FCV-F9, MNV-1</td>
<td>Azizkhani et al., 2013, Elizaquível et al., 2013, Gilling et al., 2014a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clove</td>
<td>Eugenol, eugenile acetate, β-caryophyllene</td>
<td>FCV-F9, MNV-1</td>
<td>Azizkhani et al., 2013, Elizaquível et al., 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zataria</td>
<td>Carvacrol, γ-terpinene, α-pinene, eucalyptol, globulol</td>
<td>FCV-F9, MNV-1</td>
<td>Azizkhani et al., 2013, Elizaquível et al., 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Essential oils</td>
<td>Allspice oil, lemongrass oil, citral</td>
<td>MNV-1</td>
<td>Gilling et al., 2014b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.1 Representative structures of flavan-3-ol monomers and their dimers (B-type PACs). When $R_1=OH$ and $R_2=H$, the monomer is (-)-epicatechin. When $R_1=H$ and $R_2=OH$, then the monomer is (+)-catechin. (Adapted from Hammerstone et al., 2000).

Figure 1.2 Structure of a typical cranberry proanthocyanidin tetramer composed of epicatechin units with one A-type linkage (Adapted from Neto, 2007).
Figure 1.3. A-type and B-type PACs (Adapted from Blumberg et al., 2013)
Chapter II

Dose and time-dependence effects of cranberry juice and cranberry proanthocyanidins against hepatitis A virus and murine norovirus
Dose and time-dependence effects of cranberry juice and cranberry proanthocyanidins against hepatitis A virus and murine norovirus

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Running Head: Cranberry proanthocyanidins decrease hepatitis A virus and murine norovirus infectivity

To be submitted to: Food Microbiology

Key words: Hepatitis A virus, murine norovirus, cranberry juice, reduction

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Highlights

- Cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) decrease hepatitis A virus (HAV) infectivity.
- Increased C-PAC levels show higher activity against HAV and murine norovirus in shorter time.
- CJ and C-PAC show promise to alleviate symptoms of enteric viral disease.
Abstract

Hepatitis A virus (HAV) outbreaks that cause severe foodborne disease are on the rise in the United States. Cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) have shown antiviral activity against human norovirus surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1). Although 0.6 mg/mL C-PAC reduced FCV-9 to non-detectable levels after 1 h at room temperature, studies with increasing concentration and time at 37°C for effective MNV-1 reduction are needed. Besides, the effects of CJ and C-PAC against HAV are currently unknown. The objective of this research was to determine the dose and time-dependent effects of CJ and C-PAC on MNV-1 and HAV over 24 h at 37°C. C-PAC at 0.12, 1, 2, and 4 mg/mL, CJ (pH 3.0), malic acid (pH 3.0; control) or PBS (pH 7.2) were individually mixed with equal volumes of HAV or MNV-1 at ~5 log PFU/mL and incubated at 37°C over 24 h. Treatments were stopped in media containing fetal bovine serum. Recovered viral titers after triplicate treatments were evaluated in duplicate by standardized plaque assays and data were statistically analyzed. Transmission electron microscopy (TEM) was used to determine structural effects after treatment. CJ reduced MNV-1 and HAV to non-detectable levels after 6 h at 37°C, while C-PAC (final concentrations of 0.5 and 1 mg/mL) reduced both viruses to non-detectable levels after 5 min. TEM showed viral structural damage of the treated viruses. Increased C-PAC levels at 1 mg/mL caused higher viral reduction within shorter times. CJ and C-PAC show promise as natural antivirals against HAV and MNV-1.
1. Introduction

According to Scallan et al. (2011), 9.4 million cases of foodborne illnesses occur every year in the United States. Of these, 5.5 million (59%) are caused by human noroviruses (HNoV) that are the leading cause of foodborne illnesses across all age groups (Hall et al., 2013). Most of the viral foodborne and waterborne outbreaks have been attributed to HNoVs, hepatitis A virus (HAV), hepatitis E virus, sapoviruses, parvoviruses, rotaviruses, astroviruses, Aichi virus, other small round viruses and human enteroviruses including polioviruses, coxsackieviruses, and echoviruses (Sair et al., 2002; D’Souza et al., 2007; de Aceituno et al., 2013).

According to Hall et al. (2013), HNoVs cause an average ~570–800 deaths, 56,000–71,000 hospitalizations, 400,000 emergency department visits, 1.7–1.9 million outpatient visits, and 19–21 million illnesses per year in the US. Between 1993 and 2011, more than 900 reports of laboratory-confirmed norovirus outbreaks have been published (Matthews et al., 2012). No vaccine is yet available to prevent HNoV infection. HNoVs are non-enveloped and belong to the *Caliciviridae* family with very low infectious doses (10-100 viral particles) (Turcios et al., 2006; Siebenga et al., 2009; Widdowson et al., 2004; Teunis et al., 2008). HNoVs are known to be transmitted via the fecal-oral route (contaminated food and water as carriers) or via close personal contact with an infected person. The spread of these viruses is difficult to control due to their high stability, resistance to degradation in the environment and resistance to majority of chemical inactivation methods (Cheesbrough et al., 2000; Kuusi et al., 2002; Widdowson et al., 2004). HNoV infections from the newly emergent virulent strains are known to be life threatening particularly for elderly and immuno-compromised individuals (Siebenga et al., 2009; Su et al., 2010a). Outbreaks associated with HNoV are common in closed settings such as cruise ships, healthcare facilities, restaurants, hotels and schools (CDC, 2014). There is no reproducible cell-culture based assay available for HNoVs to date and hence cultivable surrogates (such as murine
norovirus (MNV-1), feline calicivirus (FCV-F9), or Tulane Virus (TV), etc.) are used to determine potential inactivation after treatments (Cannon et al., 2006; Su et al., 2010a, 2010b, 2010c; Hirneisen et al., 2010; Cromeans et al., 2014).

The other epidemiologically significant foodborne virus, HAV belongs to the genus Hepatovirus classified under the *Picornaviridae* family. It is a non-enveloped virus, 27-nm in diameter with an icosahedral capsid and a positive-sense single stranded RNA which is 7.5 kb long (Bidawid et al, 2000b). HAV is usually transmitted via the fecal-oral route (Lemon, 1997). The average incubation period for HAV illness is 28 days (may range from 15 to 50 days during which viral shedding in feces occur and the risk of transmission is high) that makes detection of the source of infection rather challenging (CDC, 2012a; Hollinger et al., 1996; Koff, 1998; Stapleton et al., 1994). HAV is important because of severity of the disease with typical symptoms that include fever, malaise, anorexia, nausea, abdominal discomfort, dark urine and jaundice. The illness does not usually last longer than 2 months, but some (~10%–15%) patients may demonstrate prolonged or relapsing symptoms for up to 6 months. While a 90% decrease in HAV cases (mostly attributed to vaccination of children and people at risk for HAV infection) has been observed in the US over the last 20 years, there are still an estimated 2,700 new cases of HAV illness per year (CDC, 2014).

HAV and HNoV are quite stable on produce and food contact surfaces (Bidawid et al., 2004; Croci et al., 2002; D’Souza et al., 2006; Dawson et al., 2005; Mattison et al., 2007). HAV is resistant to high temperature with reduction of 6 log PFU/mL in 5.3 min in buffered cell culture medium (Bozkurt et al., 2014). It is also resistant to inactivation by low pH, low temperature (freezing), detergents, and acids (Brundage and Fitzpatrick, 2006). Given the effect that these viruses have on the human population, it is essential to evaluate novel compounds like natural plant extracts against their spread. Natural plant extracts are preferred by consumers as they are
perceived to be safer than chemicals used in food processing and preservation, can be lower in cost, and are sustainable. Also, these extracts are suitable for consumption and hence can be evaluated as preventive or therapeutic measures against viral infections.

Cranberries (Vaccinium macrocarpon) are known to contain bioactive polyphenols (with known antimicrobial and anti-inflammatory properties) such as proanthocyanidins with unusual A-type linkages (Foo et al. 2000a, 2000b), and contrast with other foods rich in tannins which possess proanthocyanidins with B-type linkages. Cranberries have been used over centuries to prevent urinary tract bacterial infections (UTI) (Foo et. al. 2000a, 2000b; Howell et al., 2005; Gupta et al. 2007; Eydelnant and Tufenkji, 2008; Hidalgo et al. 2011). Clinical trials suggest that cranberry juice (CJ) and its components prevent the adherence of Escherichia coli to uroepithelial cells, vaginal epithelial cells (Gupta et al. 2007), and other materials such as polyvinyl chloride and polytetrafluoroethylene (Eydelnant and Tufenkji, 2008) due to the decrease in fliC adherence gene expression (Hidalgo et al. 2011). C-PAC at 16 mg/mL was effective in reducing biofilms formed by Candida albicans (a common agent of nosocomial UTI) and its adherence to polystyrene and silicone discs (Rane et al., 2014). This bioactive polyphenol can also inhibit adhesion of tooth decay causing Streptococcus mutans and S. sobrinus (Weiss et al., 2004; Steinberg et al., 2005). Cranberry extracts are also known to be effective against ulcer-causing Helicobacter pylori infections (Vattem et al., 2005) and several enteric pathogens including vancomycin resistant Enterococcus faecium, Escherichia coli O157:H7 EDL 933, Escherichia coli ATCC 25922, Listeria monocytogenes HPB 2812, Pseudomonas aeruginosa ATCC 15442; Salmonella Typhimurium SL1344 and Staphylococcus aureus ATCC 29213 (Caillet et al., 2012).

In addition to bacteria, cranberry juice (CJ) and C-PAC have shown significant antiviral activity against bacteriophages T4 and T2, the simian rotavirus SA-11, bovine reovirus (Lipson et
al., 2007) and influenza virus (Weiss et al., 2005). CJ and pomegranate juice were individually shown to be effective in decreasing the specific binding ability of HNoV to saliva when P particles of HNoV GII.4 were used as a research surrogate (Li et al, 2012). Su et al. (2010b) reported that human enteric viral surrogates, FCV-F9 were reduced by ~5 log plaque forming units (PFU)/mL after 30 min and 10 min when treated with CJ at pH 2.6 and CJ at pH 7.0, respectively (Su et al., 2010b). They also showed that MNV-1 when treated with CJ (pH 2.6), CJ (pH 7.0), 0.15 mg/mL C-PAC or 0.30 mg/mL C-PAC was reduced by ~1.9 log PFU/mL, ~1.7 log PFU/mL, ~2.2 log PFU/mL or ~2.9 log PFU/mL after 1 h, respectively (Su et al., 2010a). They reported that Transmission electron microscopy (TEM) analysis showed structural damage of CJ-treated FCV-F9 (Su et al., 2010b).

Not much is known about the effects of CJ and C-PAC against HAV or if higher concentrations of C-PAC (>0.6 mg/mL) can cause complete reduction of MNV-1 over time. Also, the effect of CJ and C-PAC against either HAV or MNV-1 at 37°C has not been evaluated. CJ and C-PAC are aimed to be consumed as therapeutic or preventive measures and it is hence essential to test their antiviral activity at human body temperature (37°C). The objectives of this study were to (1) determine the dose and time-dependence effects of C-PAC at 0.12 mg/mL (amount of C-PAC equivalent to that in CJ), 0.5 mg/mL, 1 mg/mL, and 2 mg/mL and CJ over 24 h on MNV-1 and HAV titers at 37°C; and (2) understand the mechanism of action of CJ and C-PAC by (a) evaluating the effect of CJ and C-PAC on viral adsorption and replication, and (b) determining the extent of structural change/damage that occurs to the viral particles after treatment using transmission electron microscopy (TEM). The hypothesis for this study is that CJ and C-PAC, at the chosen concentrations, will not be toxic to the host cell-lines and will be effective in reducing viral titers of HAV and MNV-1 at 37°C.
2. Materials and Methods

2.1. Viruses and cell lines

Murine norovirus (MNV-1) was received as a gift from Dr. Skip Virgin (Washington Univ., St Louis, MO) and RAW 264.7 cells were obtained from the University of Tennessee at Knoxville. Hepatitis A virus (HAV; strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were obtained from our collaborator, Dr. Kalmia Kniel (University of Delaware).

2.2. Propagation of viruses

The methods used by Su et al. (2010a) for viral propagation were followed. Briefly, RAW 264.7 cells and FRhK4 cells were grown in Dulbecco’s Modified Eagles Medium (DMEM; HyClone Laboratories, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) in an atmosphere containing 5% CO₂ at 37⁰C in 175 cm² flasks. Viral stocks of HAV and MNV-1 for the study were made by inoculating HAV and MNV-1 stocks onto confluent cell monolayers of FRhK4 and RAW 264.7 cells, respectively and incubated under 5% CO₂ until >90% cell lysis was observed. The viral suspensions obtained were freeze-thawed and centrifuged for 10 min at 5000 x g. The supernatants were then filtered through 0.2 µm membrane filters, aliquoted and stored at -80°C until use.

2.3. Determination of the Antiviral effects of CJ and C-PAC on HAV and MNV-1

Cranberry Juice (Cranberry Juice Cocktail from Ocean Spray Cranberries, Inc., CJ) was purchased from a local grocery store and cranberry proanthocyanidins (C-PAC) were obtained from Dr. Amy Howell, Marucci Center for Blueberry and Cranberry Research (Rutgers University, Chatsworth, NJ). C-PAC stock (4 mg/mL) was prepared by dissolving C-PAC in 10% ethanol and filter-sterilizing through a 0.2µm filter as described before (Su et al., 2010a). Cranberry juice
cocktail contains approximately 30% cranberry juice with C-PAC levels of about 0.12 mg/ml (Su et al., 2010a) and hence 0.12 mg/mL of C-PAC was also used in this study to determine the difference, if any, in the activity of juice and the corresponding level of purified proanthocyanidins. CJ (pH 3.0), C-PAC (0.12, 1, 2 and 4 mg/mL), or controls such as malic acid (pH 3.0), 10% ethanol, and phosphate buffered saline (PBS, pH 7.2), were individually mixed with equal volumes of each virus (titer of ~5 log PFU/mL of MNV-1 or HAV) and incubated at 37\(^\circ\)C for 5, 15, 30, 60, 180 min or 24 h. The final concentrations of C-PAC were 0.06, 0.5, 1 and 2 mg/mL and that for CJ was 50%. At the various time points, treatments were stopped/neutralized by 10-fold serial dilution in DMEM containing 10% FBS. Viral infectivity was then determined using three serial dilutions of the treated or untreated virus in standard plaque assays (D’Souza et al., 2006, 2009; D’Souza and Su, 2010; Su et al., 2009; Wobus et al., 2004). All treatments were run in duplicate and replicated thrice.

