

**Survival of Human Norovirus Surrogates
In Juices and their Inactivation Using
Novel Methods**

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ABSTRACT

Human noroviruses (HNoV) have been implicated in gastrointestinal outbreaks associated with fruits and juices. Virus survival studies and non-thermal inactivation technologies that maintain nutritional and sensory attributes of juices are needed. In the absence of culturable HNoV, cultivable murine norovirus (MNV-1) and feline calicivirus (FCV-F9) surrogates are used. This study aimed to (1) determine HNoV surrogate survival in juices (orange juice (OJ) and pomegranate juice (PJ)), juice blends (OJ-PJ), and milk over 21 days at refrigeration; (2) determine high pressure homogenization (HPH) effects alone on HNoV surrogates in milk, OJ, OJ-PJ blend or with an emulsifier (lecithin) for FCV-F9 and MNV-1 in milk and OJ; (3) determine HNoV surrogate survival in blueberry juice (BJ) after 21 days at refrigeration and HPH inactivation. Our results showed no reduction of MNV-1 after 21 days in OJ and milk, but 1.4 log reduction in PJ and complete reduction after 7 days in OJ-PJ. FCV-F9 was completely reduced after 14 days in OJ and PJ; by ~3 logs after 21 days in milk, and completely reduced after 1 day in OJ-PJ. MS2 was reduced by ~1.28 log in OJ and <1 log in milk, PJ, or OJ-PJ after 21 days. At 300 MPa HPH, FCV-F9 in milk and OJ was reduced by $\geq 4 \log_{10}$ PFU/ml and MNV-1 in milk by ~1.3 log and negligibly in OJ. In PJ-OJ, FCV-F9 was completely reduced at 250 MPa. At 300 MPa in PJ-OJ, MNV-1 and MS2 showed 1.78 and 4.1 $\log_{10}$ PFU/ml reduction. FCV-F9 showed complete reduction in OJ or milk combined with lecithin at 300 MPa. Thus, juice-blends and lecithin aid in viral reduction by HPH. In BJ, FCV-F9 was undetectable after 1 day while MNV-1 showed minimal reduction after 21 days and MS2 was undetectable after 7 days. FCV-F9, MNV-1, and MS2 survived after 21 days in PBS. FCV-F9 and MS2 showed complete reduction after minimal homogenization pressures in BJ. Overall, milk was the most protective media for HPH and virus survival. These viral survival patterns that aid in foodborne viral transmission can be used in quantitative viral risk assessment studies along with HPH to prevent/control virus outbreaks.

TABLE OF CONTENTS

CHAPTER	PAGE
I. Literature Review.....	1
Human Enteric Viruses.....	1
Processing Methods for Foodborne Viral Inactivation	2
Foodborne Virus Survival	26
Natural Plant Polyphenols for Foodborne Viral Inactivation.....	29
References	37
Appendix	55
II. Survival of Human Norovirus Surrogates in Milk, Orange and Pomegranate Juice, and Juice Blends at Refrigeration (4°C)	78
Abstract	79
Introduction	80
Materials and Methods	82
Viruses and Cell Lines	82
Virus Propagation and Preparation	82
Infectious Plaque Assays	83
MS2 Plaque Assay	83
Survival Study.....	84
Statistical Analysis	84
Results	85
Survival Characteristics of FCV-F9 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C).....	85
Survival Characteristics of MNV-1 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C).....	85
Survival Characteristics of MS2 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C)	86
Survival Characteristics of FCV-F9, MNV-1, and MS2 in Pomegranate Juice and Pomegranate Polyphenol Diluted in PBS over Time at 4°C	86
Survival Characteristics of FCV-F9, MNV-1, and MS2 in Blends of Orange Juice and PBS, Orange Juice and Pomegranate Juice or Orange Juice and Pomegranate Polyphenols	87
Discussion	88
Acknowledgements	97
References	98
Appendix	102
III. Application of High Pressure Homogenization for the Inactivation of Human Norovirus Surrogates in Milk, Orange Juice, and Pomegranate Juice Blends	115
Abstract	116
Introduction	117
Materials and Methods	120
Viruses and Cell Lines	120
FCV-F9 Plaque Assay.....	121
MNV-1 Plaque Assay	121
MS2 Plaque Assay	122
Preparation of Lecithin	122

	HPH Unit and Treatments.....	123
	Statistical Analysis.....	124
	Results	124
	Effect of HPH on FCV-F9 in PBS, Milk, Orange Juice, and Lecithin Combinations	124
	Effect of HPH on MNV-1 in PBS, Milk, Orange Juice, and Lecithin Combinations	125
	Effect of HPH on FCV-F9 and MNV-1 in Juice Combinations	125
	Effect of HPH on MS2 in PBS, Milk Orange Juice, and Pomegranate Juice Combinations	126
	Discussion	126
	Acknowledgements.....	134
	References	135
	Appendix.....	138
IV.	Survival of Human Norovirus Surrogates in Blueberry Juice at Refrigeration (4°C) and their Inactivation in Blueberry Juice using High Pressure Homogenization	143
	Abstract	144
	Introduction.....	145
	Materials and Methods	148
	Viruses and Cell Lines	148
	FCV-F9 Plaque Assay.....	149
	MNV-1 Plaque Assay	150
	MS2 Plaque Assay.....	150
	Survival Study.....	150
	HPH Unit and Treatments	151
	Statistical Analysis.....	151
	Results.....	152
	Survival Trends of FCV-F9, MNV-1, and MS2 in PBS (pH=7.2 and pH=2.77) and Blueberry Juice over 21 Days at Refrigeration temperature (4°C)	152
	Effect of HPH on FCV-F9, MNV-1, and MS2 in Blueberry Juice	153
	Discussion	153
	Acknowledgements.....	159
	References	160
	Appendix.....	164
Vita		169

LIST OF TABLES

TABLE	PAGE
1.1: Foodborne Viral Outbreaks.....	56
1.2: The Effect of Heat Treatment on Foodborne Viruses	58
1.3: Effect of Ultrasound Treatment on Foodborne Microorganisms	60
1.4: Classification of Irradiated Foods	62
1.5: Gamma Irradiation Inactivation of Foodborne Microorganisms.....	63
1.6: Pulsed Electric Field Inactivation of Foodborne Microorganisms.....	66
1.7: Effect of High Intensity Pulsed Light Treatment on Foodborne Microorganisms.....	68
1.8: Effect of High Hydrostatic Pressures on Viruses	70
1.9: Effect of High Pressure Homogenization on Foodborne Microorganisms	72
1.10: The Efficacy of Decontamination Procedures of Fresh Produce to Reduce the Level of Viruses	74
1.11: Dietary Sources of Plant Phenolics	76
1.12: Natural Plant Components, Health Benefits, and Antimicrobial Effects.....	77
2.1: Reduction in Titers of FCV-F9 (~6 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS).....	103
2.2: Reduction in Titers of MNV-1 (~5 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS).	104
2.3: Reduction in Titers of MS2 (~6 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS)	105
2.4: Reduction in Titers of FCV-F9 (~6 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP)	106
2.6: Reduction in Titers of MS2 (~6 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP)	107
2.5: Reduction in Titers of MNV-1 (~5 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP)	108
3.1: Effect of High Pressure Homogenization on Bacteriophage MS2 in PBS (control), Milk, Orange Juice (OJ), OJ Combined with Pomegranate Juice (PJ), OJ Combined with Pomegranate Polyphenols (PP)	139
4.1: Reduction of FCV-F9 (~7 log ₁₀ PFU/ml), MNV-1(~6 log ₁₀ PFU/ml), and MS2 (~7 log ₁₀ PFU/ml) at 4°C over 21 Days in Blueberry Juice (BJ) and Phosphate Buffered Saline (PBS at pH=7.2 and pH=2.77)	165
4.2: Reduction of FCV-F9 (~6 log ₁₀ PFU/ml), MNV-1 (~5 log ₁₀ PFU/ml), and MS2 (~6 log ₁₀ PFU/ml) after High Pressure Homogenization Processing in Blueberry Juice (BJ) and Phosphate Buffered Saline (PBS)	166

LIST OF FIGURES

FIGURE	PAGE
Figure 2.1: Recovery of FCV-F9 from Titer of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days	109
Figure 2.2: Recovery of MNV-1 from Titer of $\sim 5 \log_{10}$ PFU/ml (10^5 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days	110
Figure 2.3: Recovery of MS2 from Titer of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days.....	111
Figure 2.4: Recovery of FCV-F9 at Titers of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days	112
Figure 2.5: Recovery of MNV-1 at Titers of $\sim 5 \log_{10}$ PFU/ml (10^5 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days	113
Figure 2.6: Recovery of MS2 at Titers of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days	114
Figure 3.1: Recovery of FCV-F9 in PBS (control), Milk, Orange Juice (OJ), PBS with Lecithin, Milk with Lecithin, and OJ with Lecithin using High Pressure Homogenization (HPH) Treatments of 0, 100, 200, 250, and 300 MPa	140
Figure 3.2: Recovery of MNV-1 in PBS (control), Milk, Orange Juice (OJ), PBS with Lecithin, Milk with Lecithin, and OJ with Lecithin at 0, 100, 200, 250, and 300 MPa using High Pressure Homogenization (HPH)	141
Figure 3.3: Recovery of MNV-1 and FCV-F9 in PBS (control), Orange Juice (OJ) Combined with Pomegranate Juice (PJ) and OJ Combined with Pomegranate Polyphenols (PP) using High Pressure Homogenization	142
Figure 4.1: Recovery of FCV-F9 (from initial titers of $\sim 7 \log_{10}$ PFU/ml), MNV-1 ($\sim 6 \log_{10}$ PFU/ml), and MS2 ($\sim 7 \log_{10}$ PFU/ml) from Blueberry Juice (BJ) at Refrigeration Temperature (4°C) over 21 Days	167
Figure 4.2: Recovery of FCV-F9 (from initial titers of $\sim 7 \log_{10}$ PFU/ml), MNV-1 ($\sim 6 \log_{10}$ PFU/ml), and MS2 ($\sim 7 \log_{10}$ PFU/ml) from Phosphate Buffered Saline (PBS; pH 7.2 and 2.77) at Refrigeration Temperature (4°C) over 21 Days	168

CHAPTER I

LITERATURE REVIEW

Human Enteric Viruses

Throughout the world, foodborne illnesses are one of the foremost health problems. Foodborne illnesses can be of viral, bacterial, parasitic, or chemical origin. Surprisingly, viruses are currently the major cause of foodborne illness, followed by bacteria and parasites. It is estimated that every year in the United States, 31 pathogens cause 9.4 million foodborne illnesses, 55,961 hospitalizations, and 1,351 deaths (Scallan et al., 2011). Viruses alone reportedly cause an estimated 5.5 million foodborne illnesses (59%), 27% of hospitalizations, and 12% of deaths (Mead et al., 1999; 2000; Scallan et al., 2011). Viruses can be transmitted through the fecal-oral route by contaminated food and water, as well as through person- to- person and contact with contaminated surfaces. Foods at risk, associated with contamination, include shellfish, fresh produce, berry fruits and juices, and other ready-to-eat (RTE) foods that do not undergo further processing (Sair et al., 2002a). Viral contamination of foods occurs often by infected workers with poor hygiene practices, which is preventable. Another source of contamination is from fecally polluted waters that are drained into large bodies of water (oceans) or flow into farmlands, commonly as a result of flooding.