2.4. Infectious plaque assays

Standard protocols were followed for HAV and MNV-1 plaque assays (D’Souza et al., 2006, 2009; D’Souza and Su, 2010; Su et al., 2009; Wobus et al., 2004). FRhK4 and RAW 264.7 cells were infected with 0.5 ml of serially diluted treated or control samples of HAV and MNV-1 (as mentioned earlier) respectively and incubated for 2 to 3 h to allow for infection.Viruses were then aspirated and the cells were overlaid with 2 ml complete DMEM containing 0.75% agarose (for MNV-1) or 1% agarose (for HAV) and incubated at 37\(^\circ\)C under 5% CO\(_2\). After an incubation of three days for MNV-1 and eight days for HAV, the cell monolayers were stained using neutral red and further incubated at 37\(^\circ\)C until plaques were visible; usually one day for both HAV and MNV-1. Plaques were then enumerated as PFU/mL.
2.5. Effect of CJ and C-PAC on Adsorption and Replication of Viruses

The effect of CJ and C-PAC on the adsorption or replication of HAV was evaluated by modifying the method stated by Su et al. (2010a; 2010b) and Oh et al. (2012). To evaluate if CJ and C-PAC interfere with adsorption of viruses onto cell lines by blocking the cell receptors, confluent FRhK4 cells were first treated with 0.25 mg/mL C-PAC or 50% neutralized CJ (final pH 7.0, neutralized with 1 M NaOH) or DMEM with 2% FBS (as a control) for 30 min, the media was aspirated, followed by infection with HAV and incubated for 3 h. Neutralized CJ was used as CJ at acidic pH was found to be cytotoxic to the host cells when added directly. Plaque assays were performed as described earlier and plaques were counted and compared to controls. Reductions in titers for the viruses, in terms of PFU/mL were determined in comparison to the controls. A reduction in titer would suggest that C-PAC and CJ exert an inhibitory effect on the adsorption/internalization of the viral particles to the host cells by blocking the cell receptors. Effect of CJ and C-PAC on adsorption and replication of MNV-1 was not tested in this study as it was previously evaluated by Su et al (2010b). To further understand if C-PAC or CJ inhibit the adsorption of viruses by either affecting the structure of the viral particle or preventing the internalization of the viruses onto host cells, co-treatment of virus was carried out (Oh et al., 2012). Co-treatment included addition of the viruses diluted in 0.25 mg/mL PAC or 50% neutralized CJ directly/simultaneously added onto confluent monolayers of host cells followed by incubation for 3 h (for HAV) or 2 h (MNV-1) (Oh et al., 2012).

To determine if C-PAC and CJ have an inhibitory effect on viral replication, the confluent monolayer of FRhK4 cells was infected with HAV for 3 h, followed by the addition of 0.25 mg/mL PAC or 25% CJ or DMEM with 2% FBS (as a control) for 30 min. Plaque assays were performed
as described earlier to determine any reduction in infectivity of viral particles. Each experiment was conducted in duplicate and replicated thrice.

2.6. Statistical analysis

A completely randomized design with sampling was used. Each experiment was run in duplicate (samples) and replicated thrice. Analysis of variance was conducted with Statistical Analysis Software (SAS, version 9.2, SAS Institute, Cary, NC, USA) and Tukey’s test at p<0.05 to determine statistically significant differences between the recovered titers of the treatments and controls.

2.7. Transmission electron microscopy (TEM) studies

With an aim to determine the mechanism of action of CJ and C-PAC on HAV and MNV-1 structure, TEM studies were carried out at the UT-Knoxville Advanced Microscopy and Imaging Center following the protocol of Su et al. (2010b). HAV or MNV-1 was treated with equal volume of CJ, 0.5 mg/mL C-PAC or PBS and incubated at 37°C for 45 min. Ten-µl of each sample was then applied to a freshly glow discharged, formvar and carbon coated copper grid (Electron Microscopy Sciences, Hatfield, PA). Excess sample was removed after 1 min. The grid with sample was then stained with uranyl acetate for 1 min, excess stain removed and the grid allowed to dry. The stained viruses were viewed using a 200kV Zeiss LIBRA 200MC.

3. Results

3.1. Dose and Time-Dependent Effects of CJ and C-PAC on MNV-1 and HAV infectivity

Regardless of time, C-PAC at 0.5 mg/mL was as effective as C-PAC at 2 mg/mL at 37°C against both HAV and MNV-1 (Tables 2.1 and 2.2). CJ reduced MNV-1 to undetectable levels (detection limit of plaque assay is 2 log PFU/mL) after 6 hat 37°C, with 0.84±0.01, 1.33±0.05 and 1.46±0.01 log PFU/mL reduction after 1 h, 2 h and 3 h, respectively (Table 2.1). C-PAC at 0.5
mg/mL and 1 mg/mL reduced MNV-1 to undetectable levels within 5 min at 37°C (Fig 2.3). C-PAC at 0.06 mg/mL reduced MNV-1 by 0.46±0.01 and 1.46±0.02 log PFU/mL in 1 and 3 h, respectively. Ethanol (10% v/v with water) used as a solvent for C-PAC as well as malic acid (pH 3.0) used as pH control for CJ, showed no detectable reduction of MNV-1 (Table 2.1, Figure 2.3 and Figure 2.4) at all the time points tested except the 2 h time point where significant reduction was obtained with 10% ethanol. However, this reduction by 10% ethanol was less than that obtained with 0.5, 1 and 2 mg/mL of C-PAC in the same time.

Similar results were obtained for HAV treated with CJ and C-PAC. C-PAC at 0.06 mg/mL reduced HAV to undetectable levels in 10 min with reduction of 2.45±0.07 log PFU/mL in 5 min. CJ reduced HAV to undetectable levels after 2 h at 37°C while C-PAC at 0.5 mg/mL and 1 mg/mL showed reduction to undetectable levels within 5 min at 37°C. CJ caused reduction of HAV by 1.61±0.04 log PFU/mL within 1 h. Both 10% ethanol showed no reduction for HAV over 3 h (able 2.2, Figure 2.5 and Figure 2.6). Malic acid (pH 3.0) also did not reduce HAV titers as significantly as CJ over 3 h (p<0.05). For the 2 h time point significant reduction of 0.3 log PFU/mL was obtained for HAV with malic acid which was lesser than compared to reduction obtained for HAV with CJ within the same time.

3.2. Effect of CJ and C-PAC on Adsorption and Replication of Viruses

In the adsorption assay for HAV, reductions of 0.28±0.12 and 0.12±0.10 log PFU/mL were obtained with 50% neutralized CJ and 0.25 mg/mL C-PAC, respectively. The results are summarized in Table 2.3. However, these reductions were not statistically significant (p>0.05). No significant reduction (p>0.05) was obtained for HAV in the replication assay. Co-treatment of cells with HAV and 50% neutralized CJ and 0.25 mg/mL C-PAC showed reductions of 1.49±0.16 and 1.40±0.01 log PFU/mL, respectively.
3.3. TEM Results

In order to understand mechanism of action of both CJ and C-PAC against HAV and MNV-1 capsid structure, TEM studies were conducted. HAV or MNV-1 treated with equal volumes of CJ, 0.5 mg/mL C-PAC or PBS at 37°C for 45 min were used for sample preparation. Figure 2.1b shows that the HAV particles treated with CJ were distorted in shape. Some of the viral particles exhibited damage to the capsid in comparison with the intact viral particle in PBS as seen in Figure 2.1a. TEM analysis for MNV-1 treated with CJ (Figure 2.2b) showed results similar to those obtained for HAV hence indicating a similar mode of action against both viruses i.e. via structural deformation of the viral capsid. TEM images of viruses treated with C-PAC (Figure 2.1c and Figure 2.2c) generated high background due to C-PAC particles and did not give conclusive information regarding the effect of C-PAC on viral particles.

4. Discussion

With an aim to test if the antiviral activity of CJ is due to low pH, malic acid (pH 3.0) was used as a pH control, as the pH of CJ is 3. Solutions of C-PAC were made in 10% ethanol and hence 10% ethanol solution was also used as a control. For all the time points tested, malic acid and 10% ethanol did not cause significant reductions compared to the reductions in viral titers for both HAV and MNV-1 by CJ and C-PAC at the same time points. Thus, the antiviral activity of CJ or C-PAC cannot be attributed to low pH or ethanol content alone.

To the best of our knowledge, this study was the first to show that CJ and C-PAC have antiviral activity against HAV at 37°C and against MNV-1 at 37°C, while only results for MNV-1 at room-temperature were previously reported (Su et al., 2010a; Su et al., 2010b). CJ and C-PAC (at 0.5, 1.0 and 2 mg/mL) reduced both HAV and MNV-1 to undetectable levels within 6 h at 37°C. C-PAC at 0.5 mg/mL reduced MNV-1 and HAV to undetectable levels in 1 h and 5 min.
respectively while CJ demonstrated the same effect after 2 h against HAV and 6 h against MNV-1. C-PAC at 0.06 mg/mL, approximately the same amount as present in CJ, reduced HAV to undetectable levels within 10 min compared to 2 h required by CJ. This difference in reduction time could be attributed to the complex nature of the CJ matrix where the bioactive components are present amidst a multi-component system, thus making it less available for action on the viral particles. However, no significant difference in reduction was obtained for MNV-1 when treated with 0.06 mg/mL of CPAC or CJ, even after 3 h of incubation. This suggests that C-PAC is more effective against HAV at lower concentration than against MNV-1, at 37°C.

Su et al. (2010a) reported reductions of 2.63, 2.75 and 2.95 log PFU/mL for MNV-1 when treated for 1 h at RT with C-PAC at 0.15, 0.30 and 0.60 mg/mL respectively. The results obtained in this study are consistent with this finding as reduction to undetectable levels was observed for MNV-1 when treated with C-PAC. However, we observed this reduction within 5 min of treatment with 0.5 mg/mL C-PAC at 37°C. Thus, increased concentration of C-PAC from 0.15 mg/mL or 0.30 mg/mL, as was used in prior research (Su et al., 2010a; Su et al., 2010b), to 0.5 mg/mL resulted in greater reduction of MNV-1 within a shorter time. The time required to achieve ~3 log PFU/mL reduction decreased from 1 h to 5 min. The difference in time required to achieve similar reduction by 0.60 mg/mL C-PAC (Su et al., 2010a) and 0.5 mg/mL C-PAC in this study could be attributed to the change in temperature of treatment from RT to 37°C, suggesting that the reduction in titers by CJ and CPAC is temperature-dependent. At higher temperatures, it can be speculated that the binding activity of the cranberry bioactives could be enhanced or that the viral capsid is more susceptible to binding by the bioactives.

Su et al (2010a) observed reductions of ~2.2 log PFU/mL and ~2.9 log PFU/mL when MNV-1 was treated with 0.15 mg/mL and 0.30 mg/mL of C-PAC, respectively at RT for 1 h. In
comparison to our study, higher concentrations of C-PAC showed MNV-1 reduction to non-detectable levels within shorter times.

Among a variety of fruits juices, polyphenols and plant extracts that have been evaluated by researchers for their antiviral activity, CJ and C-PAC have demonstrated promising results. Su et al. (2010c) showed that for high (~7 log PFU/mL) titers of FCV-F9, MNV-1, and MS2, reductions of 1.20, 0.06, and 0.63 log PFU/mL respectively were observed when treated with pomegranate juice for 1 h at RT. When treated with 8, 16, and 32 mg/mL of pomegranate polyphenols for 1 h at RT, both high and low titers of FCV-F9 were reduced to undetectable levels while MNV-1 (initial titer of ~ 5 log_{10} PFU/mL) was reduced by 1.30, 2.11, and 3.61 log PFU/mL and MNV-1 (high initial titer) reduced by 1.56, 1.48, and 1.54 log PFU/mL respectively (Su et al., 2010c). Black raspberry juice at concentrations of 3% and 6% reportedly demonstrated greater antiviral activity against MNV-1 than the results obtained with cranberry juice used in our study (Oh et al., 2012). When treated with grape seed extract (GSE) at 0.25, 0.5, and 1.0 mg/mL at 37°C for 2 h, reductions of 1.49, 1.72, and 1.97 log PFU/mL for MNV-1 and 1.86, 2.26, and 2.89 log PFU/mL for HAV, respectively were observed by Su et al. (2011). As per the results obtained in our study, the lowest concentration of C-PAC required to reduce ~5 log PFU/mL of MNV-1 by ~3 log PFU/mL in 5 min was found to be 0.5 mg/mL which is significantly lower than the concentration of pomegranate polyphenols of 32 mg/mL. This shows that C-PAC is more effective than pomegranate polyphenols or GSE in causing MNV-1 titer reduction. Also, C-PAC at 0.5 mg/mL reduced HAV (~5 log PFU/mL) by ~3 log PFU/mL in 5 min compared to GSE at 0.5 mg/mL which reduced HAV by 2.26 log PFU/mL in 2 h at 37°C. Thus, C-PAC appears to be a more potent antiviral than GSE against HAV.
A study to test the effect of essential oils from oregano, clove and zataria conducted by Elizaquível et al. (2013) reported reductions of 1.04, 1.17 and 1.62 log Tissue culture infectious dose 50/mL (TCID\(_{50}\)/mL) for MNV-1 when treated for 2 h at 37°C with 0.5, 1 and 2% of oregano essential oils respectively with no reduction observed at 4°C at the tested concentrations and time. When treated with 0.1, 0.5 and 1% of clove essential oil for 2 h at 37°C less than 0.7 log TCID\(_{50}\)/mL reductions were observed. MNV-1 showed no reduction when treated with zataria essential oils (0.01% to 0.1%) at 37°C but showed reductions of 0.55 and 1 log TCID\(_{50}\)/mL when treated with concentrations of 0.08 and 0.1% respectively at 4°C. Hence, C-PAC shows greater reduction of MNV-1 within shorter times when compared with essential oils.