Viruses differ from bacteria in that they are intracellular parasites, needing a host cell to infect and replicate. According to Sair (2000b), virus particles have properties causing them to be environmentally stable and resistant to extreme pH and enzymes, such as in the gastrointestinal tract. Thus, foods are a common vehicle for viral transmission due to the ability of viruses to withstand many food processing and storage conditions. Along with the ability of foodborne

viruses to survive adverse conditions, they reportedly have a low infectious dose, where as few as 10 virus particles can make a person sick (CDC, 2010b). Viruses that are associated with foodborne and waterborne diseases include rotaviruses, human noroviruses, hepatitis A virus, adenoviruses, sapoviruses, astroviruses, Aichi virus (AiV), and other enteroviruses (CDC, 2010b; Hirneisen et al., 2010). Notable foodborne viral outbreaks are reported in Table 1.1. Much research has been done on the taxonomy and properties of foodborne viruses, as well as their transmission, prevention, and inactivation methods through processing and chemical means. This review will provide an update of the current literature on foodborne viruses and novel processing technologies to control outbreaks.

In terms of non-bacterial gastroenteritis cases and foodborne outbreaks, human noroviruses are the most frequently detected pathogen. Norovirus is termed from a 1968 school outbreak that occurred in Norwalk, Ohio. This outbreak was placed on the radar due to its rapid transmission and wide spread nature. Infected school children returned home that resulted in secondary infection of family members that eventually led to an epidemic outbreak. The nomenclature of the virus depended on the progress of scientific understanding; once called *winter vomiting disease*, the name changed to “Norwalk-like virus”, and recently named Norovirus and classified based on morphology and phylogeny (Lopman et al, 2008). Human norovirus initially visualized and identified as a small, round-structured virus in 1972 virus by Kapikian and others, is a member of the *Caliciviridae* virus family, due to its “cup-like” shape (Kapikian et al., 1996; Green et al., 2001; D’Souza et al., 2007). Currently, the *Caliciviridae* family is comprised of four genera based on taxonomy; *Vesivirus*, *Lagovirus*, *Norovirus* (in which human norovirus is included), and *Sapovirus* (D’Souza et al., 2007). Recently, a fifth and sixth genera in the *Caliciviridae* family, named *Nebovirus* and *Recovirus*, have been proposed

that show significantly different properties from the four current genera (Scipioni et al., 2008; Plavsic et al., 2010). Generally, the *Sapoviruses* and *Noroviruses* are the genera most responsible for epidemic gastroenteritis, whereas the *Vesiviruses* and *Lagoviruses* are considered animal caliciviruses and currently appear to pose no human risk (D'Souza et al., 2007).

Human noroviruses are non-enveloped RNA viruses, approximately 27 to 35 nm in diameter, which to-date cannot be cultivated in cell cultures, posing a problem for experimental and foodborne research purposes (Grove et al., 2006). Noroviruses are icosahedral in shape and contain single-stranded positive-sense RNA genomes ~7.5kb in size (Jiang and Estes, 1990). Excluding the 3' end of the genome which contains a poly A tail, the norovirus genome sequence is 7,642 nucleotides in length (D'Souza et al., 2007). This genome contains three open reading frames (ORFs); ORF1, ORF2, and ORF3; which encode structural and non-structural genes (Donaldson et al., 2008). ORF1 comprised the first two-thirds of the genome (nucleotides 146 to 5,359) is over 5 kb in size and encodes a ~200 kDa non-structural polyprotein essential for viral replication (D'Souza et al., 2007; Donaldson et al., 2008). ORF2 (nucleotides 5,346 to 6,935) encodes the 57 kDa structural capsid protein VP1, and is 1.8 kb in size (D'Souza et al., 2007; Donaldson et al., 2008). Lastly, ORF3 (nucleotides 6,938 to 7,573) is 0.6 kb in size and encodes a small 22 kDa structural protein (VP2) theorized to package the genome into virions (D'Souza et al., 2007; Donaldson et al., 2008, Glass et al., 1999). It is speculated that the viral protein (VPg), that is covalently linked to the viral RNA and caps the 5' end, may function in transporting to negative strand synthesis sites (Donaldson et al., 2008).

Complete sequencing of the norovirus capsid gene has allowed for the identification and classification of 40 different virus strains, which are divided into five genogroups (G) based on sequence similarity (Frankhauser et al., 2002; Green et al., 1995; Karst et al., 2003; Oliver et al.,

2003, Donaldson et al., 2008). Genogroup I (GI), GII, and GIV consist primarily of human pathogens, while GIII and GV are bovine and murine infecting viruses, respectively. The genogroups are further numerically divided into genoclusters. The human infecting virus genogroups; GI, GII, and GIV, contain eight, seventeen, and one genoclusters, respectively (Zheng et al., 2006). GI (Norwalk virus) and GII (Snow Mountain virus) account for the majority of human norovirus cases, although the Alpatron and Ft. Lauderdale viruses that are categorized in GIV also cause increased outbreaks (D'Souza et al., 2007; La Rosa et al., 2008). Koopmans and others (2003) found that NoV GII is the most predominant group, accounting for the most outbreak cases in their molecular epidemiologic studies (Koopmans et al., 2003). Chan and others (2006) found that genogroup II (GII) cDNA viral load is ≥ 100 fold higher than GI, due to the higher transmissibility of GII through the fecal-oral route (Chan et al., 2006). The majority of human norovirus (hNoV) outbreaks are caused by GII.4 genocluster and its variants. In 2002, a GII.4 variant, named the Farmington Hill strain, was associated with 80% of acute NoV outbreaks in the United States (Fankhauser et al., 2002). The Hunter strain G II.4 variant was circulating in Australia, Europe, and Asia in 2004, but was replaced by the Sakai (detected in Southeast Asia) and Minerva (detected in United States and the Netherlands) strains in 2006 (Donaldson et al., 2008; Phan et al., 2006; Bull et al., 2006; Kroneman et al., 2006; Siebenga et al., 2007; Okada et al., 2006). In October of 2008, a local university in Los Angeles, California, 30 persons became ill from norovirus, later classified as GII.6 strain Seacroft (CDC 2010a). Although no person or food was found to cause the outbreak, Seacroft strain that had not been found previously in California (CDC 2010a). The human norovirus strains are a worldwide hindrance and continually evolving, where the emerging GII.4 variants are known to cause death in the elderly and immunocompromised (Siebenga et al., 2007)

Food workers are often implicated in human norovirus outbreaks due to the contamination of hands, food, non-food contact surfaces or utensils through fecal transfer or aerosolized vomitus onto food or surfaces. This can lead to infection of workers and patrons in food service establishments (Todd et al., 2008). The high rate and ease of transmission may account for the widespread disease outbreaks in closed settings; such as hospitals, cruise ships, and day-care centers (Widdowson et al., 2005). Human noroviruses possess numerous characteristics that aid their endemic spread including: (1) the low infectious dose; (2) the extended duration of viral shedding, even after infected persons are asymptomatic; (3) the stability of norovirus at a wide range of temperatures (freezing to 60°C); (4) the stability of norovirus at high chlorine concentrations; and (5) the insufficient long-term immunity to norovirus, in which repeated infections and exposures can occur throughout life (Patel et al., 2009; Teunis et al., 2008; Duizer et al., 2004). Norovirus infection is characterized as a self-limiting gastrointestinal infection in humans with symptoms that include nausea, vomiting, diarrhea, and fever (Grove et al., 2006; Cliver et al., 2002; Grohmann et al., 1981). Severe symptoms of non-bloody diarrhea and vomiting can occur, but typically resolve after 2-3 days (Patel et al., 2009). Alarming, viruses can be shed in stool before symptoms occur, up to three weeks after symptoms have abated, and in asymptomatic carriers (D'Souza et al., 2007). Unlike hepatitis A virus infection, individuals do not develop immunity to norovirus infection, making it a recurring threat. The susceptibility of an individual to norovirus infection seems to be a result of acquired immunity and genetic determinants (D'Souza et al., 2007). Persons outside of blood group O and persons lacking a histo-blood group antigen which serves as a receptor for norovirus binding are least susceptible to infection (D'Souza et al., 2007). The most susceptible individuals are children, the elderly, and the immunocompromised, and severe cases may require

hospitalization. In norovirus challenge studies, volunteers did develop a short-lived immunity (lasting 6 to 14 weeks), but, if symptomatic, could be reinfected with the same inoculum 2 to 3 years later (Patel et al., 2009; Wyatt et al., 1974; Parrino et al., 1977; Johnson et al., 1990). A notable restaurant outbreak described by Marks and others (2000), came about when a diner vomited on the floor (Marks et al., 2000). Even though it was quickly cleaned, 52 people later became ill, emphasizing transmission by aerosolized vomit. Although it is unlikely for a vaccination to be developed due to the high cost for a self-limiting illness, findings do suggest that noroviruses are under heavy selective pressure and that the norovirus capsid seems apt to evolve (Lopman, 2008). Human noroviruses, though not cultivable in the laboratory, are analyzed and sequenced using reverse transcriptase-polymerase chain reaction (RT-PCR). PCR assays have the great disadvantage of not being able to distinguish live, infectious virus particles from inactivated ones (Grove et al., 2006; Slomka and Appleton, 1998). The next best hurdle to tackle would be cultivating the human noroviruses in the laboratory, so that they can better be studied.

Human noroviruses are quite resistant to chlorine at low concentrations (0.5-1 mg chlorine/L) but inactivated at concentrations >2 mg chlorine/L (Koopmans et al., 2002). These non-enveloped viruses are less susceptible to adverse environmental conditions, such as reduced pH, and therefore pose a risk in handled or processed foods. Human noroviruses appear sensitive to heat and are inactivated under normal cooking conditions, so foods that have not undergone heat treatment and were handled, such as salads and boxed lunches, are implicated (Grove et al., 2006; Cliver et al., 2002).

The second largest cause of viral enteric gastroenteritis is hepatitis A virus (HAV). HAV is a member of the genus *Hepatovirus* in the *Picornaviridae* family and is transmitted

through the ingestion of fecally contaminated material (Sattar et al., 2000). HAV is a non-enveloped RNA virus, 27-32 nm in diameter, with an icosahedral shaped capsid (D'Souza et al., 2007). The positive-sense RNA genome is linear and ~7.5 kb in size. The capsid consists of three major proteins (VP1, VP2, and VP3) and the genome is divided into three regions, including a large open reading frame (D'Souza et al., 2007). HAV detection typically utilizes reverse transcription (RT) coupled with polymerase chain reaction (PCR) (Kwon et al., 2000). HAV isolates have been grouped into seven genotypes (GI to GVII), where GI, GII, GIII, and GVII are associated with human disease, and GIV, GV, and GVI are simian diseases (D'Souza et al., 2007, Arauz-Ruiz et al., 2001; Robertson et al., 1992). The most prevalent genotype, genotype I, and its sub-genotypes, encompass 80% of human strains (Kokkinos et al., 2010). In America, subgenotype IA is the major HAV of concern, whereas subgenotypes IA and IB circulate in Europe (Kokkinos et al., 2010; Rodrigues et al., 2007).

. Once ingested, HAV illness spans four phases. The incubation period lasts an average of 28 to 30 days, during which the virus is shed from the body, followed by a phase of viral replication in the body without symptoms (D'Souza et al. 2007; Grove et al., 2006). The second phase, prodromal, is characterized by an onset of symptoms including anorexia, vomiting, fatigue, jaundice, and is correlated with the greatest viral excretion in stool (Brundage and Fitzpatrick, 2006). The third phase is characterized by the onset of jaundice and an enlarged liver lasting up to 28 days. During the final phase, symptoms subside and liver function returns to normal. A major outbreak of hepatitis A occurred in Egypt among tourists in 2004, where a total of 351 persons from 9 countries were infected with a single strain after ingesting orange juice (Frank et al., 2007). Later studies revealed the juice was most likely contaminated during the manufacturing process by an infected worker due to unhygienic conditions (Frank et al., 2007).

Within the United States, an outbreak occurred in Pittsburgh, PA in 2003. Green onions imported from Mexico served in a restaurant were the cause of 650 illnesses and 4 deaths (Marler-Clark, 2011). Hepatitis A virus is challenging to study because tissue culture is only available for lab-adapted strains, encouraging the use of PCR techniques to identify and analyze the wild-type virus (Grove et al., 2006).

HAV is among one of the toughest enteric viruses known, withstanding high temperatures (85°C), low pH, saline conditions, freezing, detergents, and acids (Brundage and Fitzpatrick, 2006). Foods can become contaminated in numerous ways; produce items irrigated with fecally contaminated water, shellfish harvested in contaminated waters, and the handling of food items by infected individuals in processing plants and food establishments (Sattar et al., 2000). Foods commonly associated with HAV are shellfish, salads, vegetables, juices, ice-cream, breads, and raw undercooked foods (Sattar et al., 2000). The chemical treatments of formalin and chlorine are effective at inactivating HAV (WHO, 2000). In the United States, 120,000 acute hepatitis A cases were estimated to have occurred from 1987-1997 (Grove et al., 2006; CDC, 2010d). Though the national incidence of HAV has dramatically decreased (92%) since the introduction of the vaccine in 1995, thousands of incidents are reported annually (2,585 cases in 2008) (CDC, 2010c). It is also thought to be greatly underreported due to asymptomatic carriers. Once infected, individuals acquire life-long immunity against HAV, explaining the low number of infections in adults greater than 40 years of age. Hepatitis A vaccination is recommended for persons who are at increased risk for infection, for persons who are at increased risk for complications from Hepatitis A, and for all children at age 1 year (CDC, 2009a). HAVRIX® (manufactured by GlaxoSmithKline) and VAQTA® (manufactured by Merck & Co., Inc) are the two formalin-killed whole virus vaccines available in the United States and licensed by the Food

and Drug Administration (FDA) (D'Souza et al., 2007; CDC, 2009a; Lemon, 1997). The HAV vaccines are two-dose series, which a booster dose is administered 6-12 months after the primary immunization (CDC, 2009a).

Viruses within the *Picornaviridae* family are known as picornaviruses. Along with hepatitis A virus, many other viruses within this large family are invasive to humans. The *Picornaviridae* family consists of five genera: *Enteroviruses*, *Rhinoviruses*, *Cardioviruses*, *Aphthoviruses*, and *Hepatoviruses* (Lin et al., 2009). Picornaviruses are small (27 to 30 nm), non-enveloped icosahedral-shaped viruses containing a plus sense, single-stranded RNA genome, approximately 7,500 nucleotides in length (Lin et al., 2009). Human enteroviruses cause many illnesses including poliomyelitis, hand, foot and mouth disease, myocarditis, and meningitis. Poliomyelitis, although nearly eradicated after the 1955 introduction of the vaccine, was once a pandemic in countries throughout the world. Poliomyelitis is an acute viral disease that affects motor neurons in the brain stem and spinal cord, in some cases causing paralysis and death. Poliovirus is transmitted by close personal contact and via the fecal-oral route by ingesting contaminated food and water, and circulates in a seasonal pattern with highest incidences in summer and autumn (Kew et al., 2005). Polio was eradicated from the United States in 1979, but it is still documented in many developing countries and many global eradication initiative programs are in place (de Quadros et al., 1992). Today, the poliovirus is a useful model system for the study of RNA viruses.

A virus that affects nearly every infant and child by the age 5 is rotavirus. Rotaviruses which form one genus of the family *Reoviridae*, is the leading cause of infantile diarrhea in the United States, with 1 in 7 cases requiring a clinical visit, 1 in 70 cases requiring hospitalization, and death in 1 in 200,000 cases (Bocchini et al., 2007; Glass et al., 1996; Tucker et al., 1998).

Children in child-care centers are at the highest risk, for an infected child sheds more than 1 billion virus particles per gram of stool (Flewett, 1983; Bishop 1996). Rotaviruses are non-enveloped icosahedral-shaped viruses, approximately 75 nm in diameter, with genomes of double-stranded RNA (dsRNA) (Estes and Cohen, 1989). Rotavirus is transmitted from person to person, primarily by the fecal-oral route. The rotavirus incubation period lasts 1 to 3 days, characterized by fever and vomiting, followed by 3 to 8 days of watery diarrhea (Bocchini et al., 2007; Statt et al., 2002). Multiple rotavirus serotypes (G and P) have been identified and 4 strains (G1, G3, G4 combine with P1A, and G2 combined with P1B) comprise 96% of the identified strains (Bocchini et al., 2007; Santos and Hoshino, 2005). Most children are infected with rotavirus more than once, with initial infections more likely to result in severe gastroenteritis. After rotavirus infection, protective immunity develops and subsequent infections are usually milder or may even be asymptomatic (Bocchini et al., 2007). Two different rotavirus vaccines, RotaTeq® (RV5) and Rotarix® (RV1), are currently licensed for use in infants in the United States, to be administered in the first year of infancy (Bocchini et al., 2007).

A virus known to be transmitted through contaminated water, particularly in tropical and sub-tropical regions is hepatitis E virus (HEV). It is considered a leading cause of acute hepatitis in developing countries, including North Africa, Asia, Central America, and the Middle East (CDC, 2009b). HEV, the only member of the *Hepeviridae* family and placed in genus *Hepevirus*, are non-enveloped spherical particles measuring 27–34 nm in diameter (Aggarwal, 2011). Hepatitis E virus contains a single-stranded RNA genome that is approximately 7.2-Kb long with short noncoding regions at each end, and three open reading frames (ORFs) (Guu et al., 2009). Hepatitis E is similar to hepatitis A in that the disease is self-limiting, attacks liver cells, and causes jaundice, but HEV includes 4 genotypes; all of which infect humans (Aggarwal, 2011;

CDC, 2009b). Older persons, the immunocompromised, and pregnant women have the highest case fatality rate. Transmission through contaminated foods (undercooked) and water (not drinking water) has been reported. One of the worst HEV outbreaks through contaminated water occurred in New Delhi, India in 1955 in which 30,000 people fell ill (Viswanathan, 1957). Vaccines are being developed, but several more years of research and financial aid are needed before a widespread HEV vaccination program can be put into place.

Human adenoviruses (HAdV), first isolated by Hilleman and Rowe in the 1950's, are responsible for many acute respiratory, gastrointestinal, and ocular infections (Nemerow et al., 2009; Hilleman and Werner, 1954; Rowe et al., 1955). Adenovirus, a member of the *Adenoviridae* family and belonging to the genus *Mastadenovirus*, is a large and complex non-enveloped virus with icosahedral symmetry (Nemerow et al., 2009). Adenoviruses, representing the largest of the non-enveloped viruses, possess a linear double-stranded DNA (dsDNA) genome. Based on their DNA sequence similarity, over 52 different serotypes of HAdV assigned to 7 different species (A-G) have currently been recognized (Kajon et al., 2010). Acute respiratory disease (ARD) is often caused by the serotypes of subspecies B1 (HAdV-3, HAdV-7, HAdV-16, HAdV-21, and HAdV-50), whereas urinary tract infections and opportunistic infections of immunocompromised hosts are associated with the serotypes of subspecies B2 (HAdV-11, HAdV-14, HAdV-34, and HAdV-35) (Kajon et al., 2010; Wold and Horwitz, 2007). In 2007, a US Air Force training facility reported an outbreak severe respiratory illnesses caused by human adenovirus serotype 14; with an estimated 551 trainees infected, 23 were hospitalized with pneumonia, 4 required admission to an intensive care unit, and 1 died (Tate et al., 2009). Vaccines were developed for adenovirus serotypes 4 and 7, but were only available for military

recruits. Vaccine production was stopped in 1996 and, currently, strict attention to good infection-control practices, is effective for stopping outbreaks of adenovirus (Kajon et al., 2010).

Viral surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) are cultivable in the laboratory and are genetically similar to human noroviruses. Details about FCV-F9 and MNV-1 are described in sections below.

Unlike human noroviruses, some animal caliciviruses can be cultivated in the laboratory. Feline calicivirus (FCV-F9) and Murine norovirus (MNV-1) are genetically similar to human noroviruses and are used as surrogates to better understand human noroviruses survival, infectivity, and transmission. Feline calicivirus is a pathogen of cats and belongs to the family *Caliciviridae*, making it genetically similar to human norovirus. FCV-F9 is a single-stranded RNA virus, belonging to the genus *Vesivirus*, is transmitted via the nasal, oral or conjunctival routes (Radford et al., 2007). There are a large number of strains of FCV-F9 presenting a range of clinical symptoms, but FCV-F9 is an upper respiratory infection typically characterized by oral ulcerations, limping syndrome, and ocular and nasal discharge (Thiel and Konig, 1999). After infection, cats can shed virus particles for up to 30 days (Radford et al., 2007). This virus is widespread in the cat population and particularly prevalent in households with a large number of cats and animal shelters. Broad-spectrum antibiotics are administered to infected cats and live-attenuated and inactivated vaccines are available and administered to prevent outbreaks. Recently, highly virulent and strains have emerged with high mortality in outbreaks (Hurley et al., 2004). Feline calicivirus, unlike other members of the *Caliciviridae* family (norovirus and saprovirus), can be cultivated in cell cultures and are a model for calicivirus molecular biology. Infected cells show a characteristic cytopathic effect in cell culture associated with membrane blebbing (Radford et al., 2007; Knowles, 1988). FCV-F9 is easily inactivated by chemical

treatments. D'Souza and Su (2010) found that high-titer FCV-F9 was completely inactivated ($6.84 \log_{10}$ PFU/ml reduction) after a 30 second contact time with 10% bleach (0.6% sodium hypochlorite, 5000 ppm available chlorine) and 2% trisodium phosphate (TSP) (D'Souza and Su, 2010). The survivability of FCV-F9 is surprisingly high on surfaces and at room temperature was detectable for up to 28 days (Doulton et al., 1999). A study by D'Souza and others (2006) observed that FCV-F9, from an initial titer of $9 \log_{10}$ PFU/ml, at room temperature (22°C) on various environmental surfaces (formica, stainless steel, and ceramic) had a 2-3 $\log_{10}$ PFU/ml reduction after 1 h and 6-7 $\log_{10}$ PFU/ml reduction after 7 days (D'Souza et al., 2006). Noroviruses have the potential to be infective for up to a month in the environment, in similar to other caliciviruses, with high concentrations of disinfectants needed to eliminate them (Doulton et al., 1999).