In order to gain insights into the mechanism of action of CJ and C-PAC against HAV, confluent FRhK4 cells were first treated with 0.25 mg/mL PAC or 50% neutralized CJ for 30 min followed by treatment with HAV and incubated for 3 h, where reductions of 0.12±0.10 log PFU/mL or 0.28±0.12 log PFU/mL were obtained, respectively. This suggests that C-PAC and CJ exert some, although minimal and not statistically significant (p>0.05), inhibitory effects on the adsorption/internalization of the viral particles to the FRhK4 cells by blocking the cell receptors. Su et al. (2010c) observed that pomegranate juice (20%) and pomegranate polyphenols (0.4 mg/mL) exerted greater effect on prevention of adsorption of FCV-F9 and MNV-1 than on their replication and suggested that pomegranate juice and the polyphenols could be preventing the adsorption of the viral particles either by blocking receptors on the host cell or those on the viral particle. Increased reductions of 1.49±0.16 log PFU/mL and 1.40±0.01 log PFU/mL were observed for HAV in cells co-treated with C-PAC or CJ, respectively. Similar to the results of our current study, maximum inhibition was obtained by Oh et al. (2012) when black raspberry juice and virus were added together to the RAW 264.7 cells. They suggested that black raspberry juice could be
preventing the attachment of the viral protein to the receptors of the host cells or could be inhibiting the internalization of the viral particles into the host cells. Our results, hence, suggest the role of C-PAC and CJ in preventing adsorption of HAV onto FRhK4 cells by either damaging the viral capsid (as is suggested by the results of TEM for HAV treated with CJ), or by affecting the cell receptors (less likely as minimal reduction was observed when cells were treated with C-PAC or CJ prior to infection with virus). In the study conducted by Su et al. (2010b), reductions of 0.51 and 0.91 log PFU/mL were observed for MNV-1 when RAW 264.7 cells were first treated with 50% neutralized CJ and 0.3 mg/mL C-PAC, respectively, suggesting that CJ and C-PAC exert some effect on preventing the adsorption of MNV-1.

When the confluent monolayer of FRhK4 cells were incubated with HAV for 3 h followed by addition of 0.25 mg/mL C-PAC or 50% neutralized CJ for 30 min, no reduction in viral titers was observed. This suggests that both CJ and C-PAC did not exhibit any effect on HAV replication post-adsorption. For MNV-1, however, CJ and C-PAC were found to prevent viral replication to some extent as reductions of 0.80 and 0.42 log PFU/mL with 50% neutralized CJ and 0.3 mg/mL C-PAC, respectively, were reported (Su et al., 2010b). Blueberry proanthocyanidins, at 0.5 mg/ml demonstrated no effect on the adsorption or replication of HAV (Joshi et al., 2014). Hence, the role of both cranberry and blueberry proanthocyanidins in preventing adsorption or replication of HAV cannot be established. This study also suggests that CJ and C-PAC could have different mechanism of action against HAV and MNV-1. This difference could possibly be due to the difference in the capsid structure of the two viruses.

5. Conclusion

As of date (during writing and submission of this manuscript), this is the first study that demonstrates the antiviral effects of CJ and C-PAC on HAV at 37 °C. This research lays the
foundation for the potential use of CJ and C-PAC as natural and easily available alternatives in the prevention of severe enteric foodborne illnesses. These bioactives can be further studied as preventive and therapeutic options for HAV and HNoV illness especially in scenarios when vaccines are unavailable or cost-prohibitive. The results of this study provide the preliminary data and platform for clinical trials that could be undertaken to evaluate the effects of CJ and C-PAC in vivo. Future research involves the evaluation of the antiviral effects of CJ and C-PAC on HAV and MNV-1 in food matrices and simulated gastric fluids, as well as encapsulation of bioactive polyphenols. However, before any health claims can be made, in vivo animal feeding studies and clinical trials with CJ and C-PAC are needed as well as approval by appropriate regulatory agencies need to be obtained.

Acknowledgements

Funding provided by OceanSpray Cranberries Inc. and the University of Tennessee Institute of Agriculture (TEN # 00391) to D. D’Souza to carry out this research is gratefully acknowledged. We sincerely thank Dr. John Dunlap (UTK) for his assistance and guidance with the TEM analysis.
References


### Appendix

Tables:

Table 2.1. Reduction of murine norovirus (MNV-1) titers by cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) at 37°C over 3 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4.92±0.19abc</th>
<th>0.92±0.15abc</th>
<th>5.28±0.25ab</th>
<th>1.33±0.05ef</th>
<th>4.92±0.18</th>
<th>0.17±0.01</th>
<th>4.92±0.18 abc</th>
<th>0.17±0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (pH 7.2)</td>
<td>0.5</td>
<td>4.53±0.23bcd</td>
<td>0</td>
<td>4.92±0.19ab</td>
<td>0</td>
<td>5.28±0.25a</td>
<td>0</td>
<td>4.92±0.18abc</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJ (pH 3.0)</td>
<td>0.5</td>
<td>4.35±0.11cde</td>
<td>0.19±0.12</td>
<td>4.08±0.2de</td>
<td>0.84±0.01</td>
<td>3.95±0.19ef</td>
<td>1.33±0.05</td>
<td>3.46±0.17f</td>
<td>1.46±0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malic acid (pH 3.0)</td>
<td>0.5</td>
<td>4.7±0.04bc</td>
<td>0</td>
<td>4.68±0.04bc</td>
<td>0.24±0.15</td>
<td>5.33±0.04a</td>
<td>0</td>
<td>4.75±0.30bc</td>
<td>0.17±0.1</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C-PAC 0.06 mg/mL</td>
<td>0.5</td>
<td>Not tested</td>
<td>4.46±0.18c</td>
<td>0.46±0.01</td>
<td>Not tested</td>
<td>3.46±0.16f</td>
<td>1.46±0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-PAC 0.5 mg/mL</td>
<td>0.5</td>
<td>ND</td>
<td>≥2.53±0.23</td>
<td>ND</td>
<td>≥2.92±0.19</td>
<td>ND</td>
<td>≥3.28±0.25</td>
<td>ND</td>
<td>≥2.92±0.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-PAC 2 mg/mL</td>
<td>0.5</td>
<td>ND</td>
<td>≥2.53±0.23</td>
<td>ND</td>
<td>≥2.92±0.19</td>
<td>ND</td>
<td>≥3.28±0.25</td>
<td>ND</td>
<td>≥2.92±0.18</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ethanol (10%)</td>
<td>0.5</td>
<td>4.70±0.09bc</td>
<td>0</td>
<td>4.68±0.09bc</td>
<td>0.24±0.1</td>
<td>4.69±0.07bc</td>
<td>0.58±0.01</td>
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<td>0.23±0.11</td>
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</table>

Data are reported as Mean ± Standard deviation
ND: None detected (Limit of detection of the plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Table 2.2. Reduction of hepatitis A virus (HAV) titers by cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) at 37 C over 3 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered titer (log PFU/mL)</td>
<td>Reduction (log PFU/mL)</td>
<td>Recovered titer (log PFU/mL)</td>
<td>Reduction (log PFU/mL)</td>
</tr>
<tr>
<td>PBS (pH 7.2)</td>
<td>4.55±0.10&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0</td>
<td>4.72±0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>CJ (pH 3.0)</td>
<td>Not tested</td>
<td>0</td>
<td>3.11±0.14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.61±0.04</td>
</tr>
<tr>
<td>Malic acid (pH 3.0)</td>
<td>4.61±0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0</td>
<td>4.61±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>C-PAC 0.5 mg/ml</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.55±0.10</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.72±0.10</td>
</tr>
<tr>
<td>C-PAC 1 mg/ml</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.55±0.10</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.72±0.10</td>
</tr>
<tr>
<td>C-PAC 2 mg/ml</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.55±0.10</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>≥2.72±0.10</td>
</tr>
<tr>
<td>Ethanol (10%)</td>
<td>4.69±0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0</td>
<td>4.65±0.13</td>
<td>0.07±0.03</td>
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</table>

Data are reported as Mean±Standard deviation
ND: None detected (Limit of detection of the plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Table 2.3. Effect of CJ (neutralized, 50%) and C-PAC (0.25 mg/mL) on adsorption and replication of hepatitis A virus (HAV).

<table>
<thead>
<tr>
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<th>Adsorption</th>
<th>Replication</th>
<th>Co-treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Recovered titer (log PFU/mL)</td>
<td>Reduction (log PFU/mL)</td>
<td>Recovered titer (log PFU/mL)</td>
</tr>
<tr>
<td>Control</td>
<td>4.75±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>4.71±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CJ</td>
<td>4.47±0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.28±0.12</td>
<td>4.81±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C-PAC</td>
<td>4.63±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12±0.10</td>
<td>4.92±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns.
Figures:

Figure 2.1. Transmission Electron Microscopy (TEM) images of HAV after 1 h treatment at 37°C with (a) water, (b) cranberry juice (CJ, pH 3.0), or (c) 0.5 mg/mL cranberry proanthocyanidins (C-PAC). HAV was stained with uranyl acetate.

Figure 2.2. Transmission Electron Microscopy (TEM) images of MNV-1 after 1 h treatment at 37°C with (a) water, (b) cranberry juice (CJ, pH 3.0), and (c) 0.5 mg/mL cranberry proanthocyanidins (C-PAC). MNV-1 was stained with uranyl acetate.
Figure 2.3. Transmission Electron Microscopy (TEM) images of (a) cranberry juice (CJ, pH 3.0), (c) 0.5 mg/mL cranberry proanthocyanidins (C-PAC)

Figure 2.4. Reduction of MNV-1 titers at ~5 log PFU/mL by cranberry proanthocyanidins (C-PAC) over 3 h at 37°C
PBS: phosphate-buffered saline; C-PAC: cranberry Proanthocyanidins
Each data set is an average of three replicates and error bars denote standard deviation.
Figure 2.5. Reduction of MNV-1 titers at ~5 log PFU/mL by CJ over 3 h at 37°C
PBS: phosphate-buffered saline; CJ: cranberry juice; Molarity of Malic acid: 10mM
Each data set is an average of three replicates and error bars denote standard deviation.
Figure 2.6. Reduction of HAV titers at ~5 log PFU/mL by cranberry proanthocyanidins (C-PAC) over 3 h at 37°C
PBS: phosphate-buffered saline
Each data set is an average of three replicates and error bars denote standard deviation.

Figure 2.7. Reduction of HAV titers at ~5 log PFU/mL by CJ over 3 h at 37°C
PBS: phosphate-buffered saline; CJ: cranberry juice; Molarity of Malic acid: 10mM
Each data set is an average of three replicates and error bars denote standard deviation.
Chapter III

Reduction of Aichi virus titers by Cranberry Juice and Cranberry Proanthocyanidins
Reduction of Aichi virus titers by Cranberry Juice and Cranberry Proanthocyanidins

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Running Head: Cranberry Proanthocyanidins decrease Aichi virus infectivity

To be submitted to: Food Microbiology

Key words: Aichi virus reduction, cranberry juice, proanthocyanidins

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Highlights

- Aichi virus (AiV) is an emerging global enteric pathogen
- Effective preventive measures and treatment options for AiV infections are being researched
- Cranberry juice and cranberry proanthocyanidins show potential as antivirals against AiV
Abstract

Aichi virus (AiV) is an emerging enteric pathogen, causing gastroenteritis in humans worldwide. There is limited literature on effective control measures to prevent AiV spread or treat AiV infections. The demand for natural products to prevent and treat infections is increasing, especially in the absence of available vaccines. Cranberry juice (CJ) and cranberry proanthocyanidins (C-PAC) have health benefits including antimicrobial effects. CJ and C-PAC have shown antiviral activities against human norovirus surrogates, murine norovirus and feline calicivirus. The objective of this study was to determine the effect of CJ and C-PAC on AiV over 1 h at 37°C and to gain insights on their mode of action. CJ (pH 3.0), C-PAC (0.12 and 1 mg/mL), 10 mM malic acid (pH 3.0, control), 10% ethanol (control) or PBS (pH 7.2) were individually mixed with equal volumes of AiV (~5 log PFU/mL), incubated over 1 h, serially diluted in media containing fetal bovine serum and plaque assayed. Transmission electron microscopy (TEM) and binding studies were undertaken to understand their mechanism of action. All treatments were run in duplicate, replicated thrice, and data were statistically analyzed. C-PAC at 0.12 and 1 mg/ml reduced AiV to undetectable levels within 5 and 1 min, respectively. After 15 min, CJ reduced AiV by ~3 log PFU/mL while malic acid caused ~1.5 log PFU/mL in 60 min. TEM showed damage of CJ-treated viral capsids. C-PAC decreases AiV titers by preventing viral binding/adsorption to host cells. CJ and C-PAC show promise as natural antivirals against AiV in vitro.
1. Introduction

Aichi virus (AiV) is an emerging human pathogen of the *Kobuvirus* genus in the *Picornaviridae* family (Reuter et al., 2011). It was first isolated in March 1989 at Aichi Prefecture in Japan from stool specimens of patients who were suffering from nonbacterial gastroenteritis after consuming raw oysters (Yamashita et al., 1991). Kobuviruses are one of the five genera in the *Picornaviridae* family being spherical, non-enveloped, ~30 nm in diameter with single-stranded, positive sense RNA (Yamashita et al., 1991; Yamashita et al., 2003; Reuter et al., 2009, Drexler et al., 2011). Kobuviruses have higher amounts of pyrimidines U and C than found among other picornaviruses (Reuter et al., 2011). The genome of AiV is comprised of 8250 nucleotides, excluding the ones in the poly-A tail (Yamashita et al., 1998) with three genotypes, A, B and C (Yamashita et al., 2000, Ambert-Balay et al., 2008). Gastroenteritis caused by AiV is characterized by diarrhea, abdominal pain, nausea, vomiting and fever (Yamashita et al., 1991; Yamashita et al., 2001). The incubation period for AiV infection is 3-7 days and the illness can last for 3-10 days (Švraka-Latifovic, 2011). AiV is shed in feces and transmitted via the fecal-oral route (Reuter et al., 2011). Hence, water and shellfish harvested from water bodies contaminated with sewage are the primary source for infection (Yamashita et al., 2000, Ambert-Balay et al., 2008; Le Guyader et al., 2008). AiV has a cytopathic effect on host BSC-1 and Vero cells, both obtained from the kidney of normal adult African green monkey (*Cercopithecus aethiops*) and hence these cell lines are used for AiV propagation and detection assays (Yamashita et al., 1991).