Murine norovirus (MNV-1), also a member of the *Caliciviridae* family, is even more genetically similar to norovirus than FCV-F9, for it is within the *Norovirus* genus. Murine norovirus is the only norovirus that replicates in cell culture and in a small animal, making experimentation relatively versatile and inexpensive (Wobus et al., 2006). The MNV-1 model system provides the first opportunity to understand the relationship between basic mechanisms of norovirus replication in tissue culture and pathogenesis in a natural host (Wobus et al., 2006). MNV-1, the first norovirus to infect mice, was first described in 2003 and was initially isolated from brain tissue (Wobus et al., 2006; Karst et al., 2003). Mice infected through the peroral and intranasal routes either succumbed to the infection (if immunocompromised) or infected a large proportion of laboratory mice throughout the nation (Huang et al., 2005). MNV-1, along with human norovirus, is transmitted via the fecal-oral route. The clinical onset of symptoms in with norovirus infection is similar to that of humans, including diarrhea, fever, nausea, and abdominal

pain (Green et al., 2001). MNV-1 RNA can be detected in fecal shedding for up to 8 weeks after inoculation. MNV-1 shares many features with human noroviruses including the size (28 to 35 nm in diameter), the shape (icosahedral), and buoyant density (Green et al., 2001; Karst et al., 2003). Such similarities give hope to utilizing the MNV-1 model system to better understand the biology and pathogenesis of a leading cause of human disease.

Another human enteric virus surrogate is the bacteriophage MS2. A bacteriophage is a virus that only infects bacterial cells and MS2 infects *Escherichia coli* American Type Culture Collection (ATCC) 15597B in particular. MS2 is classified as a single-stranded RNA virus with icosahedral symmetry within the *Leviviridae* family that belongs to group 1 of the RNA coliphages (Calender, 1988; Dawson et al., 2005). MS2, commonly found in sewage, is between 27-34 nm in diameter and is adapted to the intestinal tract (Dawson et al., 2005). MS2, along with other norovirus surrogates has been previously studied for inactivation by chemical and processing means, as well survivability. Black and others (2010) found that MS2 was very resistant to pressure, in comparison to other coliphages, and was inactivated by $<1 \log_{10}$ PFU/ml at pressures of 500 MPa for 5 minutes at 20°C (Black et al., 2010). These experiments proved MS2 as a suitable surrogate for hepatitis A virus (HAV). D'Souza and Su (2010) found that low concentrations of trisodium phosphate (TSP) effectively inactivated MS2 and other surrogates. High-titer MS2 was reduced by $\geq 6 \log_{10}$ PFU/ml at 5% TSP and a $4.5 \log_{10}$ PFU/ml reduction was achieved with 1% TSP after 30 seconds of contact time (D'Souza and Su, 2010). Due to produce items being a common source of contamination, Dawson and others (2005) studied the survivability of MS2 bacteriophage on fresh iceberg lettuce, baton carrot, cabbage, spring onion, curly leaf parsley, capsicum pepper, tomato, cucumber, raspberries, and strawberries. MS2 survived for a very long time and even extended the shelf life of the produce, for $<1 \log_{10}$

PFU/ml reduction was observed after 50 days at 4 and 8°C (Dawson et al., 2005). MS2 is an ideal surrogate for human norovirus and hepatitis A virus, for they are able to survive for prolonged periods on environmental surfaces.

Processing Methods for Foodborne Viral Inactivation

Foodborne virus outbreaks are a setback within the food industry and many control measures can be taken to prevent contamination and possible illness. The most common means of killing or inactivating pathogens in foods is thermal processing. The effect of heat treatment on viruses is summarized in Table 1.2. The disadvantages of thermal processes are that it can change the properties of foods resulting in negative sensory effects and even nutrient loss. A way to get around these drawbacks is to look into novel processing methods to kill pathogens that are mostly non-thermal, that having minimal negative effects on the quality of foods. Some of these methods include high intensity ultrasound (sonication), irradiation, pulsed electric field, high intensity pulsed light, high hydrostatic pressure, and high pressure homogenization.

High intensity ultrasound (HIUS) processing, also known as sonication, is the transmission of sound waves at varying frequencies to kill microorganisms. Villamiel and others (1999) defined HIUS as sound wave frequencies greater than 20 kHz (Villamiel et al., 1999). This process is not typically related to food preservation, but it is commonly used within the medical field (equipment sanitation) and in the laboratory (compound extraction). In medicine, HIUS with a frequency range of 20 kHz- 2 MHz and power of 100-500 W/cm² is destructive, causing microbial inactivation, enzyme inactivation, and biocomponent separation (Su et al., 2010c). Ultrasound treatments are divided into low-power frequency (non-destructive in food processes) and high-power frequency (sanitation, microbial inactivation and enzyme alteration)

(Suslick 1988; McClements and Povey, 1989; McClements, 1995). When used in food systems, the advantages over heat pasteurization include: minimal flavor loss, greater homogeneity, and considerable energy savings (Crosby, 1982). The mechanism behind sonication to kill microorganisms is the energy waves travelling through material cause vibrations, which causes displacement of surrounding particles (Sala and others, 1995). This displacement of particles creates cycles of expansion and compression, which in turn form bubbles. The formation of bubbles, called cavitation, and the resulting action of the bubbles rapidly expanding and collapsing is dependent on frequency, medium viscosity, temperature and pressure (Suslick, 1988; Betts et al., 1999; Piyasena et al., 2003). The lethal effect of cavitation on microorganisms is due to shear disruption of cell membranes, localized heating, and free radical formation (Earnshaw, 1998). Studies show that bacterial spores are more resistant than vegetative cells, and Gram positive bacteria are more resistant to sonication than Gram negative bacteria (Barbosa-Canovas and Rodriguez, 2002; Raso et al., 1998, Earnshaw, 1998). Few reports demonstrate the effect of sonication on viruses, but Su et al., (2010c) found that FCV-F9, MS2, and MNV-1 in PBS, at titers of $\sim 4 \log_{10}$ PFU/ml, required 5, 10, and 30 minute treatments at 20 kHz, respectively, for complete inactivation (Su et al., 2010c). The effect of ultrasound treatment on a variety of microorganisms is reported in Table 1.3. Unfortunately this process has disadvantages and limitations. It currently can only be executed on a very small scale, limiting commercialization, and high intensities are required to sterilize foods. However, with the combination of heat, pH, and pressure; sonication could be used to minimally process foods as in hurdle approaches (Barbosa-Canovas and Rodriguez, 2002; Mason et al., 1996; Guerrero et al., 2001).

Food irradiation, a food preservation process used since the 1940s, exposes foods to doses of ionizing radiation. The two types of radiation used in food processing are cobalt-60 gamma sources and electron beam generators (Hirneisen et al., 2010). Irradiation treatment excites electrons within foods above their ionization potential (Barbosa-Canovas et al., 1998) and causes microbial death by inhibiting DNA synthesis at a cellular level (Farkas, 1988). The dose, or quantity of energy absorbed by the foods, is measured in grays (Gy). Food irradiation treatments can be divided into three categories (Wilkinson and Gould, 1996): radurisation, radicidation, and radappertisation (Barbosa-Canovas and Rodriguez, 2002). The radurisation process targets spoilage microorganisms, the dosage typically below 10 kGy and radicidation targets non-spore forming bacterial pathogens, generally 2.5-10 kGy. Radappertisation aims to inactivate spore-forming pathogenic bacteria and viruses, typically 10-50 kGy. Irradiated foods are categorized into five groups (Kume et al., 2009), described in Table 1.4. Currently, the FDA limits the dosage to control microbes to a maximum of 7 kGy for frozen uncooked meat foods, 4.5 kGy for refrigerated uncooked meat foods, and 1 kGy for inhibition of microorganisms of fresh foods, and 4 kGy for fresh iceberg lettuce and spinach (FDA, 2008). Surprisingly, viruses are the most resistant microorganisms, followed by yeasts and spore-forming bacteria (Barbosa-Canovas and Rodriguez, 2002; Wilkinson and Gould, 1996). The effectiveness of gamma irradiation on viruses depends on factors including; virus size, type of food product, and temperature (Patterson, 1993). Molds and Gram positive bacteria are more resistant to irradiation than Gram negative bacteria (van Gerwen et al., 1999). This is thought to be due to the thicker cell and peptidoglycan layer of Gram positive bacteria in comparison to Gram negative bacteria. The effect of irradiation on a variety of microorganisms is documented in Table 1.5. Although

irradiation is a safe and effective means of controlling pathogens, its commercial use has been limited due to cost, low consumer acceptance, and public apprehension around the world.

Pulsed electric field (PEF) is a non-thermal process applied to foods to inactivate microorganisms in which foods are treated with high intensity electric field pulses. PEF parameters typically include pulsed field intensity of 15-50 kV cm⁻¹, pulse width of 1-5 μ s, and pulse frequency of 200-400 Hz (pulses/s) (Wan et al., 2009). Minimal quality, sensory, and nutritive property changes occur due to the relative low temperature attained at <60°C (Wan et al., 2009). This is a continuous system, making it ideal for the processing of liquid food products. Although the exact mechanisms of microbial inactivation by PEF are unclear, electroporation-induced cell inactivation, due to the osmotic imbalance across the cell membrane is an accepted theory (Tsong, 1991; Zimmerman, 1986; Wan et al., 2009). Electroporation is a significant increase in electroconductivity and permeability across a cell membrane, proposed to induce pore formation, lethally injuring the cells (Barbosa-Canovas et al., 1999). In studies, vegetative cells are more sensitive than spores (Barbosa-Canovas and Rodriguez, 2002; Yonemoto et al., 1993; Pothakamury et al., 1996) and Gram positive cells are more resistant to PEF than Gram negative (Barbosa-Canovas and Rodriguez, 2002; Pothakamury et al., 1995). To date, very little research on has been shown on the effectiveness of PEF at inactivating viruses. Khadre and Yousef (2002) found that rotavirus was resistant to PEF treatment of 20 to 29 kV/cm for 145.6 μ s, which may be explained by the presence of a protein capsid on enteric viruses compared to the lipid membranes of bacterial cells (Khadre and Yousef, 2002). The effect of PEF on a variety of microorganisms is summarized in Table 1.6. There are disadvantages to this system that include the difficulty of processing at a large scale and the inability to standardize lethal PEF treatment for a broad range of microbes, for inactivation is affected by electric field strength,

pulse length, pulse shape, number of pulses, and start temperature (Barbosa-Canovas et al., 1999). Also, PEF treatments are yet to be implemented commercially for the system designs do not assure uniform treatment of the food product.

Another novel non-thermal food process is the use of high intensity pulsed light. This food preservation method involves the use of intense, short-duration pulses of broad spectrum light (Elmnasser et al., 2007). The topical decontamination depends on the intensity of light (measured in $J \cdot cm^2$) and the number of pulses delivered. Numerous mechanisms of action have been proposed to explain the lethal effect of pulsed light, all related to its photochemical and photothermal effect (Rowan et al., 1999). The photochemical mechanism targets nucleic acids and cleaves cellular DNA, resulting in lethal injury to the microorganisms (Elmnasser et al., 2007). The photothermal mechanism disrupts cells by rapid overheating. Large amounts of thermal energy (exceeding $0.5 J \cdot cm^{-2}$) results in an “explosion” of the cell and a release of its contents (Wekhof, 2000). Studies show that spores are more resistant to pulsed light than vegetative cells, but fungi are more resistant than bacteria (Rowan et al., 1999). Rowan et al (1999) also reported that Gram positive bacteria are more resistant than Gram negative. Pulsed light is very effective at inactivating viruses in PBS, with a $\log_{10}$ PFU/ml reduction ranging from 4.8-7.2 (Roberts and Hope, 2003). The effect of high intensity pulsed light on a variety of microorganisms can be seen in Table 1.7. Though a promising method, there are many disadvantages and limitations to high intensity pulsed light. The decontamination of the food product depends on the packaging the food is in and the surface texture of the product. The light pulses only hit the surface of the food and therefore need to be in clear packaging and the surface of the food needs to be smooth. If there are crevices and an uneven surface where bacteria may hide, the light will not reach it. Laboratory studies have shown promising inactivation of

microorganisms on agar (flat surface) but not much reduction on actual food surfaces. It has also been shown that pulsed light is more effective on solid surfaces than liquids (Elmnasser et al., 2007; Marquenie et al., 2003). It is also difficult to standardize the intensity of light and the number of pulses to inactivate a broad range of microorganisms.

The application of pressure to foods has been acknowledged as a way to preserve foods and ensuring their safety while maintaining the physical and nutritive properties. This technology has become popularized in the food industry because it does not use heat, yielding higher quality products with virtually unaffected sensory and nutritional attributes, in comparison to traditional thermal methods (San Martin et al., 2002). The concept of using pressure to affect microorganisms is far from novel. Publications and experiments using the technology to extend food shelf-life date back over one-hundred years ago when Hite (1899) reported the effect of high pressure on microorganisms in milk and later, fruits and vegetables (San Martin et al., 2002). Over the years this technology has improved and it is well known how high pressure affects food products and its constituents, bacteria, fungi, and viruses. High hydrostatic pressure (HHP) processing is applied to foods, frequently at room temperature, for reasons including reducing or inactivating foodborne microorganisms and prolonging the shelf life of food products (Kovac et al., 2010; Guerrero-Beltran et al., 2005; Korhonen et al., 1998). “According to the Pascal or isostatic principle, pressure is transmitted uniformly and immediately through the pressure-transferring medium, and thus effects of pressure are independent of product size and geometry” (Guerrero-Beltran et al., 2005; Knorr, 1993). The process can subject foods up to 900 MPa pressure, but in commercial systems the pressures used typically range from 400 to 700 MPa (San Martin et al., 2002). The major components of a HHP system are a pressure vessel, a pressurization system, devices for temperature control, and product handling devices (San Martin

et al., 2002). HHP is a cyclic batch-wise process, although semi continuous lines may be built, that takes three steps: an increase in the working pressure, a holding time of this pressure, and depressurizing time (Denys et al., 2000, Guerrero-Beltran et al., 2005). A batch HHP system process involves filling the process vessel with product, closing the vessel, increasing the vessel to pressure, decompressing the vessel and, lastly, removing the product (FDA, 2009). Semi-continuous HHP systems are currently used for treating liquids. The process entails: (1) filling the pressure vessel using a food pump (2) when filled, the free piston is displaced and high pressure process water is brought in to compress the liquid food (3) pressure is increased (4) the high pressure process water is released, decompressing the system and (5) the treated liquid food is discharged from the pressure vessel (FDA, 2009). Pressure within a vessel is generated by one of two primary means; direct or indirect compression. Direct compression involves the reduction of the volume of the treatment chamber, or vessel, with the use of a piston by action of a hydraulic pump. This directly increases the pressure of the vessel (San Martin et al., 2002, Guerrero-Beltran et al., 2005). Indirect compression systems use high-pressure pumps, or intensifiers, pumps the pressure medium through a tubing system and directly into the vessel until desired pressure is achieved (San Martin et al., 2002, Guerrero-Beltran et al., 2005). The transmission of pressure is instantaneous, no matter the size and geometry of the food product, (Barbosa-Canova and Rodriguez, 2002). In the United States, food products that have been brought to market for high pressure processing include raw oysters, fruit jellies, fruit juices, salad dressings, raw quid, smoothies, rice cakes, ham, quacamole, and salsa (FDA, 2009). The pressure required to inactivate microbes is dependent on the product stability and target organism. Generally, vegetative bacteria are the most sensitive to HHP, then yeasts and molds, while the most resistant microbes are viruses and bacterial spores (Kovac et al., 2010; Patterson

et al., 2007). The site of pressure damage to bacteria and fungi is the cytoplasmic membrane (Grove et al., 2006). The effect of HHP on viruses depends on the virus structure, and non-enveloped viruses demonstrate a broad range of sensitivities (Kovac et al., 2010; Manas and Pagan, 2005; Grove et al., 2006). Many authors suggest that HHP denatures the virus capsid proteins, therefore preventing host cell binding and infection (Kovac et al., 2010; Hogan et al., 2005; Khadre and Yousef, 2002; Kingsley et al., 2002; Buckow and Heinz, 2008; Li et al., 2009; Tang et al., 2010). A parameter to be considered that could have an effect on virus inactivation by HPH is the food medium in which the virus is found. Food components such as proteins, lipids, or carbohydrates can have a protective effect (Kovac et al., 2010; Simpson & Gilmour, 1997; Garcí'a- Graells et al., 1999; Kingsley & Chen, 2009; Murchie et al., 2007). The effect of high hydrostatic pressure on a number of viruses can be seen in Table 1.8. The disadvantage to this system is that the inactivation of microorganisms is time-dependent and also dependent on the initial number (titer) of microbes. In addition, this system is not continuous and has a slight elevation in temperature, making it inadequate for processing fluid foods. Although promising, issues that should be addressed are the differences in resistance of viruses to HHP and the evaluation of foodborne viruses that cannot grow in cell culture medium (Kovac et al., 2010).

The most promising novel non-thermal processing method for fluid foods is high pressure homogenization (HPH). Homogenization is the process in which you take a food of varying particle sizes and disperse them into uniform particle sizes, making a homogenous solution. Homogenization was invented by Auguste Gaulin in 1900, and was exhibited at the World Fair in Paris (Diels and Michiels, 2006). This process is used extensively in the food industry, particularly for creating dairy and food emulsions, such as milk, cream, and butter, to improve texture, flavor, and shelf-life attributes (Diels and Michiels 2006; Dickinson and Stainby, 1988).

Recently, the addition of pressure to homogenization systems has been developed. High pressure homogenization (HPH) systems are continuously operated and can be subdivided into valve homogenizers (sometimes referred to as radial diffusers), counterjet dispersators, and axial nozzle aggregates (Schultz et al., 2004). The most common HPH system used in industry is the valve homogenization. This HPH system design consists of a positive-displacement pump that forces fluid into a homogenization valve (Middelberg, 1995). Numerous homogenizing valve configurations (for the disruption of microbial cells) are available, including the standard valve, cell rupture valve, grooved valve, knife edge valve, conical valve, and ball cell disruption valve (Engler, 1990). The fluid in the homogenization valve is then forced under pressure through a small opening between the valve and the valve seat (Diels and Michiels, 2006). The fluid then exits the valve at high radial velocities and strikes an impact ring (Middelberg, 1995). Lastly, the fluid exits the homogenizer. The pressure is controlled by altering the distance between the valve and the valve seat, thus adjusting the force of the valve (Diels and Michiels, 2006; Middelberg, 1995). High pressure homogenization (HPH), today, is used in the food, pharmaceutical, cosmetic, and chemical industries including emulsion preparation and cell disruption of yeasts or bacteria (Diels and Michiels, 2006). HPH has been theorized to inactivate microorganisms due to the combined effects of pressure, cavitation, turbulence, high temperature, and sheer stress (Taylor et al, 2007). Doulah and others (1975) hypothesized that turbulence, created by velocity fluctuations of the liquid, causes microbial cell wall disruption (Doulah et al., 1975). This theory was rejected by Engler and Robinson (1981), for they postulated that impingement of a high velocity jet of suspended cells on a stationary surface leads to cell disruption (Engler and Robinson, 1981). Save and others (1994) proposed that cavitation, the rapid process of gas cavity growth and collapse in a liquid, and resulting wave and

pressure impulses are responsible for cell disruption (Save et al., 1994). Studies have also shown that increasing fluid viscosities will (1) inhibit cavitation by suppressing underpressures caused by vortices and (2) decrease cavitation collapse pressures by reducing high localized fluid velocities (Diels and Michiels, 2006; Svedberg et al., 1999; Keller, 2001; Rosina and Tistruga, 2001; Totten et al., 1999). The primary mechanism that HPH kills microorganisms is mechanical destruction of the cell wall (Diels and Michiels, 2006). Studies have shown that Gram-negative bacteria are more sensitive to HPH than Gram-positive bacteria, due to the thinner peptidoglycan layer (Kelemen and Sharpe, 1979; Wuytack et al., 2002). The effect of HPH on a variety of microorganisms is reported in Table 1.9. HPH has been applied to apple juice contaminated with *E.coli* K-12 and resulted in a 7.5 log₁₀ PFU/ml reduction at 250 MPa (Pathanibul et al., 2009). Moroni and others (2002) found, using electron microscopy, that lactococcal bacteriophages (c2, sk1, and u136) were most likely inactivated after HPH by the breaking of the phage heads, and release of genetic material (Moroni et al., 2002). Norovirus surrogates have also been inactivated using HPH. Bacteriophage MS-2 showed 3.3 log₁₀ PFU/ml reduction at pressures of 250 MPa or more and MNV-1 showed slight reduction (0.8 log₁₀ PFU/ml at 300 MPa) (D'Souza et al., 2009). Although little is known of the effect HPH has on viruses, D'Souza and others (2009) theorized that the process causes changes or disruption in viral capsid structure and different viruses may have differing resistance levels due to dissimilarity in their coat proteins as well as morphological differences (D'Souza et al., 2009). Though a relatively costly process currently needing further research, HPH shows potential for the commercialization for processing safe fluid foods.