AiV has been reported to be prevalent in various parts of the world. AiV was detected in the feces of a 3-year-old child from Hungary who was suffering from diarrhea and respiratory illness (Reuter et al., 2009b). AiV has also been found in human stool samples in Japan, Germany, Brazil, Bangladesh, Thailand, Vietnam, France, Tunisia, Hungary, China and Finland (Yamashita...
et al., 1991, Yamashita et al., 1993; Oh et al., 2006; Pham et al, 2007; Ambert-Balay et al., 2008; Sdiri-Loulizi et al., 2008; Reuter et al., 2009b; Yang et al., 2009; Kaikkonen et al., 2010). Studies conducted in Japan, Germany, France and Spain suggest that around 80-95% of the population between 30–40 years demonstrate the presence of antibodies against AiV which is indicative of infection at some stage in their life (Yamashita et al., 1993; Oh et al., 2006; Goyer et al., 2008; Ribes et al., 2010).

There is not much reported in literature on methods to control or treat AiV infections. Typically, non-enveloped viruses exhibit longer persistence in the environment than enveloped viruses (Kotwal and Cannon, 2014). Like other picornaviruses, AiV is stable *in vitro*, reported to survive in acidic conditions of pH 3.5 and resistant to treatments by chloroform, ether and non-ionic detergent, but is susceptible to inactivation by heating at 60°C for 30 min (Yamashita et al., 1998). A recent study conducted by Cromeans et al. (2014) reported AiV to be more resistant than feline calicivirus (FCV), murine norovirus (MNV), porcine enteric calicivirus (PEC) and Tulane virus (TV) to generally used disinfection and inactivation treatments like pH, heat, alcohols, chlorine and high hydrostatic pressure (HHP). Treatment of lettuce, green onions and strawberries with ultraviolet light at 240 mWs cm⁻² was reported to reduce AiV by 4.59, 2.49 and 1.87 log Tissue Culture Infectious Dose₅₀ (TCID₅₀)/ml, respectively (Fino and Kniel., 2008). High pressure processing treatment of AiV at 600 MPa in minimum essential growth medium with 10% fetal bovine serum for 5 min was found to be ineffective in reducing AiV titers (Kingsley et al., 2004). With respect to use of natural plant products for inactivation of AiV, essential oil extracts from herbal parts of *Origanum acutidens* (Lamiaceae) were tested *in vivo* but were found to be ineffective in preventing AiV replication (Sokmen et al., 2004).
However, as the demand for natural antimicrobials increase, research continues to focus on plant extracts to control the spread of infections. Cranberries (*Vaccinium macrocarpon*) contain bioactive polyphenols with known antimicrobial and anti-inflammatory properties and have been used to prevent bacterial urinary tract infections (UTI) caused by *Escherichia coli* adherence to uroepithelial cells in humans (Gupta et al. 2007). The bioactive polyphenols in cranberries include proanthocyanidins (C-PAC) with A-type linkages (Foo et. al. 2000a, 2000b), which are different from other foods rich in tannins that possess proanthocyanidins with B-type linkages. The successive monomeric flavan-3-ol units in B-type proanthocyanidins (PACs) are linked by C-C interflavan bonds between the C4 of the upper unit and the C6 (4β →6) or C8 (4β →8) of the lower unit (Hammerstonne et al., 2000; Gu et al., 2004; Dixon et al., 2005). In A-type PACs the flavan-3-ol units are linked by a mixture of C-C bonds and interflavonoid bonds by C-O oxidative coupling between both C2 and C4 of the upper unit and the oxygen at C7 and positions C6 or C8, respectively, of the lower unit (4β →8 and 2β →O→7) (Côté et al., 2010). Fifty-one to ninety-one % of the total PAC content in cranberries consists of at least one A-type PAC (Foo et al., 2000; Feliciano et al., 2012). In addition to antibacterial effects, CJ and C-PAC have also demonstrated significant antiviral activity against bacteriophages T4 and T2, bovine reovirus, the simian rotavirus SA-11 (Lipson et al., 2007) and influenza virus (Weiss et al., 2005). Studies show that CJ and pomegranate juice are effective in decreasing the specific binding ability of human norovirus (HNoV) to saliva when human norovirus GI.4 P particles were used as a research surrogate for HNoV (Li et al, 2012). Su et al. (2010a) also reported that in vitro, CJ and C-PAC at 0.15, 0.3 and 0.6 mg/mL were effective in reducing the infectivity of human enteric viral surrogates, namely, MNV-1, FCV-F9, bacteriophage MS2 and bacteriophage φ-X174, after 1 h at room temperature (RT). At initial titers of ~5 log PFU/mL. FCV-F9 was reported to be reduced to
undetectable levels with CJ or C-PAC at the tested concentrations. While for MNV-1, these researchers reported reductions of 2.63, 2.75, and 2.95 log PFU/mL with 0.15, 0.30, and 0.60 mg/mL C-PAC, respectively and 2.06 log PFU/mL with CJ. Transmission electron microscopy (TEM) analysis of FCV-F9 treated with CJ and C-PAC at 0.30 mg/mL showed structural damage to viral particles indicating that the viral capsid was the target for inactivation by CJ and C-PAC (Su et al., 2010b).

Given the reported prevalence of AiV around the globe and the reported antiviral effects of plant extracts, it becomes necessary to evaluate natural alternatives to prevent or treat AiV infections. The primary objective of this study was to evaluate the effectiveness of CJ and C-PAC in reducing AiV titers and to gain insights into their mode of action using binding studies on viral adsorption and replication as well as transmission electron microscopy.

2. Materials and Methods

2.1. Viruses and cell lines

AiV was kindly provided by Dr. David Kingsley (USDA ARS, Delaware) along with the Vero host cells.

2.2. Propagation of viruses

Vero cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone Laboratories, Logan, UT) supplemented with 2% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) in an atmosphere containing 5% CO₂ at 37°C in 175 cm² flasks as described earlier (Su et al., 2010a; Fino and Kniel, 2008). Viral stocks of AiV for the study were made by inoculating AiV onto monolayers of confluent Vero cells, and incubated under 5% CO₂ until >90% cell lysis was observed. The viral suspensions obtained were freeze-thawed thrice and centrifuged
for 10 min at 5000 x g. The supernatants were then filtered through 0.2 µm membrane filter, aliquoted and stored at -80°C until use.

2.3. Determination of the Antiviral effects of CJ and C-PAC on AiV

Cranberry Juice Cocktail (CJ, 27% cranberry juice) was purchased from a local grocery store and cranberry proanthocyanidins (C-PAC) were obtained from Dr. Amy Howell, Marucci Center for Blueberry and Cranberry Research (Rutgers University, Chatsworth, NJ). C-PAC stock (4 mg/mL) was prepared by dissolving in 10% ethanol and filter-sterilizing through a 0.2-micron filter. CJ (pH 3.0), C-PAC (0.12 and 1 mg/mL), or controls such as 10 mM malic acid (pH 3.0), 10% ethanol, and phosphate buffered saline (PBS, pH 7.2), were individually mixed with equal volumes of AiV (titer of ~5 log PFU/mL) and incubated at 37°C for 1, 5, 15, 30 or 60 min. At the various time points, treatments were stopped/neutralized by a 10-fold serial dilution in DMEM containing 10% FBS. Subsequent 10-fold serial dilutions were made in DMEM containing 2% FBS. These dilutions were then used to determine viral infectivity by standard plaque assays. All treatments were run in duplicate and replicated thrice.

2.4. Infectious plaque assays

Standard protocols as described by Fino and Kniel (2008) were followed for AiV plaque assays. Vero cells were infected with 0.5 ml of treated or control samples of AiV and incubated for 2 h to allow for infection. Viruses were then aspirated and the cells were overlaid with 2 ml complete DMEM containing 0.75% agarose and incubated at 37°C under 5% CO2. After an incubation of three days, the cell monolayers were stained using neutral red and further incubated at 37°C until plaques were visible. Plaques were then enumerated.
2.5. Effect on adsorption and replication of viruses

To evaluate if CJ and C-PAC affect AiV adsorption or replication, binding assays as stated by Su et al. (2010a; 2010b) and Oh et al. (2012) were used. Briefly, confluent Vero cells were first treated with 0.25 mg/mL C-PAC or 25% CJ (these concentrations are not cytotoxic to the host cells used), neutralized CJ (pH 7.0, neutralized with 4M NaOH) or DMEM with 2% FBS (as a control) for 30 min followed by infection with AiV and incubated for 2 h. Plaque assays were performed as described earlier and plaques were counted and compared to controls. Any titer reduction obtained would suggest that C-PAC and CJ exert an inhibitory effect on the adsorption/internalization of the viral particles to the host cells by blocking the host-cell receptors.

The host cells were also subjected to co-treatment i.e. simultaneous treatment with AiV serially diluted in neutralized CJ (50%) or 0.25 mg/mL C-PAC for 2 h so as to further determine their mechanism of action in prevention of adsorption of the viral particles (Oh et al., 2012).

To determine if C-PAC and CJ have an inhibitory effect on viral replication, the confluent monolayer of Vero cells were first infected with AiV for 2 h, followed by the addition of 0.25 mg/mL PAC or 25% CJ or DMEM with 2% FBS (as a control) for 30 min. Plaque assays were performed as described earlier and plaques were counted and compared to controls. Each experiment was conducted in duplicate and replicated thrice.

2.6. Transmission electron microscopy (TEM) analysis

TEM studies following the protocol described by Su et al. (2010b) were carried out at the UT-Knoxville Advanced Microscopy and Imaging Center. AiV was treated with equal volumes of CJ, 0.5 mg/mL C-PAC or PBS and incubated at 37°C for 5 min. Ten-µl of each sample was then applied to a freshly glow discharged, formvar and carbon coated copper grid (Electron Microscopy Sciences, Hatfield, PA). Excess sample was removed after 1 min. The grid with sample was then
stained with uranyl acetate for 1 min, excess stain was removed and the grid was allowed to dry. The stained viruses were viewed using the 200kV Zeiss LIBRA 200MC as described earlier (Su et al., 2010b).

2.7. Statistical Analysis

A completely randomized design with sampling was used. Each experiment was run in duplicate (samples) and replicated thrice. Analysis of variance was conducted with Statistical Analysis Software (SAS, version 9.2, SAS Institute, Cary, NC, USA) and Tukey’s test at p<0.05 to determine statistically significant differences between the recovered titers of the treatments and controls.

3. Results

3.1. Reduction of AiV titers with CJ and C-PAC

Reduction in AiV titers were observed with both CJ (pH 3.0) and C-PAC (Table 3.1). CJ was found to reduce AiV to undetectable levels (detection limit of plaque assay is 2 log PFU/mL) within 15 min at 37°C, with 1.26±0.12 and 2.16±0.07 log PFU/mL reduction after 1 min and 5 min, respectively. Malic acid (10 mM, pH 3.0) used as the pH control was shown to cause reductions of 0.50±0.06 log PFU/mL after 15 min but caused no reduction after 1 min or 5 min when compared to the PBS control. Cranberry juice cocktail contains approximately 30% cranberry juice with C-PAC levels of about 0.12 mg/mL (Su et al., 2010a). C-PAC at concentrations of 0.12 mg/mL and 1 mg/mL were used for 1:1 treatment with AiV. Hence, the final concentrations of C-PAC were 0.06 mg/mL and 0.5 mg/mL which reduced AiV to undetectable levels within 5 min and 1 min, respectively at 37°C. The ethanol (10%) control used as a solvent for C-PAC showed no significant (p>0.05) reduction in AiV titer as compared to the
PBS control over 1 h. This showed that the effects were mainly due to C-PAC and not due to solvent or pH alone.

3.2. Effect on CJ and C-PAC on viral adsorption and replication

C-PAC (0.25 mg/mL) added prior to and following viral infection of Vero cells decreased the infectivity of AiV by 2.10±0.04 and 2.50±0.25 log PFU/mL, respectively (Figure 3.2). Neutralized CJ (50%, pH 7.0) when used for the treatment of Vero cells, prior to or post infection with AiV resulted in reductions of 1.38±0.16 and 0.16±0.12 log PFU/mL, respectively. Co-treatment of Vero cells with 0.25 mg/mL C-PAC or neutralized CJ (50%) reduced AiV infectivity by 2.58±0.22 or 1.24±0.15 log PFU/mL, respectively.

3.3 TEM analysis

AiV treated with an equal volume of CJ, 1 mg/mL C-PAC or PBS and incubated at 37 °C for 5 minutes when viewed by TEM (Figure 3.3), showed that viral particles treated with CJ (image b) had damaged viral capsids leading to distorted particles as compared to those in image (a) which showed intact viral particles in the PBS control. Image (c) of the viral particles treated with C-PAC lead to inconclusive results given the high background of the C-PAC particles. Hence, from TEM analysis, it can be suggested that the primary target of CJ against AiV is the viral capsid.

4. Discussion

Recently, many control strategies have been evaluated for the inactivation of AiV. A study conducted by Cromeans et al. (2014) established the resistance of AiV to pH, heat, alcohols, chlorine and HHP. AiV exhibited resistance to extreme pH with no significant reduction obtained when subjected to pH 2, 3, 9 and 10 for 30 min at 37°C (Cromeans et al., 2014). They also reported that AiV was rapidly inactivated when subjected to heat treatment at 60°C and 63°C (Cromeans et al., 2014) and that at 56°C, 4 log PFU reduction (maximum measurable reduction being 5 log

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PFU) in infectivity was obtained for AiV in 20 min (Cromeans et al., 2014). Treatment with 70% isopropanol, 90% isopropanol, 70% ethanol or 90% ethanol resulted in less than 0.5 log reduction in AiV titers in 1 and 5 min indicating its resistance to alcohols that are generally used as sanitizers (Cromeans et al., 2014). When viruses dried on stainless steel discs were exposed to 200 or 1,000 ppm chlorine for 5 min reductions of 0.9 ± 0.2 log and 1.3 ± 0.9 log PFU, respectively were obtained for AiV (Cromeans et al., 2014). When nucleic acids of the samples treated with 1000 ppm chlorine were analyzed using RT-qPCR, reduction of 2.5 log PFU was obtained for FCV RNA while AiV RNA was reduced by <0.5 log. HHP was found to inactivate FCV, MNV, and PEC completely at ≥300 mega Pascal (MPa), Tulane virus (TV) at 600 MPa while AiV demonstrated complete resistance to pressures up to 800 MPa (Cromeans et al., 2014).