Pre-cut bagged vegetables and fruits are in high consumer demand due to their convenience and availability (Baert et al., 2009). Antimicrobial agents, such as disinfectants and

sanitizers, are used in food processing to reduce or eliminate microbial populations and to prevent the spread of microorganisms. Produce items are often sprayed, washed, or immersed in water post-harvest, followed by either dipping or spraying with an antimicrobial on the surface (Hirneisen et al., 2010; Beuchat, 1998). It is important to know how these chemical substances affect virally contaminated produce items. The efficacy of washing produce with water and various chemical agents to lessen the viral load can be seen in Table 1.10. Washing produce items with water generally reduces the viral load by 1-2 $\log_{10}$ PFU/ml, depending on the produce item (Beuchat, 1998). Dawson and others (2005) reported that bacteriophage MS2 was reduced between 0.08 and 0.79 $\log_{10}$ PFU/ml by washing tomatoes, pepper, cucumbers, onion, carrots, lettuce, parsley and strawberries with tap water (Dawson et al., 2005). Another study found that FCV-F9 on lettuce and strawberries was reduced by 2 $\log_{10}$ PFU/ml after 10 minutes of washing with water (Gulati et al., 2001). The sanitizing agent chlorine has been shown to be somewhat effective in reducing virus titers on produce, but the experimental setup factors are important in defining decline rates (Baert et al., 2009). Bacteriophage MS2 was reduced between 0.3 and 2.1 $\log_{10}$ PFU/ml on leafy vegetables after a 5 minute treatment of 100 ppm chlorine (Dawson et al., 2005). Gulati and others (2001) FCV-F9 on strawberries treated with 200 ppm chlorine was not additionally reduced when compared to a tap water wash (Gulati et al., 2001). MS2 and HAV were reduced by 1.7 $\log_{10}$ PFU/ml on strawberries, tomatoes, and lettuce treated with 20 ppm chlorine. Baert and others (2009) indicated that FCV-F9, MS2, and HAV may react similarly to chlorine, due to the findings of Allwood and others (2004) and Casteel and others (2008). Shin and Sobsey (2008) found that norovirus was reduced by 2 $\log_{10}$ PFU/ml (intital titer of $\sim 4.5 \log_{10}$ PFU/ml) after 3 minutes of contact time of 1 mg/L free chlorine, which disputes the study of Duizer and others (2004) in which norovirus was found to be highly resistant to chlorine (Shin

and Sobsey, 2008; Duizer et al., 2004). Although higher chlorine levels would cause a greater reduction in viral titers, this application would cause negative sensory aspects. Allwood and others (2004) found bleach (50 ppm) effective by reducing MS2 and FCV-F9 by $\sim 2 \log_{10}$ PFU/ml on lettuce, but similar to chlorine, this chemical causes negative sensory effects.

Foodborne Virus Survival

Although many studies have shown means to inactivate viruses, whether by chemical or processing means, it's also important to focus on the untreated survivability of viruses on different foods or surfaces and at different temperatures. The low infective dose of some viruses, even with poor survivability in the environment, suggests that viruses are able to persist for days, weeks, or months in numbers adequate to cause infection (Vasickova et al., 2010; Barker et al., 2001; Boone and Gerba, 2007). Virus particles are preserved by refrigeration or freezing temperatures, as well as being able to persist for days or months over a range of temperatures (Cliver, 2009). Virus survival in the environment is influenced by a combination of biological (i.e., type of virus, envelope, other microorganisms), physical (i.e., temperature, surface type, relative humidity, sunlight and UV radiation), and chemical (i.e., pH, salt and ionic strength, adsorption, organic matter, antiviral chemicals) factors (Vasickova et al., 2010). D'Souza and others (2006) detected human norovirus by RT-PCR on formica, stainless, steel, and ceramic up to 7 days after inoculation and transferred the virus from each surface to lettuce, confirming norovirus persistence on surfaces and ease of transfer (D'Souza et al., 2006). Another study determined the survivability of norovirus GI and GII on produce in freezing temperatures (Butot et al., 2008). Both genotypes had minimal reduction ($< 1 \log_{10}$ PFU/ml) after 2 days of storage on blueberries, raspberries, and strawberries. Norovirus GII had the greatest reduction ($2.3 \log_{10}$ PFU/ml) after 90 days in frozen storage, but there was no more than $1 \log_{10}$ PFU/ml difference in

the reductions for the two norovirus genotypes (Butot et al., 2008). The sturdy Hepatitis A virus, associated with shellfish, sandwiches, fruits, vegetables, and ready-to-eat foods, can readily survive freezing, can persist in fresh or salt water for up to 12 months, and is quite resistant to numerous environmental conditions (Sattar et al., 2000; Sobsey et al., 1988; Mbithi et al., 1992). Mbithi and others (1992) found that after 4 hours, up to 30% of recovered HAV remained detectable on human hands, suggesting its potential to retain its infectivity during a food handler's work shift (Sattar et al., 2000; Mbithi et al., 1992). The virus has also shown an ability to survive at varying temperatures and relative humidities (RHs). On stainless steel disks, HAV survival was inversely proportional to RH (Mbithi et al., 1991). HAV's half life was nearly 7.8 days at 5°C and a low RH; and a little over 2 h at a high RH and 35°C (Mbithi et al., 1991). HAV also has the ability to survive in acidic environments. HAV in marinated mussels survived for 4 weeks, with no decrease in titer, in acid marinade with a pH of 3.75 (Hewitt and Greening, 2004). HAV has been shown to endure at various temperatures on produce items. Fino and Kniel (2008) found that HAV had a 78, 83, and 62% recovery after 12 h incubation at 4°C on strawberries, green onions, and lettuce, respectively (Fino and Kniel, 2008). Croci and others (2002) found that HAV on produce items (lettuce, carrots, and fennel) remained vital after 9 days of storage at 4°C (Croci et al., 2002). 2.46 log₁₀ PFU/ml HAV remained after 9 days on lettuce before washing and this study proved that washing does not guarantee virus reduction or inactivation (Croci 2002). As far as freezing temperature, HAV remained stable for 90 days on frozen raspberries, blueberries, strawberries, parsley, and basil (Butot et al., 2008). Poliovirus was inoculated on strawberries, raspberries, lettuce, green onions, and cabbage and stored for 15 days at 4°C (Kurdziel et al., 2001). No significant reduction was achieved on green onions and raspberries, but a 1 log₁₀ PFU/ml reduction was seen on lettuce, cabbage, and strawberries after

11.6, 14.2, and 8.4 days, respectively (Kurdziel et al., 2001). The survival of rotavirus was studied in contaminated fruit juice (pH 2.98) during storage at 4°C for 3 days, and showed an ~3 log₁₀ PFU/ml reduction, indicating the potential of rotavirus to survive in low pH and refrigeration temperatures (Mahony et al., 2000).

Other, less resistant viruses have also been studied for their survivability. D'Souza and others (2006) observed that FCV-F9 on various environmental surfaces (formica, stainless steel, and ceramic) at room temperature (22°C) had a 2-3 log₁₀ PFU/ml reduction after 1 h and 6-7 log₁₀ PFU/ml reduction after 7 days, from an initial titer of 9 log₁₀ PFU/ml (D'Souza et al., 2006). Another study found that FCV-F9 survived on brass and computer keyboard keys up to 12 hours at room temperature, although a 1 log₁₀ PFU/ml reduction was observed in less than 4 h (Clay et al., 2006). One study observed that FCV-F9 was stable (> 72% recovery) on strawberries, green onions, and lettuce after 12 h incubation at 4°C (Fino and Kniel, 2008). Mattison and others (2007) found that at 4°C, a 2 log₁₀ PFU/ml reduction was observed in FCV-F9 on lettuce after 7 days; and more than 2.5 log₁₀ PFU/ml reduction on strawberries after 6 days (Mattison et al., 2007). FCV-F9 infectivity was reduced by 0.34 log₁₀ PFU/ml after 5 cycles of freeze-thawing (Duizer et al., 2004). The cultivable norovirus surrogate MNV-1 is more stable and survives longer in various environmental conditions than FCV-F9. Bae and Schwab (2008) found that over a 3-5 weeks in environmental waters at 25°C, MNV-1 had a 0.09 log₁₀ PFU/ml reduction in infectivity per day, while the less stable FCV-F9 had a 0.18 log₁₀ PFU/ml reduction per day (Bae and Schwab, 2008). Cannon and others (2006) found that wet and dry suspensions of MNV-1 on stainless steel survived 7 days with < 2 log₁₀ PFU/ml reduction at 4°C (Cannon et al., 2006). In the same study, a wet suspension of MNV-1 survived 7 days with < 2 log₁₀ PFU/ml reduction at room temperature (Cannon et al., 2006). MNV-1 was also reported to be resistant to

freezing, for no reduction of infectivity was observed after 6 months on frozen onions and frozen spinach (Baert et al., 2008).

Natural Plant Polyphenols for Foodborne Viral Inactivation

Virus survival of a variety of fruits and vegetables determined not only by the many factors mentioned above, but also antiviral compounds the food may naturally possess. Until the beginning of the century, medicine practice depended largely on the use of plants. In ancient China, one of the earliest recorded medicines was the use of the dried shoot of the “Ma Huang” plant (Haslam, 1989). This active component of this plant, ephedrine, was used as a cough treatment and cardiac stimulant, and later introduced to Western medicine in the 1920s (Haslam, 1989). Plants rich in vegetable tannins (polyphenols) were used in Chinese and Japanese folk medicine to treat inflammation, liver injury, kidney problems, hypertension, blood pressure, stomach disorders, ulcers, and many others (Haslam, 1989). Plants produce a large number of secondary metabolites that have a range of functions; including signal compounds (to attract pollinating or seed dispersing animals), defense (against herbivores, microbes, viruses, or competing plants), and protection from ultraviolet radiation and antioxidants (Lattanzio et al., 2008). Phenolic compounds, also referred to as “plant polyphenols”, are the most widely distributed group of secondary metabolites in the Plant Kingdom (Lattanzio et al., 2008). Haslam (1998) comprehensively defined plant polyphenols (also referred to as *vegetable tannins*) as water-soluble phenolic compounds having molecular masses between 500 and 3,000-4,000 Da, possessing 12-16 phenolic groups and 5-7 aromatic rings per 1000 relative molecular mass, and expressing special properties such as the ability to precipitate proteins and alkaloids (Haslam, 1998). Plant phenolics, due to their various structures, are categorized based on their basic skeleton. The phenolic classes include: C₆ (simple phenols, benzoquinones), C₆-C₁ (phenolic

acids), C₆-C₂ (acetophenones, phenylacetic acids), C₆-C₃ (hydroxycinnamic acids, coumarins, phenylpropenes, chromones), C₆-C₄ (naphthoquinones), C₆-C₁-C₆ (stilbenes, anthraquinones), C₆-C₃-C₆ (flavonoids, isoflavonoids), (C₆-C₁)₂ (hydrolysable tannins), (C₆-C₃)₂ (lignans, neolignans), (C₆-C₃-C₆)₂ (biflavonoids), (C₆-C₃)_n (lignans), (C₆)_n (catechol melanins), and (C₆-C₃-C₆)_n (condensed tannins) (Harborne, 1980; Hattenschwiler and Vitousek, 2000; Iwashina, 2000). Plant phenolics, usually accumulated in the central vacuoles of epidermal and guard cells, have key roles as the major red, blue, and purple pigments (Lattanzio et al., 2008). Recently, biomedical research has disclosed that dietary phenolics, due to their free radical scavenging and antioxidant properties, play major roles in the prevention of many chronic diseases (Kutchan, 2001). Dietary sources of plant phenolics can be seen in Table 1.11. Antimicrobial effects of plant phenolics can be seen in Table 1.12. The natural antimicrobial substances (phenolic compounds) from fruits; including pomegranate, blueberry, cranberry, grapefruit, grapeseed, orange, and other berry fruits are the focus of this section.