In comparison to the processing methods described above, CJ and C-PAC (final concentration 0.5 mg/mL) reduced AiV to undetectable levels (~3 log PFU/mL reduction) within 15 min and 1 min, respectively at 37°C. A difference of 10 min was observed in the time taken to reduce AiV titers to undetectable levels by CJ (containing C-PAC at 0.12 mg/mL as per Su et al., 2010a) and C-PAC at 0.12 mg/mL (final concentration 0.06 mg/mL). C-PAC, by itself, was found to be more effective than CJ. The enhanced activity of C-PAC could be due to the absence of other complex components like sugars that are present in CJ, making the bioactive components more freely available for the antiviral effect. MNV-1 when treated with 0.15, 0.30, and 0.60 mg/mL C-PAC was reduced by 2.63, 2.75, and 2.95 log PFU/mL, respectively after 1 h at RT (Su et al., 2010a). This suggests that CJ and C-PAC have a greater effect on reduction of AiV than MNV-1 titers. However, the difference in the temperature of the treatments must be noted (effect of CJ and C-PAC on AiV was tested at 37°C while that on MNV-1 was tested at RT).
C-PAC was shown to demonstrate higher potential than pomegranate and grape polyphenols in reducing viral titers of MNV-1 (Su et al., 2010c). High (~7 log PFU/mL) titers of FCV-F9, MNV-1, and MS2 were reported to be reduced by 1.20, 0.06, and 0.63 log PFU/mL respectively when treated with pomegranate juice for 1 h at room temperature. However, when these viruses were treated with 8, 16, and 32 mg/mL of pomegranate polyphenols for 1 h at room temperature, both high and low titers of FCV-F9 were reduced to undetectable levels, while MNV-1 (low initial titer of ~ 5 log PFU/mL) was reduced by 1.30, 2.11, and 3.61 log PFU/mL and MNV-1 (high initial titer) was reduced by 1.56, 1.48, and 1.54 log_{10} PFU/mL respectively. When treated with grape seed extract (GSE) at 0.25, 0.5, and 1.0 mg/mL at 37°C for 2 h, MNV-1 reductions of 1.49, 1.72, and 1.97 log PFU/mL, respectively were observed by Su et al. (2011). Also, as mentioned earlier, AiV was shown to exhibit greater resistance to pH, heat, alcohols, chlorine and HHP than MNV (Cromeans et al., 2014). As MNV-1 is typically more resistant than FCV to most treatments, the increased sensitivity of AiV to CJ and C-PAC than MNV-1 is noteworthy.

TEM results showed structural damage to the viral capsid when treated with CJ which is similar to that observed for FCV-F9 (Su et al., 2010b). The results for TEM of viral particles treated with C-PAC were inconclusive owing to the high background noise of the C-PAC particles. To understand the mode of action of C-PAC, TEM analysis using lower concentrations of C-PAC would be helpful as it would decrease the background caused by C-PAC particles.

The mode of action studies for AiV revealed that C-PAC (0.25mg/mL), on pre-treatment, co-treatment and post-treatment of Vero cells, decreased the infectivity of AiV by 2.10±0.04, 2.58±0.22, and 2.50±0.25 log PFU/mL, respectively. This suggests the role of C-PAC in inhibition of viral adsorption as well as replication. This observation is in agreement with that reported by Su et al (2010b) where neutralized 50% CJ (pH 7.0) or C-PAC (0.3 mg/mL) decreased the
infectivity of MNV-1 using both pre- or post-infection treatment of RAW 264.7 cell monolayers suggesting the effect of CJ and C-PAC on both adsorption and replication of MNV-1. In the case of FCV-F9, infectivity was inhibited only by the post infection treatment with CJ and C-PAC suggesting a small effect on replication of the virus (Su et al., 2010b). Treatment of Vero cells with 50% neutralized CJ prior to and post infection with AiV resulted in reductions of 1.38±0.16 and 0.16±0.12 log PFU/mL. When the cells were subjected to co-treatment with 50% neutralized CJ, reduction of 1.24±0.15 log PFU/mL in comparison to the control was observed. This suggests that CJ does inhibit the adsorption of AiV to host cells by either causing structural changes in the viral capsid (as is evident in the TEM analysis) or by blocking the host cell receptors. However, this study cannot conclusively depict/delineate the role of CJ in preventing replication of AiV.

This study reports interesting findings on the potential application of CJ and the bioactive polyphenols from cranberries to either prevent foodborne viral infection or to alleviate the disease symptoms caused by AiV. This is important as vaccines are currently unavailable for this emerging viral pathogen as reported above and hence alternate natural preventive options are being sought.

5. Conclusion

The results of the current study with CJ and C-PAC are interesting and show promise for evaluation as alternative preventive and control options for AiV. However, the stability and efficacy of CJ and C-PAC as antivirals against AiV in model food systems and simulated gastric fluids need to be studied. In vivo studies using animal models and challenge studies (or human feeding studies, when possible) would be needed together with appropriate regulatory approvals before any claims are made regarding the antiviral activity of CJ and C-PAC against AiV.
Acknowledgements

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References


Appendix

Tables:

Table 3.1. Recovered AiV titers (from initial 5 log PFU/mL) by cranberry juice (CJ) and cranberry proanthocyanidins (0.06 and 0.5 mg/mL CPAC) over 60 min at 37°C.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>PBS, pH 7.2</th>
<th>CJ, pH 3.0</th>
<th>Malic acid, pH 3.0</th>
<th>C-PAC, 0.5 mg/mL</th>
<th>C-PAC, 0.06 mg/mL</th>
<th>Ethanol, 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.53±0.07b</td>
<td>3.15±0.12g</td>
<td>4.77±0.07a</td>
<td>NDi</td>
<td>4.53±0.07b</td>
<td>4.53±0.07b</td>
</tr>
<tr>
<td>5</td>
<td>4.06±0.04e</td>
<td>2.3h</td>
<td>4.47±0.10bc</td>
<td>NDi</td>
<td>NDi</td>
<td>4.06±0.04e</td>
</tr>
<tr>
<td>15</td>
<td>4.30±0.06cd</td>
<td>NDi</td>
<td>3.79±0.13i</td>
<td>NDi</td>
<td>NDi</td>
<td>4.30±0.06cd</td>
</tr>
<tr>
<td>30</td>
<td>4.25±0.08de</td>
<td>NDi</td>
<td>3.24±0.11f</td>
<td>NDi</td>
<td>NDi</td>
<td>4.29±0.13cd</td>
</tr>
<tr>
<td>60</td>
<td>4.31±0.18cd</td>
<td>NDi</td>
<td>3.20±0.10g</td>
<td>NDi</td>
<td>NDi</td>
<td>4.39±0.08bcd</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
ND: None detected (Limit of detection of the plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Table 3.2. Effect of cranberry juice (CJ, neutralized, 50%) and cranberry proanthocyanidins (C-PAC, 0.25 mg/mL) on adsorption and replication of AiV.

<table>
<thead>
<tr>
<th>Titer in log PFU/mL</th>
<th>Adsorption</th>
<th>Replication</th>
<th>Co-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (DMEM)</td>
<td>C-PAC (0.25 mg/mL)</td>
<td>CJ</td>
</tr>
<tr>
<td>Recovered titer</td>
<td>4.40±0.04 (^a)</td>
<td>2.30 (^c)</td>
<td>3.02±0.20 (^b)</td>
</tr>
<tr>
<td>Reduction</td>
<td>-</td>
<td>2.10±0.04</td>
<td>1.38±0.16</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
ND: None detected (Limit of detection of the plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Fig. 3.1. Reduction of AiV titers at ~5 log PFU/mL by cranberry juice (CJ) over 1 h at 37°C. PBS: phosphate-buffered saline; CJ: cranberry juice; Molarity of Malic acid: 10mM
Each data set is an average of three replicates and error bars denote standard deviation.
Fig. 3.2. Reduction of AiV titers from initial ~5 log PFU/mL by cranberry proanthocyanidins (C-PAC) over 1 h at 37ºC

PBS: phosphate-buffered saline

Each data set is an average of three replicates and error bars denote standard deviation.
Fig. 3.3. Transmission Electron Microscopy (TEM) images of (a) AiV after 5 min incubation with Phosphate-buffered saline (PBS), (b) AiV after 5 min incubation with cranberry juice (CJ, pH 3.0) (c) AiV after 5 min incubation with cranberry proanthocyanidins (C-PAC, 0.5 mg/mL) (d) only PBS, (e) only CJ, (f) only C-PAC and staining with uranyl acetate.
Chapter IV

Survival of hepatitis A virus and Aichi virus in cranberry-based juices at refrigeration (4°C)
Survival of hepatitis A virus and Aichi virus in cranberry-based juices at refrigeration (4°C)

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Running Head: Cranberry juice effects survival of hepatitis A virus and Aichi virus

Key words: Hepatitis A virus, Aichi virus, cranberry juice, titer reduction

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**Highlights**

- Hepatitis A virus and Aichi virus retain infectivity in two cranberry-based juices over 21 days of storage at 4°C
- Storage temperature plays a role in viral survival and antiviral activity of cranberry bioactives
- Data is provided for risk assessment associated with contaminated cranberry juice consumption
Abstract

Viral foodborne illness continues to be a cause of concern globally. Hepatitis A virus (HAV) outbreaks in fruits and juices have been reported. Aichi (AiV) virus is an emerging pathogen with limited data on its epidemiology. Both HAV and AiV are resistant to low pH and can survive under adverse environmental conditions leading to ease of transmission and outbreaks. The objective of this study was to evaluate the survival of HAV and AiV in cranberry-based juices (Cranberry juice cocktail, CJ and a 100% juice with cranberry, MJ) over 0, 1, 2, 3, 4, 5, 6, 7, 14, and 21 days at refrigeration (4°C). The juices inoculated with each virus (final titer of 6 log PFU/mL) were stored at refrigeration over 21 days. At each time interval, the inoculated juices were serially diluted in cell culture media and standard plaque assays were used to determine the infectivity of the viral particles. Each experiment was carried out in duplicate and replicated thrice. Reductions of 0.72±0.06 to 2.3±0.18 log PFU/mL and 0.63±0.02 to 1.84±0.14 log PFU/mL were obtained over 21 days for AiV with MJ and CJ respectively. Reductions ranging from 0.67±0.03 to 1.09±0.1 and 0.93±0.27 to 1.49±0.18 log PFU/mL were obtained for HAV over 21 days at refrigeration in MJ and CJ, respectively. Both AiV and HAV were found to survive storage at refrigeration. The data obtained from this study will help determine AiV survival and transmission risk from cranberry-based juices and provide data for risk-modeling and risk assessment studies.
1. Introduction

Foodborne illnesses are a major threat to human health around the globe with 9.4 million cases of foodborne illness, attributed to 31 major pathogens, occurring every year in the United States, 59% of which are caused by viruses (Scallan et al., 2011). Human noroviruses, hepatitis A virus (HAV), hepatitis E virus, Aichi virus (AiV), sapoviruses, rotaviruses, paroviruses, astroviruses, other small round viruses and human enteroviruses including polioviruses, echoviruses, and coxsackie viruses contribute to most foodborne and waterborne viral infections (Sair et al., 2002; D’Souza et al., 2007; de Aceituno et al., 2013). Scallan et al. (2011) reported that 27% of the~55,961 estimated hospitalizations and 12% of the 2,612 deaths due to foodborne illnesses caused annually in the United States are due to viruses.

HAV is one of the epidemiologically significant viruses, due to severity of the disease with typical symptoms that include fever, malaise, anorexia, nausea, abdominal discomfort, dark urine and jaundice. The illness does not usually last longer than 2 months, but some (~10%–15%) patients may demonstrate prolonged or relapsing symptoms for up to 6 months. HAV belongs to the *Picornaviridae* family and does not possess a lipid envelope and is stable in the gastrointestinal tract after it is excreted from the liver of an infected person into the bile (Lemon, 1997). HAV is very sturdy/resilient to environmental stress, can persist for long durations in fresh as well as salt water and soil, and temperatures higher than 85°C are required for inactivation (Sattar et al., 2000; Mbithi et al., 1992; Sobsey et al., 1988). D-values (min) for HAV in buffered cell culture medium were reported to be 56.22 ± 1.95, 8.40 ± 0.43, 2.67 ± 0.42, 1.73 ± 0.98, and 0.88 ± 0.11 at 50°C, 56°C, 60°C, 65°C, and 72°C, respectively (Bozkurt et al., 2014a). When tested in blue mussel homogenate, D-values (min) for HAV were observed to be 54.17±4.94, 9.32±3.26, 3.25±0.72, 2.16±0.17, and 1.07±0.24 min at 50°C, 56°C, 60°C, 65 °C, and 72°C, respectively (Bozkurt et al.,
HAV also exhibits resistance to inactivation methods that include use of detergents, freezing and low pH (pH 2) (Brundage and Fitzpatrick, 2006; Grove et al, 2006). Orange juice, contaminated by HAV during the manufacturing process, was identified as the cause for an outbreak among 351 tourists from nine European countries visiting Egypt (Frank et al., 2007). HAV outbreaks with fruits including frozen berries used in smoothies (Lassen et al., 2013), as vehicles of infection have also been reported. A multistate (10 states) outbreak of HAV (first in ~10 years) traced back to consumption frozen pomegranate arils (imported from Turkey) occurred in the United States in May 2013 and resulted in 165 illnesses, 69 hospitalizations, 2 cases of fulminant hepatitis and 1 case in which liver transplant was needed (Collier et al., 2014). While a 90% decrease in HAV cases (mostly attributed to vaccination of children and people at risk for hepatitis A) has been observed in the US over the last 20 years, there are still an estimated 2,700 new cases of HAV illness per year (CDC, 2014).

AiV is an emerging foodborne virus belonging to the *Kobuvirus* genus in the *Picornaviridae* family (Reuter et al., 2011) that causes gastroenteritis (symptoms include diarrhea, nausea, vomiting, abdominal pain and fever) in humans, globally (Yamashita et al., 1991; Yamashita et al., 2001). It is spherical (~30 nm in diameter), non-enveloped, with single-stranded, positive sense RNA(Yamashita et al., 1991; Yamashita et al., 2003; Reuter et al., 2009, Drexler et al., 2011). Shellfish, contaminated with water from sewage have been identified as the major source of AiV infections (Yamashita et al., 2000, Ambert-Balay et al., 2008; Le Guyader et al., 2008). AiV, *in vitro*, exhibits stability in acidic conditions as low as pH 2. It is resistant to conventional methods of inactivation including heat, alcohols, chlorine, high hydrostatic pressure, chloroform, ether and non-ionic detergents (Yamashita et al., 1998; Cromeans et al. 2014).
AiV was identified as one of the causative agent in an outbreak of gastroenteritis (205 cases) in France associated with consumption of oysters from a flooded shellfish production lagoon (Le Guyader et al., 2008). Between 1987 and 1998, AiV was detected in 20% of the 268 fecal samples obtained from patients suffering from gastroenteritis due to consumption of oysters in Japan (Yamashita et al., 2000). AiV has shown to be prevalent globally as it has been detected in human stool samples across Asia (Japan, Thailand, Bangladesh, Vietnam, and China), Europe (Germany, France, Hungary, and Finland), South America (Brazil), and Africa (Tunisia) (Yamashita et al., 1991 and 1993; Oh et al., 2006; Pham et al, 2007.; Ambert-Balay et al., 2008; Sdiri-Loulizi et al., 2008; Reuter et al., 2009b; Yang et al., 2009; Kaikkonen et al., 2010). Not much is known about the epidemiology for AiV in the United States.

Given the fecal-oral route of transmission for these viruses, contamination of foods and beverages can occur due to improper handling and cause illness. It is hence essential to study the survival of foodborne viruses in fruit juices. Juices are generally stored at refrigeration and hence evaluation of the survival of these viruses under refrigeration over time is important. Cranberry juice and proanthocyanidins have been reported to exhibit antiviral activity against the influenza virus (Weiss et al., 2005), simian rotavirus SA-11, bacteriophages T4 and T2, bovine reovirus, (Lipson et al., 2007) and human norovirus surrogates, namely murine norovirus (MNV-1), feline calicivirus (FCV-F9), bacteriophage MS2 and bacteriophage φ-X174 Su et al. (2010a). Commercial cranberry juice cocktail (CJ, 27% cranberry juice) has also been observed to be effective in reducing titers of HAV and AiV at 37°C to undetectable levels within 2 h and 15 min, respectively (Sewlikar et al., a and b, Manuscript in preparation). However, the effect of CJ on reduction of viral titers for HAV and AiV at refrigeration temperature has not been studied. Cranberry with 100% juice (MJ) for survival analysis can be a good matrix to understand if the
synergistic effect of a combination of fruit juices (including cranberry, grape, apple and pear) can enhance viral reduction over time. Hence, the objective of this study was to determine the survival of HAV and AiV at refrigeration temperatures over 21 days of storage in CJ and MJ. This would mimic household conditions of storage and use and provide information needed regarding the risk associated with consumption of contaminated juice or due to cross-contaminated juice consumption. The data obtained from this study will thus provide input for risk-modeling and risk assessment studies.

2. Materials and Methods

2.1. Viruses and cell lines

HAV (strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were obtained from our collaborator, Dr. Kalmia Kniel (University of Delaware). Aichi Virus (AiV) was kindly provided by Dr. David Kingsley (USDA ARS, Delaware) for use with the Vero host cells.

2.2. Propagation of viruses

FRhK4 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone Laboratories, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) while Vero cells were grown in DMEM with 2% FBS and 1% Anti-Anti and incubated in an atmosphere containing 5% CO₂ at 37⁰C in 175 cm² flasks as described earlier (Su et al., 2010a; Fino and Kneil, 2008). Viral stocks of HAV and AiV for the study were made by inoculating HAV or AiV stocks onto monolayers of host FRhK4 or Vero cells, respectively and incubated under 5% CO₂ until >90% cell lysis was observed. The viral suspensions obtained were freeze-thawed thrice and centrifuged for 10 min at 5000 x g. The supernatants were then filtered
through 0.2 µm membrane filters, aliquoted and stored at -80°C until use as described earlier (Su et al., 2010a).

2.3. Infectious plaque assays

Standard protocols were followed for HAV and AiV plaque assays as described earlier (Su and D’Souza, 2011b; Fino et al., 2008). FRhK4 or Vero cells infected with 0.5 ml of treated or control samples (and their serial dilutions) of HAV or AiV respectively and incubated for 2 to 3 h to allow for infection. Viruses were then aspirated and the cells were overlaid with 2 ml complete DMEM containing 0.75% agarose (for AiV) or 1% agarose (for HAV) and incubated at 37°C under 5% CO₂. After an incubation of three days for AiV and eight days for HAV, the cell monolayers were stained using neutral red and further incubated at 37°C until plaques were visible.

2.4. Survival of viruses in CJ over storage at refrigeration for 3 weeks

Commercially available cranberry juice cocktail (CJ, 27% cranberry juice) and 100% juice with cranberry (MJ, containing cranberry juice, grape juice, apple juice and pear juice) were purchased from a local grocery store. Survival of HAV and AiV, each at initial titers of 7 log PFU/mL, was evaluated in CJ, MJ or PBS following the procedure previously described by Horm et al. (2011). Briefly, each virus (0.2 ml) were added aseptically to 1.8 ml commercial CJ, MJ or PBS and were stored for 0, 1, 2, 3, 4, 5, 6, 7, 14, and 21 days at refrigeration temperature (4°C). After each time point, each virus was serially diluted in DMEM containing 10% or 2% FBS and enumerated using plaque assays as described above.

2.5. Statistical analysis

A completely randomized design with sampling was used. Each experiment was run in duplicates (samples) and replicated thrice. Analysis of variance was conducted with Statistical Analysis Software (SAS, version 9.2, SAS Institute, Cary, NC, USA) and Tukey’s test at p<0.05.
to determine if any statistically significant difference exists between the viral titers recovered from PBS, CJ or MJ over time as compared to the day zero controls.

3. Results

3.1. Survival of AiV in CJ and MJ

Viral titers for both HAV and AiV were found to gradually decrease in CJ and MJ over time. In PBS over 21 days, AiV titers decreased significantly (p<0.05) but the reduction was less than that obtained in CJ or MJ.

The results are detailed in Table 4.1. For AiV in PBS, reduction of only 0.59±0.26 was obtained after 21 days of storage. Reduction for AiV with MJ increased from 0.72±0.06 (1 day of storage) to 2.3±0.18 log PFU/mL (21 days of storage) over the three week period. CJ reduced AiV titers by 0.63±0.02 after 1 day of storage and the reduction continued to increase with the time of storage causing the final reduction of 1.84±0.14 log PFU/mL after 21 days. However, complete reduction was not observed for AiV in either PBS, MJ or CJ even after 21 days at refrigeration.

3.2. Survival of HAV in CJ and MJ

HAV did not show significant reduction (p>0.05) in PBS over 21 days of storage. Reductions of 0.67±0.03 and 0.93±0.27 log PFU/mL were observed after 1 day of storage for HAV in MJ and CJ, respectively. Increased reduction in HAV titers were observed over time (Table 4.2). HAV was reduced by 1.09±0.1 and 1.49±0.18 log PFU/mL after 21 days of storage in MJ and CJ, respectively. Similar to AiV, complete reduction for HAV was not observed even after 21 days at refrigeration.
4. Discussion

The studies on survival of enteric viruses in fruit juices at refrigeration are limited. As both HAV and AiV are transmitted via the fecal-oral route, juices could be cross-contaminated during handling by infected individuals or from water used in preparation. Juices are stored under refrigeration and hence evaluation of the survival of these viruses in juices would help in determining the risk of infection associated with consumption of contaminated juices.

Storing food at low temperatures is a conventional method used to preserve food and to prevent growth of spoilage organisms or pathogens (Zuber et al., 2013). However, HAV in mineral water packed in plastic bottles, was found to survive better for 9 months at 4°C (0.5 log reduction) than at RT (4.1 log reduction) (Biziagos et al., 1988; Butot et al., 2007). Storage of carrots and fennel at 4 °C for 7 days caused >2.5 log reduction for HAV while 2 log reduction was observed in lettuce under the same conditions (Croci et al., 2002). When stored at 5 °C for 15 days, and at -17.5 °C (freezing) for 12 weeks, poliovirus (more sensitive to environmental conditions than other enteric viruses) on oysters was reduced by just 1 log (Di Girolamo et al., 1970; Abad et al., 1994; Zuber et al., 2013). Hence, it has been shown that low temperature storage may not reduce viral loads in foods to a great extent (Zuber et al., 2013).

In this study, we observed that both HAV and AiV were sturdy and survived in CJ (pH 3.0) and MJ (pH 3.0) at 4°C, with the viruses retaining infectivity after 21 days. It must be reemphasized that HAV and AiV are stable at low pH. Also, as can be seen from Fig. 4.1 and Fig. 4.2, not much difference is seen in the survival characteristics of either HAV or AiV in CJ or MJ. MJ is a mixture of cranberry, grape, apple and pear juices while CJ contains only cranberry juice. This indicates that it could be the temperature of incubation that aids the antiviral activity of fruit juices/bioactives in the fruit juices. AiV appeared to show higher reduction by approximately 1 log than HAV over
21 days, showing that HAV is more resilient and could survive the low pH and bioactive components of CJ better than AiV.

Sewlikar et al. (a and b, Manuscripts in preparation) observed that HAV and AiV were reduced to undetectable levels (reduction of ~3 log PFU/mL) when treated with equal volumes of CJ at 37°C within 2 h and 15 min, respectively. However, in the current study reductions of 1.15±0.12 and 1.45±0.05 log PFU/mL were obtained for HAV and AiV, respectively when incubated with CJ for 21 days at refrigeration. It can be deduced that titer reductions of HAV and AiV in CJ are also dependent on temperature of storage. Su et al. (2010b) reported that high (~7 log PFU/mL) titers of human norovirus surrogates, feline calicivirus (FCV-F9), and murine norovirus (MNV-1), and bacteriophage MS2 were reduced by 1.20, 0.06, and 0.63 log PFU/mL, respectively when treated with pomegranate juice (PJ) for 1 h at room temperature (RT). Low titers (~5 log PFU/mL) of FCV-F9 and MNV-1 were reduced by 2.56 and 1.32 log PFU/mL, respectively after the 1 h treatment with PJ at RT. Horm et al. (2011) reported that FCV-F9 in PJ showed reduction of only 1 log PFU/mL in 2 days, 1.3 log PFU/mL in 7 days, 2.35 log PFU/mL in 14 days and to undetectable levels in 21 days at refrigeration. It can be seen that greater reduction was obtained for high titer FCV-F9 with PJ at RT in 1 h (1.20 log PFU/mL) than was obtained at refrigeration in 2 days. A similar trend was reported for the survival of MNV-1 in PJ. Reductions of 0.33, 0.85, 1.8, and 2.02 log PFU/mL were obtained for MNV-1 when inoculated in PJ and stored at refrigeration for 2, 7, 14, and 21 days, respectively. The reductions obtained for MNV-1 with PJ at refrigeration temperature in 2 and 7 days are less than that caused by the treatment with PJ for 1 h at RT. Horm et al. (2012) also evaluated the survival of human norovirus surrogates (MNV-1, FCV-F9 and MS2) in blueberry juice at refrigeration (4°C). FCV-F9 (initial titer of ~5 log PFU/mL) and MS2 (initial titer of ~6 log10 PFU/mL) were found to be undetectable in BJ
after 1 and 7 days, respectively. MNV-1 (initial titer of $\sim 4$ log PFU/ml) was observed to be reduced by 1.95 log PFU/mL only after 21 days. However, when tested in BJ at 37°C, FCV-F9 (initial titer of $\sim 5$ log PFU/mL) was reduced to undetectable levels within 3 h (Joshi et al., 2014). This suggests that the activity of bioactive compounds present in juices responsible for reducing viral titers of FCV-F9 and MNV-1 is dependent on the temperature of incubation, with greater reductions obtained at higher temperatures. The results obtained in the present study with CJ support this observation, where HAV and AiV retained infectivity for 21 days in CJ and MJ at refrigeration temperature (4°C) in comparison to reduction to undetectable levels in 2 h and 15 min, respectively in CJ at 37°C (Sewlikar et al., a and b, Manuscript in preparation).

5. Conclusion

The data reported in this study will help in assessment of risk associated with consumption of contaminated cranberry-based juices. This data will also help to increase awareness of the survival and transmission routes of these viruses during storage of cranberry based juices.
References


Appendix

Tables:

Table 4.1. Reduction of Aichi virus (AiV) in cranberry juices at refrigeration (4°C) over storage of 3 weeks.

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>PBS</th>
<th>100% juice with cranberry (MJ)</th>
<th>Cranberry juice cocktail (CJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered titer (log PFU/ml)</td>
<td>Reduction (log PFU/ml) compared to the day 0 control</td>
<td>Recovered titer (log PFU/ml)</td>
</tr>
<tr>
<td>0</td>
<td>5.69±0.06bc</td>
<td>0</td>
<td>5.69±0.06a</td>
</tr>
<tr>
<td>1</td>
<td>5.74±0.10b</td>
<td>-0.05±0.04</td>
<td>4.97±0.12b</td>
</tr>
<tr>
<td>2</td>
<td>5.74±0.10b</td>
<td>-0.05±0.04</td>
<td>4.94±0.15b</td>
</tr>
<tr>
<td>3</td>
<td>5.59±0.04bd</td>
<td>0.1±0.02</td>
<td>4.71±0.19bc</td>
</tr>
<tr>
<td>4</td>
<td>5.40±0.07cd</td>
<td>0.29±0.01</td>
<td>4.43±0.13cd</td>
</tr>
<tr>
<td>5</td>
<td>6.05±0.05a</td>
<td>-036±0.01</td>
<td>4.29±0.16d</td>
</tr>
<tr>
<td>6</td>
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<td>0.18±0.02</td>
<td>4.47±0.12cd</td>
</tr>
<tr>
<td>7</td>
<td>5.32±0.09d</td>
<td>0.37±0.03</td>
<td>3.85±0.30e</td>
</tr>
<tr>
<td>14</td>
<td>5.32±0.10d</td>
<td>0.37±0.04</td>
<td>3.87±0.28e</td>
</tr>
<tr>
<td>21</td>
<td>5.10±0.32e</td>
<td>0.59±0.26</td>
<td>3.39±0.24f</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
Different superscript letters denote significant differences (p < 0.05), when compared across the columns
Table 4.2. Reduction of hepatitis A virus (HAV) in cranberry juices at refrigeration (4°C) over storage of 3 weeks.