Pomegranate (*Punica granatum*) is a widely cultivated deciduous shrub, or small tree, in the Mediterranean countries, India, Japan, China, Russia, and the United States (California) (Haidari et al., 2009). The fruit of this tree has a tough reddish rind and contains many edible seeds (arils), each enclosed in a juicy, mildly acidic, red pulp. Pomegranate fruit arils are popular for their taste, rich color, and high antioxidant content. Manufacturers commonly add this fruit to juices, jellies, ice-creams, and truffles (Kanatt et al., 2010). In the past decade, interest and research in the pomegranate has increased due to its multifunctionality as a medicinal and nutritional product (Jaiswal et al., 2010). Pomegranates are a rich source of polyphenols, including flavonoids (anthocyanins), condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins and gallotannins) (Jaiswal et al., 2010; Gil et al., 2000; Hernandez et al.,

1999; Santagati et al., 1984). The most abundant antioxidant pomegranate polyphenols (PP) are the anthocyanins, ellagitannins, and gallotannins (Gil et al., 2000; Madrigal-Carballo et al., 2009). Ellagitannins, which account for 92% of the antioxidant activity in pomegranate juice (PJ), are condensed in the membranes, peel, and piths of the fruit (Basu and Penugonda, 2009). Anthocyanins are water-soluble pigments that appear red, purple, or blue in plants, and are associated with a wide range of biological activities that may positively affect health (Burdulis et al., 2009). Studies have shown that anthocyanins may reduce the risk of heart disease, protect arterial endothelial cells, decrease the risk of cancer, and modulate immune response (Burdulis et al., 2009). When evaluated for free-radical scavenging and iron-reducing capacities, PJ was shown to be three times higher than red wine and green tea (Gil et al., 2000). PJ was also shown to have considerably higher antioxidant levels, compared to grape, cranberry, grapefruit, or orange juice (Basu and Penugonda, 2009; Azadzo et al., 2005; Rosenblat et al., 2006). PJ and its potent antioxidant activity may provide protection against cardiovascular diseases, strokes, and other health ailments (Aviram et al., 2002). A study by Aviram and others (2002) found that PJ significantly reduced atherosclerotic lesion areas in mice, compared to a water-treated group (Aviram et al., 2002). Pomegranates have also been studied for their antibacterial and antiviral qualities. Kanatt and others (2010) found that pomegranate extract (PE) showed excellent antioxidant and iron-chelating capacity, and inhibited the growth of *Staphylococcus aureus* and *Bacillus cereus* (Kanatt et al., 2010). Although the antibacterial mechanisms remain unclear, it is theorized that extracts containing phenolics disrupt the cellular membranes (Kanatt et al., 2010). Haidari and others (2009) studied the effect of purified PE on influenza virus. It was found that the hydrolysable tannin punicalin blocked replication of the virus RNA, inhibited agglutination of chicken red blood cells by the virus, and, when used in combination with the antiviral

oseltamivir, increased the anti-influenza effect of oseltamivir (Haidari et al., 2009). Recently, Su et al. (2010b) showed that (PJ) and (PP) could reduce the viral titers of MS2, FCV-F9 and MNV-1. After one hour incubation at room temperature (25°C) in PJ, low titer ($\sim 10^5$ log₁₀ PFU/ml) MS2, FCV-F9 and MNV-1 were reduced by 0.32, 2.56, and 1.32 log₁₀ PFU/ml, respectively (Su et al., 2010b). In the same study, MS2, FCV-F9 and MNV-1 were reduced by 0.41, 4.54, and 1.30, respectively, after a 1 hour incubation at 25°C in PP (4 mg/ml), with MS2 being the most resistant to treatment and FCV-F9 the most susceptible (Su et al., 2010b). The authors suggested that PJ and PP may play a role in inhibiting the binding of virus to the cells by blocking cell surface receptors (Su et al., 2010b).

Another well studied fruit also known for its high antioxidant capacity is the blueberry. Blueberries are flowering plants of the genus *Vaccinium* native to North America, but now grown in New Zealand, Australia, and South American countries (Wang et al., 2010). Blueberries contain structurally related polyphenols, including anthocyanins and flavonoids. Anthocyanins from berry extracts have been studied for their antimicrobial properties. One study tested the effect anthocyanin extracts of blueberry (*Vaccinium corymbosum* L.) and bilberry (*Vaccinium myrtillus* L.) fruits have on different bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, and, *Salmonella Typhimurium*, and found that *Citrobacter freundii* and *Enterococcus faecalis* were the most sensitive (Burdulis et al., 2009). In addition, blueberries contain proanthocyanidins (PAC), also referred to as condensed tannins, that are considered to be active against carcinogenesis and bacterial adherence during urinary tract infections (Smith et al., 2000; Bomser et al., 1996; Howell et al., 1998; Ofek et al., 1991). Tannins (condensed and hydrolysable) differ from other phenolic compounds for they are able to precipitate proteins, known as astringency (Scalbert, 1991). PACs are polyhydroxyflavan

oligomers or polymers, and the monomeric flavanols differ in the stereochemistry of C-3 and their hydroxylation pattern in ring A and B (Serrano et al., 2009). Hydrolysable tannins are polyesters of a sugar moiety and organic acids, and the designation was assigned for, upon treatment with diluted acids, these compounds undergo hydrolytic cleavage to the respective sugar and acid moiety (Serrano et al., 2009). The toxicity effect of tannins on microorganisms, including fungi, bacteria, yeasts, and viruses have been examined (Serrano et al., 2009; Chung et al., 1993). These mechanisms include inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth, and direct action on microbial metabolism through inhibition of oxidative phosphorylation (Scalbert, 1991). Tannin extracts have been shown to possess antiviral properties. One study found that hydrolysable and galloylated tannin inhibited *Herpes simplex* virus absorption (Fukuchi et al., 1989). Nakashima and others (1992) observed that hydrolysable tannins inhibit the cytopathic effects of human immunodeficiency virus (HIV), and the expression HIV-antigen in human lymphotropic virus type 1-positive MT-4 cells (Serrano et al., 2009; Nakashima et al., 1992). Recently, Takeshita et al. (2009) found that blueberry PAC inhibits the expression and replication of hepatitis C virus (Takeshita et al., 2009). This research leads us to believe that blueberries may also possess components with a range of antiviral properties, yet, the survival of foodborne human noroviruses in blueberry juice is currently unknown.

The North American cranberry (*Vaccinium macrocarpon*) is one of only three native North American fruits and is the United States Department of Agriculture (USDA) standard for fresh cranberries and cranberry juice cocktail (Cote et al., 2010). The primary phytochemicals found in cranberries are the flavonols, flavan-3-ols, the anthocyanins, and the tannins (ellagitannins and proanthocyanidins) (Cote et al., 2010.) Flavonoids (including flavonols and

flavan-3-ols) possess two six-carbon aromatic centers (A and B rings) and a three-carbon bridge (C ring) that forms a phenol bridge with oxygen (Robards and Antolovich, 1997; Haslam, 1998; Gee and Johnson, 2001). The anthocyanin concentration increases as the fruit ripens and averages 95 mg/100g in ripe cranberry (USDA, 2007). The flavonol concentration in cranberries averages 30mg/100 g fresh fruit weight and the flavan-3-ols concentration averages 7mg/100g fresh fruit (USDA, 2007). Raw cranberries have an average PAC concentration of 410mg/100g, which is generally located in the vacuoles of plant cells (USDA, 2004; Cote et al., 2010). Many *in vitro* studies have shown that berry phenolics have antioxidant and free radical-scavenging properties, as well as metal chelation, anticarcinogenic, antibacterial, anti-inflammatory, and antiviral properties (Cote et al., 2010; Lee, 2000; Merken and Beecher, 2000a; 2000b; Nijveldt et al., 2001; Higdon, 2007). Heinonen (2007) concluded that daily dose of cranberry PAC may reduce the occurrence of urinary tract infections (UTIs), for this polyphenol reduced the adhesion of certain *E. coli* bacteria to the urinary tract (Heinonen, 2007). Bodet and others (2008) found that cranberry inhibited *Streptococcus mutans* growth, a bacteria involved in periodontal diseases and dental caries (Bodet et al., 2008). Cranberry PAC could be affecting the growth of bacteria by a number of mechanisms, including destabilizing the cytoplasmic membrane and deprivation of substrates required for microbial growth (Heinonen, 2007). Su and others (2010a) tested the effect of cranberry juice (CJ) and CJ PAC on the following viruses: MNV-1, FCV-F9, MS2, and Φ -X174. After 1 h in CJ (pH 2.6), FCV-F9, MNV-1, Φ -X174, and MS2 showed ~5, 1.9, 1.6, and 0.1 log₁₀ PFU/ml reductions, respectively (Su et al., 2010a). Weiss and others (2005) found that high molecular weight materials in CJ inhibited influenza adhesion to cells and subsequent infectivity (Weiss et al., 2005). Another study found that bacteriophage T2 was no longer detectable after exposure to CJ cocktail (Lipson et al., 2007). In the same study, treatment of the

simian rotavirus SA-11 with a 20% CJ suspension was sufficient to inhibit hemagglutination (Lipson et al., 2007). This effect appeared to be related to the adsorption stage of the viruses' replication cycle (Lipson et al., 2007).

Fruits from tropical regions, particularly the citrus fruits, contain phenolic compounds in their essential oils. Citrus fruits, native to tropical regions of Asia, belong to six genera; *Fortunella*, *Eremocitrus*, *Clymendia*, *Poncirus*, *Microcitrus* and *Citrus* (Chanthaphon et al., 2008). The *Citrus* genus includes the major commercial fruits oranges, mandarins, limes, lemons, and grapefruits. Citrus essential oils, commonly used as a fragrance, are extracted from the peel by methods including methods such as water, steam, organic solvent extraction, as well as cold pressing (Lin et al., 2010). Citrus fruit oils and extracts can provide a natural way to kill microorganisms and extend the shelf life of foods. Citrus oils consist mainly of monoterpene hydrocarbons and other volatile compounds (Sawamura et al., 2004). Lin and others (2010) found, through gas chromatography (GC), the three main chemicals of sweet orange essential oil are limonene (88.4%), linalool (3.49%), and β -pinene (0.49%). Grape fruit seed extract (GFSE) is obtained by grinding the grapefruit seeds and pulp, then mixing them with glycerin (Bevilacqua et al., 2010; von Woedtke et al., 1999). The main constituents of GFSE and pulp are flavonoids, limonoids and sterols, ascorbic acid, tocopherols, and citric acids (Bevilacqua et al., 2010; Tirillini, 2000; Armando et al., 1998; Braddock and Bryan, 2001). Studies have shown that GFSE possess antibacterial, antifungal, antiviral, and antiparasitic properties (Reagor et al., 2002; Xu et al., 2007). Reagor and others (2002) tested the effect of GFSE on 67 different microorganisms, and found that they were all susceptible, with the various

strains of staphylococci and enterococci averaging the greatest susceptibilities. The exact mechanism is not known, but it is attributed to the phenolic compounds, including catechins, epicatechin, and trimeric and tetrameric procyanidins (Saito et al., 1998).