<table>
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<tr>
<th>Days of storage</th>
<th>PBS</th>
<th>100% juice with cranberry (MJ)</th>
<th>Cranberry juice cocktail (CJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered titer (log PFU/ml)</td>
<td>Reduction (log PFU/ml) compared to the day 0 control</td>
<td>Recovered titer (log PFU/ml)</td>
</tr>
<tr>
<td>0</td>
<td>6±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>6±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1</td>
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<td>0.06±0.07</td>
<td>5.33±0.04&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>6.11±0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-0.11±0.08</td>
<td>5.66±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>6.04±0.16&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>-0.04±0.15</td>
<td>5.73±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>5.89±0.22&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.11±0.21</td>
<td>5.08±0.32&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
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<td>0.28±0.21</td>
<td>5.54±0.13&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>5.97±0.14&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.03±0.13</td>
<td>5.19±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>21</td>
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<td>0.04±0.12</td>
<td>4.91±0.11&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
Different superscript letters denote significant differences (p < 0.05), when compared across the columns
Figures:

![Graph](image1)

**Figure 4.1.** Reduction of Aichi virus (AiV) in cranberry juices at refrigeration.

![Graph](image2)

**Figure 4.2.** Reduction of hepatitis A virus (HAV) in cranberry juices at refrigeration.
Chapter V

Chemical inactivation of murine norovirus, hepatitis A virus and Aichi virus
Chemical inactivation of murine norovirus, hepatitis A virus and Aichi virus

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Running Head: Chemical sanitizers to control foodborne viruses

To be submitted to: Food Microbiology

Key words: Norovirus, hepatitis A virus, Aichi virus, chemical sanitizers, inactivation

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Highlights

- Improved sanitizers to prevent the spread/transmission of foodborne viruses are needed
- Chemical sanitizers were tested against murine norovirus, hepatitis A virus, and Aichi virus
- Tested sanitizers required varied contact times for foodborne virus inactivation
Abstract

Human enteric viruses (noroviruses, hepatitis A virus (HAV), and Aichi virus (AiV)) are increasingly associated with foodborne outbreaks. There is a growing need for potent and cost-effective strategies for their control. The objective of this study was to determine the effect of commercially available alkaline (AlkW, containing calcium hydroxide, sodium lactate, ethanol, lactic acid and water) and levulinic acid-sodium dodecyl sulfate (LA-SDS) chemicals as washes against murine norovirus (MNV-1), HAV, and AiV. AlkW or PBS (pH 7.2 as a control) were individually mixed with equal volumes of each virus (~5 log PFU/mL), while 0.5% LA-0.5% SDS, 0.5% LA or 0.5% SDS was mixed at 10:1 at room temperature (RT) for 1, 5, 10, 30, 60, or 180 min. At each time-point, treatments were stopped by 10-fold dilution in cell-culture media containing 10% fetal bovine serum. Viral infectivity was determined using standard plaque assays. Data from triplicate treatments were statistically analyzed using Tukey’s test and ANOVA (p<0.05). AlkW could reduce MNV-1, AiV and HAV to undetectable levels (reduction of ~3 log PFU/mL) within 5, 10 and 30 min, respectively. LA-SDS (0.5% each) reduced AiV to undetectable levels in 5 min with no effect against HAV over 3 h. Overall, AlkW was effective against HAV, MNV-1 and AiV, while LA-SDS showed the highest effect against AiV within the shortest time. The available chemical sanitizers/washes required varied contact times for reduction of the tested foodborne viruses.
1. Introduction

Human noroviruses (HNoV) and hepatitis A virus (HAV) continue to be causes of human health concern from a food safety standpoint. Approximately 59% of the estimated 9.4 million foodborne illnesses cases that occur annually in the United States are caused by HNoV (Scallan et al., 2011). Between the years 1993 and 2011, more than 900 reports of laboratory-confirmed HNoV outbreaks have been reported (Matthews et al., 2012). Although a 90% decrease in HAV cases (mostly attributed to vaccination of children and people at risk for Hepatitis A) has been observed in the US over the last 20 years, there are still an estimated 2,700 new cases of HAV illness per year (CDC, 2014). A multistate (10 states) outbreak of HAV (first in ~10 years) that occurred in the United States in May 2013 resulted in 165 illnesses, 69 hospitalizations, 2 cases of fulminant hepatitis and 1 liver transplant case that was traced back to consumption of frozen pomegranate arils (imported from Turkey) (Collier et al., 2014). Collier et al. (2014) mentioned that decreased immunity in some adults coupled with import of foods is an important factor for such outbreaks. Aichi virus (AiV) is emerging as a foodborne pathogen causing gastroenteritis worldwide. Water and shellfish harvested from water bodies contaminated with sewage are the primary contamination sources for AiV (Yamashita et al., 2000 Ambert-Balay et al., 2008; Le Guyader et al., 2008). AiV has been detected all over the world, in human stool samples in Japan, Germany, Brazil, Bangladesh, Thailand, Vietnam, France, Tunisia, Hungary, China and Finland (Yamashita et al., 1991, 1993; Oh et al., 2006; Pham et al, 2007; Ambert-Balay et al., 2008; Sdiri-Loulizi et al., 2008; Reuter et al., 2009; Yang et al., 2009; Kaikkonen et al., 2010). Given the fecal-oral transmission route for these three viruses, food contact surfaces in addition to food products could be contaminated by infected food handlers. Chemical sanitizers/disinfectants as washes are cost-effective approaches for the control of foodborne pathogens on contaminated surfaces. Ozone,
chlorine, organic acids (tannic acid, lactic acid, per oxyacetic acid), alcohols, benzalkonium chloride, glutaraldehyde, hydrogen peroxide, trisodium phosphate, potassium monosulfate and sodium metasilicate have been evaluated for their antibacterial and antiviral properties (Allwood et al., 2004; Gehrke et al., 2004; Su and D’Souza, 2011a and 2012; Doltree et al., 1999; Moadab et al., 2001; Fraisse et al., 2011; Kim et al., 1999; Li et al., 2002; Lindsay, 1985; Anipsitakis et al., 2008; USDA FSIS, 2011). Alternative generally recognized as safe (GRAS) sanitizers are also being researched for improved efficacy.

Levulinic acid (LA) and sodium dodecyl sulfate (SDS) are GRAS substances that could be used as a synthetic flavoring and adjuvant (21 CFR 172.515) and multipurpose additive (21 CFR 172.822) respectively. Cannon et al. (2012) reported that a combination of 0.5% levulinic acid and 0.5% SDS (pH 3.8) when combined with murine norovirus (MNV-1) or feline calicivirus (FCV-F9) at 10:1 ratio resulted in reductions of 3 to 4.21 log PFU/mL within 1 min. When inoculated on stainless steel, MNV-1 reduction of >1.50 log PFU/mL after 1 min and >3.3 log PFU/mL after 5 min of exposure to a liquid or foaming solution of 5% levulinic acid plus 2% SDS was obtained. The effect of this combination was found to be unaffected by the presence of up to 10% of organic matter. However, the mechanism of action of this sanitizer is not known. The application of LA and SDS wash against HAV or AiV has not yet been reported in literature, and this research would help determine the spectrum of antiviral activity of LA-SDS combinations.

Many other chemical-based chemicals are used as antibacterial washes and sanitizers that are available commercially for use in the food processing industry. An alkaline wash (AlkW, pH 14) consisting of a mixture of calcium hydroxide, sodium lactate, ethanol, lactic acid and water has shown to be a potent disinfectant against bacterial pathogens. The primary objective of this
study was to determine the effect of commercially available chemicals (an alkaline wash, and a 0.5% LA-0.5% SDS wash) against the reduction of HAV, MNV-1 and AiV titers.

2. Materials and Methods

2.1. Viruses and cell lines

MNV-1 was received as a gift from Dr. Skip Virgin (Washington Univ., St Louis, MO) and host RAW 264.7 cells were obtained from the University of Tennessee at Knoxville (UTK). HAV (strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were obtained from our collaborator, Dr. Kalmia Kniel (University of Delaware). AiV was kindly provided by Dr. David Kingsley (USDA ARS, Delaware) and Vero cells were used as their host.

2.2. Propagation of viruses

FRhK4 cells and RAW 264.7 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone Laboratories, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY). Vero cells were grown in DMEM with 2% FBS and 1% Anti-Anti and incubated in an atmosphere containing 5% CO₂ at 37°C in 175 cm² flasks as described earlier (Su et al., 2010a; Fino and Kneil, 2008). Viral stocks of HAV, MNV-1 and AiV for the study were made by inoculating HAV, MNV-1 and AiV stocks onto monolayers of host FRhK4, RAW 264.7 and Vero cells, respectively and incubation under 5% CO₂ until >90% cell lysis. The viral suspensions obtained were freeze-thawed and centrifuged for 10 min at 5000 x g. The supernatants were then filtered through 0.2 µm membrane filters, aliquoted and stored at -80°C until use as described earlier (Su et al., 2010a).
2.3. Infectious plaque assays

Standard protocols were followed for HAV, MNV-1 and AiV plaque assays as described earlier (Su and D’Souza, 2011b; Fino et al., 2008). FRhK4, RAW 264.7 or Vero cells were infected with 0.5 ml of treated or control samples (and their serial dilutions) of HAV, MNV-1 or AiV respectively and incubated for 2 to 3 h to allow for infection. Viruses were then aspirated and the cells were overlaid with 2 ml complete DMEM containing 0.75% agarose (for MNV-1 and AiV) or 1% agarose (for HAV) and incubated at 37\(^\circ\)C under 5\% CO\(_2\). After an incubation of three days for MNV-1 and AiV and eight days for HAV, the cell monolayers were stained using neutral red and further incubated at 37\(^\circ\)C until plaques were visible. Plaques were then enumerated.

2.4. Determination of the antiviral effects of commercially available chemicals on HAV, MNV-1 and AiV

A wash containing calcium hydroxide, sodium lactate, ethanol, lactic acid and water (AlkW, pH 14), calcium hydroxide (1\% w/v, pH 14, as a control), sodium lactate (2.5\%, pH 4.6, as a control), lactic acid (2\%, pH 1.8, as a control), or PBS (as a control) were individually mixed with equal volumes of each virus (titer of ~5 log PFU/mL of HAV, MNV-1 or AiV) and incubated at room temperature (RT) for 1, 5, 30, 60, or 180 min. Levulinic acid (LA, 0.5-0.75\%) with sodium dodecyl sulfate (SDS, 0.5\%) (LA with SDS), PBS (as a control), LA (0.5-0.75\%, as a control) or SDS (0.5\%, as a control) were mixed in 10:1 ratio with each virus (titer of ~5 log PFU/mL of HAV, MNV-1 or AiV) and incubated at room temperature (RT) for 1, 5, 30, 60, or 180 min (Cannon et al., 2012). At various time points, treatments were stopped by 10-fold serial dilution in DMEM containing 10\% FBS. Viral infectivity was then determined using standard plaque assays.
2.5. Statistical analysis

A completely randomized design with sampling was used. Each experiment was run in duplicates (samples) and replicated thrice. Analysis of variance was conducted with Statistical Analysis Software (SAS, version 9.2, SAS Institute, Cary, NC, USA) and Tukey’s test at p<0.05 to determine if any statistically significant difference exists between the recovered titers for the treatments and controls.

2.6. Transmission electron microscopy (TEM) analysis

With an aim to determine the mechanism of action of AlkW and LA-SDS on HAV, AiV and MNV-1 structure, TEM studies were carried out at the UT-Knoxville Advanced Microscopy and Imaging Center following the protocol of Su et al. (2010b). HAV, AiV or MNV-1 was treated with AlkW, LA-SDS or PBS for times that gave the maximum reduction for TEM analysis. AlkW and LA-SDS were also used for analysis to understand the background in treated samples. Ten-µl of each sample was then applied to a freshly glow discharged, formvar and carbon coated copper grid (Electron Microscopy Sciences, Hatfield, PA). Excess sample was removed after 1 min. The grid with sample was then stained with uranyl acetate for 1 min, excess stain removed and the grid allowed to dry. The stained viruses were viewed using a 200kV Zeiss LIBRA 200MC.

3. Results

3.1. Effect of AlkW and LA-SDS on AiV

Results for the reduction of AiV with AlkW and LA-SDS are shown in Table 5.1 and Table 5.2. AiV titers were reduced to undetectable levels after 10 min with AlkW, while sodium lactate (2.5%, pH 4.6) and lactic acid (2%, pH 1.8) controls did not show any reduction. Calcium hydroxide (1%) was found to reduce AiV by 0.73±0.02 log PFU/mL in 10 min. LA-SDS (0.5%
each) reduced AiV to undetectable levels within 5 min, with $1.92 \pm 0.17$ log PFU/mL reduction in 1 min. LA (0.75%) + SDS (0.5%) was tested but did not enhance reduction when compared to LA-SDS (0.5% each) (data not shown). No reductions were obtained for AiV with the 0.5% LA (pH 3.0), 0.75% LA or 0.5% SDS (pH 7.0) controls.

### 3.2. Effect of AlkW and LA-SDS on HAV

AlkW reduced HAV titers to undetectable levels in 30 min. Similar to AiV, sodium lactate (2.5%, pH 4.6) and lactic acid (2%, pH 1.8) controls did not cause any reduction HAV in 30 min. However, calcium hydroxide (1%) reduced HAV by $2.35 \pm 0.03$ log PFU/mL in 30 min. The LA-SDS (0.5% each) and 0.75% LA + 0.5% SDS washes showed no effect on reduction of HAV titers over 30 min (Data not shown).

### 3.3. Effect of AlkW on MNV-1

AlkW and calcium hydroxide (1%) reduced MNV-1 to undetectable levels in 5 min (Table 5.4) with no significant reduction obtained in 5 min with the sodium lactate (2.5%, pH 4.6) and lactic acid (2%, pH 1.8) controls. LA-SDS was not studied against MNV-1 as it was reported earlier by Cannon et al., 2012.

### 4. Discussion

In this study, AiV was found to be more susceptible to reduction by chemical sanitizers than MNV-1 and HAV, and HAV was observed to be the sturdiest among the viruses tested. The LA-SDS used in this study was not as effective as the conventionally used sanitizers/disinfectants in HAV reduction. AlkW caused reductions of $1.15 \pm 0.11$, $1.74 \pm 0.16$, $2.12 \pm 0.12$ and $\geq 2.85 \pm 0.18$ PFU/mL for HAV in 5, 10, 15 and 30 min, respectively. Croci et al. (2002) observed that when cut lettuce, carrot and fennel were washed using potable water for 5 min minimal reductions in the
range of 0.1 and 1 log were obtained for HAV. Li et al. (2002) reported that complete inactivation of HAV was obtained in 30 min using chlorine at 10 or 20 mg/L. Chlorine dioxide at 7.5 mg/L caused complete reduction (~6 log) of HAV in 10 min (Li et al., 2004). Reductions of 1.7 log were obtained for HAV when tomatoes, strawberries and lettuce were treated with 20 ppm (20 mg/L) chlorine (Casteel et al., 2008). Greater than 3 log reduction (TCID$_{50}$ assays were used) was obtained for HAV when treated with 0.10% glutaraldehyde for 30 min and with 0.5% glutaraldehyde in 3 min (Passagot et al., 1987). Mbithi et al. (1990) reported 99.9% reduction of HAV on stainless steel discs using 2% glutaraldehyde in 1 min. Reduction of 7 log for HAV after 5 min was reported when treated with a 3,000 ppm (3,000 mg/L) disinfectant solution consisting of 5% glutaraldehyde and 10% quaternary ammonium but only 3 log reduction when treated on surfaces (Jean et al., 2003). HAV was reduced by 5 log using 3000 ppm (3,000 mg/L) of sodium hypochlorite at 22°C for 5 min (Jean et al., 2003). Use of a peroxycetic acid-based biocide (100 ppm) for washing lettuce did not result in significant reduction of HAV (Fraisse et al., 2011). AlkW could hence be considered for use as a produce wash to reduce contamination with HAV. However, the efficacy of AlkW on produce and in presence of organic matter needs to be tested as well as effects on sensory and quality attributes.

The LA-SDS combination effectively reduced viral titers of AiV but not HAV. Cannon et al. (2012) reported reductions of 3 to 4.21 log PFU/mL for MNV-1 and FCV-F9 within 1 min of treatment with LA-SDS (0.5% each). Liquid or foaming solutions containing 5% LA+2% SDS were shown to be effective against MNV-1 on stainless steel with reduction of >1.50 log PFU/mL after 1 min and >3.3 log PFU/mL after 5 min, with the effect being unaltered by the presence of up to 10% of organic matter (Cannon et al., 2012). Predmore and Li (2011) observed that the reduction of MNV-1 on produce was enhanced when a combination of surfactants (including SDS)
was used with chlorine at 200 ppm (200 mg/L). They suggested that SDS helped detach the virus from the surface of the produce and hence made the viral particles available for the action of the antiviral compound. However, Cannon et al. (2012) observed no difference in reduction when stainless steel discs contaminated with HNoV were subjected to static treatment with water or water containing 2% SDS. Their results also suggest that the antiviral action is dependent on the pH of the sanitizer because lower reduction was obtained for MNV-1 when pH of the sanitizer was increased to 4.0 from 2.8. When MNV-1 was incubated in buffer at pH 2.0 (100 mM citrate) only minimal reduction was obtained even after exposure for 2 h (Cannon et al., 2006). The authors suggested that the sanitizer targets the capsid of the viral particles. Cromeans et al. (2014) reported that AiV exhibited greater resistance than MNV-1 to pH, heat, alcohol, chlorine and high hydrostatic pressure treatments. Hence, the effect of the AlkW and LA-SDS against AiV is noteworthy. However, as mentioned above, for use as a produce wash, the presence of organic matter and the color/flavor that could be imparted onto the fresh produce are the factors that need to be considered.

AlkW was found to work against MNV-1, HAV as well as AiV with the fastest effect obtained against AiV. AlkW contains a mixture of calcium hydroxide, sodium lactate, ethanol, lactic acid and water. Calcium hydroxide (1%) has shown to inactivate low pathogenic avian influenza virus on a metal surface effectively after 10 min (Alphin et al., 2009). Calcium hydroxide (pH 9.5 to 10.5) treatment of wastewater resulted in a 2 log PFU/mL reduction of poliovirus (type I, Sabin) (Sattar and Ramia, 1978). Calcium hydroxide (pH 11.2) was also reported to reduce bacteria, enteric viruses, and coliphages in wastewater with the pH being regarded as the key factor for the antimicrobial activity (Grabow et al., 1978). The disinfection caused by alkali is attributed to their ability to denature proteins, even in the presence of organic matter (Agriculture and
Resource Management Council of Australia and New Zealand, 2000). Although the antiviral effects of sodium lactate have not been reported, its antibacterial activity has been studied. When dipped in 2.5% (w/v) aqueous solution of sodium lactate, the growth of *Pseudomonas* spp., hydrogen sulfide-producing bacteria, lactic acid bacteria, and *Enterobacteriaceae* on fresh salmon slices (Sallam, 2007) and on sliced poultry sausage when stored under refrigeration was inhibited (Cegielska-Radziejewska and Pikul, 2004). Using suspension tests, Straube et al. (2011) observed that 0.3% and 0.4 % D, L-lactic acid with pH 3.4-3.5 and 3.2-3.3 respectively caused significant reduction of FCV-F9 titers at 20°C after 7 days (Straube et al., 2011). The antimicrobial effect exerted by organic acids is generally attributed to the lowering of pH of the environment (In et al., 2013). Other factors that alter this antimicrobial activity include the percentage of undissociated acid, degree of branching and chain length (Doores, 1993). The effect of AlkW could hence be synergistic effect of the individual components. Sodium lactate (2.5%, pH 4.6), lactic acid (2%, pH 1.8) and calcium hydroxide (1%, pH 14) were used as controls in this study for AlkW to understand the effect of individual components on viral titer reduction. The exact composition of AlkW is not known and hence effective concentrations (from literature) of individual components were selected as controls. Calcium hydroxide was found to cause significant reductions for MNV-1, AiV and HAV in 5, 10 and 30 min, respectively while no reduction was obtained with sodium lactate or lactic acid. This suggests that calcium hydroxide could be the most active ingredient in AlkW. However, it must also be noted that for HAV and AiV, the reduction obtained with calcium hydroxide is less than that obtained with AlkW in the same time. This indicates that the antiviral activity of AlkW is due to a synergistic or combined effect of all the individual components.

TEM was used to elucidate the mechanism of action of the tested chemicals against MNV-1, HAV and AiV with an aim to determine any visual changes in the treated viral particle structure.
However, TEM studies of viral particles treated with the chemicals did not lead to any conclusive results due to interference in images caused by the treatment matrix. The mechanism of action of these chemicals hence remains unclear.

5. Conclusion

The commercially available chemicals in AlkW and LA-SDS could be used in hurdle approaches for their antiviral activity against the tested viruses. However, the efficacy of these washes against the tested viruses on model surfaces with organic load needs to be evaluated.

Acknowledgements

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References


of the picornavirus family associated with gastroenteritis in humans. Journal of Clinical Microbiology 38, 2955–2961.


Appendix

Tables

Table 5.1. Reduction of Aichi virus (AiV, initial titer of ~5 log PFU/mL) by an alkaline wash (AlkW, pH 14)

<table>
<thead>
<tr>
<th>Treatment used</th>
<th>Time of incubation/treatment (min)</th>
<th>Recovered titer in PBS (log PFU/mL)</th>
<th>Recovered titer in treatment (log PFU/mL)</th>
<th>Reduction (log PFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkW</td>
<td>5</td>
<td>4.32±0.04(^a)</td>
<td>3.04±0.25(^c)</td>
<td>1.28±0.21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.46±0.20(^{ab})</td>
<td>ND(^d)</td>
<td>≥2.46±0.20</td>
</tr>
<tr>
<td>Calcium hydroxide (1%)</td>
<td>10</td>
<td>4.66±0.19(^a)</td>
<td>3.92±0.18(^b)</td>
<td>0.73±0.02</td>
</tr>
<tr>
<td>Sodium lactate (2.5%)</td>
<td>10</td>
<td>4.66±0.19(^a)</td>
<td>4.51±0.16(^a)</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Lactic acid (2%)</td>
<td>10</td>
<td>4.66±0.19(^a)</td>
<td>4.68±0.28(^a)</td>
<td>None</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation.
ND: None detected (Detection limit of plaque assay: 2 log PFU/mL).
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns.
Table 5.2. Reduction of AiV by the combination of Levulinic acid (LA, 0.5%) and sodium dodecyl sulfate (SDS, 0.5%)

<table>
<thead>
<tr>
<th>Time of incubation/treatment (min)</th>
<th>Recovered Titer (log PFU/mL) from initial ~5 log PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LA (0.5%) + SDS (0.5%) (pH 3.8)</td>
</tr>
<tr>
<td>1</td>
<td>2.79±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
ND: None detected (Detection limit of plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Table 5.3. Reduction of hepatitis A virus (HAV, initial titer of ~5 log PFU/mL) by an alkaline wash (AlkW, pH 14)

<table>
<thead>
<tr>
<th>Treatment used</th>
<th>Time of incubation/treatment (min)</th>
<th>Recovered titer in PBS control (log PFU/mL)</th>
<th>Recovered titer in treatment (log PFU/mL)</th>
<th>Reduction (log PFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkW</td>
<td>5</td>
<td>4.49±0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.34±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.15±0.11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.49±0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.75±0.35&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.74±0.16</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.42±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3&lt;sup&gt;fg&lt;/sup&gt;</td>
<td>2.12±0.12</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.85±0.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;g&lt;/sup&gt;</td>
<td>≥2.85±0.18</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4.92±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;g&lt;/sup&gt;</td>
<td>≥2.92±0.16</td>
</tr>
<tr>
<td>Calcium hydroxide (1%)</td>
<td>30</td>
<td>4.80±0.13&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.45±0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.35±0.03</td>
</tr>
<tr>
<td>Sodium lactate (2.5%)</td>
<td>30</td>
<td>4.80±0.13&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>4.81±0.12&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>None</td>
</tr>
<tr>
<td>Lactic acid (2%)</td>
<td>30</td>
<td>4.80±0.13&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>4.81±0.21&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>None</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
ND: None detected (Detection limit of plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Table 5.4. Reduction of murine norovirus (MNV-1, initial titer of ~5 log PFU/mL) as a surrogate for human noroviruses by an alkaline wash (AlkW, pH 14)

<table>
<thead>
<tr>
<th>Treatment used</th>
<th>Time of incubation/treatment (min)</th>
<th>Recovered titer in PBS control (log PFU/mL)</th>
<th>Recovered titer in treatment (log PFU/mL)</th>
<th>Reduction (log PFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkW</td>
<td>5</td>
<td>4.95±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≥2.95±0.14</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.35±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≥3.35±0.05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.23±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≥3.23±0.22</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4.92±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≥2.92±0.19</td>
</tr>
<tr>
<td>Calcium hydroxide (1%)</td>
<td>5</td>
<td>4.95±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>≥2.95±0.14</td>
</tr>
<tr>
<td>Sodium lactate (2.5%)</td>
<td>5</td>
<td>4.95±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.92±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>Lactic acid (2%)</td>
<td>5</td>
<td>4.95±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.88±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07±0.05</td>
</tr>
</tbody>
</table>

Data are reported as Mean±Standard deviation
ND: None detected (Detection limit of plaque assay: 2 log PFU/mL)
Different superscript letters denote significant differences (p < 0.05), when compared across rows and columns
Conclusions

This research focused on evaluating the use of natural and sustainable products, namely cranberry juice (CJ) and its bioactive components, cranberry proanthocyanidins (C-PAC), as preventive or therapeutic options against enteric foodborne illnesses caused by human noroviruses (HNoVs), hepatitis A virus (HAV), and Aichi virus (AiV). Additionally, the survival of these viruses in juices at refrigeration was monitored to determine transmission routes and potential risk of transmission from cross-contamination and storage. Finally, chemical sanitizers to control and prevent their transmission on contact surfaces were evaluated.

The antiviral effects (including the mechanism of action) of CJ and C-PAC on HAV, AiV and the HNoV surrogate, murine norovirus (MNV-1) at 37°C was demonstrated. CJ was found to reduce MNV-1 and HAV to undetectable levels after 6 h and 2 h, respectively at 37°C, while C-PAC (final concentrations of 0.5 and 1 mg/mL) reduced both viruses to undetectable levels after 5 min. CJ reduced AiV to undetectable levels in 15 min while C-PAC at final concentrations of 0.5 and 0.06 mg/mL reduced AiV to undetectable levels within 1 min and 5 min, respectively. C-PAC was found to decrease AiV infection by preventing viral binding/adsorption to host cells. TEM studies for HAV and AiV showed damage of CJ-treated viral capsids, without conclusive results with C-PAC. These bioactives can be further studied as preventive and therapeutic options for HAV, HNoV and AiV illness especially in scenarios where vaccines are unavailable or cost-prohibitive. The results of this research provide the preliminary data and platform for clinical trials that could be undertaken to evaluate the effects of CJ and C-PAC in vivo. Future research involves the evaluation of the antiviral effects of CJ, C-PAC and encapsulated forms of C-PAC on HAV, MNV-1 and AiV in food matrices and simulated gastric fluids. However, before any health claims
can be made, *in vivo* animal feeding studies and clinical trials with CJ and C-PAC are needed along with approval by appropriate regulatory agencies.

Survival of HAV and AiV in cranberry-based juices at refrigeration temperatures, showed that AiV and HAV could retain infectivity at refrigeration in the two cranberry-based juices (cranberry juice cocktail and 100% juice with cranberry) over 21 days. The data obtained from this research will contribute towards assessment of risk associated with the consumption of cranberry-based juices contaminated with these viruses.

Chemical washes were tested for their antiviral activity at room temperature in suspension tests. An alkaline wash (AlkW) was found to reduce MNV-1, AiV and HAV to undetectable levels (reduction of ~3 log PFU/mL) within 5, 10, and 30 min, respectively. Levulinic acid-sodium dodecyl sulfate (LA-SDS, 0.5% each) combination wash reduced AiV to undetectable levels in 5 min with no effect against HAV over 3 h. AlkW was found to be effective against all the three viruses. This study provides useful information for the use of AlkW as an antiviral agent against enteric viruses. Further research on the efficacy of all these washes against the three foodborne viruses using carrier tests on model food contact surfaces with organic load to simulate real-world scenarios needs to be evaluated.

Overall, this research contributes towards enhancing the awareness of available prevention and control strategies for foodborne viral illnesses caused by HAV, HNoV and AiV.
Vita

Snigdha Nitin Sewlikar was born in Jodhpur, Rajasthan, India on August 4, 1989. She grew up in Loni, Maharashtra, India and graduated from Padmashree Vikhe Patil Junior College in 2006. She continued her education at the Institute of Chemical Technology, Mumbai, India where she earned a B.Tech. Degree majoring in Food Engineering and Technology. She later earned an M.S degree in Food Science and Technology with a concentration in Food Microbiology from the University of Tennessee, Knoxville. Snigdha will pursue a career in the food processing industry concentrating in Food Safety and Quality Assurance.