Based on the current literature status of foodborne viral outbreaks and novel processing methods available for inactivating pathogens to ensure food safety, hurdle approaches using processing conditions and natural phenolics may be an alternative to improve food safety and reduce commercial processing costs. In addition, determining survival of these viruses in foods will help determine appropriate mitigation strategies needed to prevent outbreaks and provide data needed to determine risks associated with foodborne viral outbreaks. Therefore, the objectives of the research were (1) to determine the survival of FCV-F9, MNV-1, and MS2 in milk and fruit juices (orange, pomegranate, and juice blends) over 21 days at refrigeration (4°C) (2) to determine inactivation of FCV-F9, MNV-1, and MS2 in fluid matrices such as milk and fruit juices using high pressure homogenization (HPH) and to determine their inactivation in milk and orange juice with the addition of lecithin using HPH and (3) to determine the survival of FCV-F9, MV-1, and MS2 in blueberry juice (BJ) over 21 days at refrigeration (4°C) and their inactivation in BJ using HPH.

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Appendix

Table 1.1: Foodborne Viral Outbreaks

Virus	Year	Food/Location	Sample Type	Detection Method	Comment	Reference
Hepatitis A	1968	Pastry icing/Bakery	NG	Epidemiology	61 persons infected from sick food handler	Schoenbaum et al., 1976
	1974	Salad, fresh fruit/ Restaurant	NG	Epidemiology	133 persons infected by personal contact with case patient	Hooper et al., 1977
	1975	Salads/Restaurant	NG	Epidemiology	33 persons infected	Denes et al., 1977
	1986	Salad/Restaurant	NG	Epidemiology	103 persons infected	Lowry et al., 1989
	1988	Iceberg lettuce/3 restaurants	NG	Epidemiology	202 person infected in Kentucky/ harvesting or processing contamination	Rosenblum et al., 1990
	1990	Frozen strawberries/2 schools	NG	Epidemiology	Harvesting or processing contamination/ 35 persons infected from schools in Georgia and Montana	Niu et al., 1992
	1991	Sandwiches/2 Restaurants	NG	Epidemiology	228 persons infected	CDC, 2003
	1992	Multiple foods/Caterer	NG	Epidemiology	43 persons infected	Dalton et al., 1996
	1994	Glazed baked goods/Bakery	NG	Epidemiology	64 persons infected	Weltman et al., 1996
	1997	Frozen strawberries	NG	Epidemiology	262 persons infected across 5 states/ harvesting or processing contamination	Hutin et al., 1999

*(adapted from D'Souza et al., 2007 and Fiore, 2004)

Table 1.1: Foodborne Viral Outbreaks (continued)

Virus	Year	Food/Location	Sample Type	Detection Method	Comment	Reference
Hepatitis A	2001	Sandwiches/ Restaurant	NG	Epidemiology	43 person infected from worker who had colostomy	Friedman et al., 2003
	2002	Blueberries	Clinical and food	RT-PCR; sequencing	Food handlers or polluted ground water	Calder et al., 2003
	2003	Green Onion	Clinical	Serological	Preharvest or postharvest contamination	CDC, 2003
	2004	Orange juice/Egyptian hotel	Clinical	RT-PCR	351 tourists infected/ poor hygiene at processing plant	Frank et al., 2007
Norovirus		Deli meats	Clinical and food	RT-PCR (nested); sequencing	Food and clinical samples had identical sequences	Daniels et al., 200, Schwab et al., 2000
		Boxed lunch	Clinical	RT-PCR; electron microscopy	Person to person transmission at a football game	Becker et al., 2000
		Potato salad	Clinical	RT-PCR; electron microscopy	Airborne transmission; kitchen worker vomited in sink	Patterson et al., 1997
		Oysters	Clinical and food	IgG antibody detection; electron microscopy; RT-PCR; sequencing	Disposal of sewage from handlers	Berg et al., 2000
		Raspberries	Clinical and food	RT-PCR; sequencing	Contaminated water	Ponka et al., 1999
		Deli sandwiches	Clinical	RT-PCR (single and nested); sequencing	Asymptomatic food handler	Parashar et al., 1998
	2009	Salad/German military base canteen	Clinical, food, environmental	RT-PCR	36 persons infected by suspected contaminated food worker	Wadl et al., 2009

*(adapted from D'Souza et al., 2007 and Fiore, 2004)

Table 1.2: The Effect of Heat Treatment on Foodborne Viruses

Virus	Matrix	Temp (°C)	Time (min)	Log₁₀ reduction	Reference
Hepatitis A	Milk	85	< 0.05	5	Bidawid et al., 2000
	Milk (skimmed; homogenized; cream)	71	6.55; 8.31; 12.67	4; 4; 4	Bidawid et al., 2000
	Milk	62.8; 71.6	30; 0.25	3; 2	Parry and Mortimer., 1984
	Milk	63	30	3	Mariam and Cliver., 2000
	1 g strawberry mashes (28°Brix; 52°Brix)	85	0.96; 4.98	1; 1	Deboosere et al., 2004
	1 g strawberry mashes (52°Brix)	80	8.94	1	Deboosere et al., 2004
	4 ml virus suspension	60; 80	10; 3	> 4.6; > 4.6	Croci et al., 1999
	4 ml shellfish homogenate	60; 80	10; 3	2; 2	Croci et al., 1999
Poliovirus	Milk	72	0.25; 0.5	0.56; > 5	Strazynski et al., 2002
	Oysters	Steaming	30	2	Di Girolamo et al., 1970
Rotavirus	Cell culture medium	60	10	7	Mahony et al., 2000
Feline calicivirus	Cell culture medium	71.3	1	3	Duizer et al., 2004
	Cell culture medium	37; 56	24 h; 8	3; 3	Duizer et al., 2004
	Cell culture medium	70	1.5	6	Buckow et al., 2008
	Cell culture medium	63	0.41	1	Cannon et al., 2006
	Cell culture medium	72	0.12	1	Cannon et al., 2006
	Cell culture medium	56; 56	3; 60	NR; 7.5	Doultree et al., 1999
	Cell culture medium	70	1; 3; 5	3; 6.5; 7.5	Doultree et al., 1999
	Cell culture medium	Boiling	1	7.5	Doultree et al., 1999

*(adapted from Baert et al., 2009)

Table 1.2: The Effect of Heat Treatment on Foodborne Viruses (continued)

Virus	Matrix	Temp (°C)	Time (min)	Log₁₀ reduction	Reference
Norovirus	Stool filtrate	60	30	Incomplete	Dolin et al., 1972
Murine norovirus 1	Raspberry puree (9.2°Brix)	65; 75	0.5; 0.25	1.86; 2.81	Baert et al., 2008
	Cell culture medium	80	2.5	6.5	Baert et al., 2008
	Cell culture medium	63; 72	0.44; 0.17	1; 1	Cannon et al., 2006

*(adapted from Baert et al., 2009)

Table 1.3: Effect of Ultrasound Treatment on Foodborne Microorganisms

Microorganism/ Medium	Frequency (kHz)	Acoustic Power (W)	Acoustic Intensity (W/cm ²)	Temp (°C)	Time (min)	Log ₁₀ Reduction or *D- value	Reference
<i>Listeria monocytogenes</i> / UHT ¹ milk	20	150	118	57	18	5	D'Amico et al., 2006
<i>E.coli</i> O157:H7/apple cider	20	150	118	57	18	6	D'Amico et al., 2006
<i>Listeria innocua</i> /whole milk	24	400	2.85	63	30	2.5	Bumudez-Aguirre and Barbosa-Canovas, 2008
<i>Listeria innocua</i> /fat-free milk	24	400	2.85	63	30	4.9	Bumudez-Aguirre and Barbosa-Canovas, 2008
<i>Listeria innocua</i> /raw whole milk	24	400	2.85	63	10	>5	Bumudez-Aguirre et al., 2009
Total mesophilic bacteria/pulp-free orange juice	23	600	NG	88	15	1.7	Valero et al., 2007
<i>L. monocytogenes</i> / orange juice with citral (75 ppm)	20	600	NG	45	15	2	Ferrante et al., 2007
<i>L. monocytogenes</i> / orange juice with vanillin (1000 ppm)	20	600	NG	45	15	1.8	Ferrante et al., 2007
<i>Alicyclobacillus acidoterrestris</i> / apple juice	24	600	NG	RT	-	*40.17	Yuan et al., 2009
<i>Pseudomonas fluorescens</i> / TSB	20	150	NG	76.1	CF ²	4.2	Villamiel and Jong, 2000
<i>Streptococcus thermophilus</i>	20	150	NG	75.9	CF ²	2.7	Villamiel and Jong, 2000

¹ Ultrahigh-temperature

² Continuous flow system

*D-value

Table 1.3: Effect of Ultrasound Treatment on Foodborne Microorganisms (continued)

Microorganism/ Medium	Frequency (kHz)	Acoustic Power (W)	Acoustic Intensity (W/cm ²)	Temp (°C)	Time (min)	Log ₁₀ Reduction or *D- value	Reference
Murine norovirus	20	750	NG	< 21	30	0.07	Su et al., 2010c
Feline calicivirus	20	750	NG	< 21	30	2.67	Su et al., 2010c
Bacteriophage MS2	20	750	NG	< 21	30	4.62	Su et al., 2010c

¹ Ultrahigh-temperature

² Continuous flow system

*D-value

Table 1.4: Classification of Irradiated Foods

Group	Food Items
1	Disinfection of spices and dry vegetables
2	Disinfection of grains and fruits
3	Disinfection of meat and seafood
4	Sprout inhibition of root crops and bulbs
5	Other food items

*(adapted from Kume et al., 2009)

Table 1.5: Gamma Irradiation Inactivation of Foodborne Microorganisms

Microorganism	Product	Temp (°C)	Atmosphere	D ₁₀ (kGy)	Reduction (log ₁₀)	Reference
Gram positive bacteria						
<i>Bacillus cereus</i> (vegetative cells)	Roast beef	Not stated	Air	0.173 ± 0.157		Grant and Patterson, 1992
	Gravy	Not stated	Air	0.181 ± 0.167		Grant and Patterson, 1992
	Cauliflower (cooked, crushed)	Not stated	Air	0.207 ± 0.099		Grant and Patterson, 1992
	potato	Not stated	air	0.199 ± 0.056		Grant and Patterson, 1992
<i>Campylobacter jejuni</i>	Filet American	18-20	Micro-aerophilic	0.08 – 0.11		Tarkowski et al., 1984
	Ground beef	18-20	Micro-aerophilic	0.14 – 0.16		Tarkowski et al., 1984
	Ground turkey	-30 ± 10	air	0.293		Lambert 1992
<i>Clostridium botulinum</i> (spores)	Phosphate buffer (pH 7.0)	-30 ± 3	vacuum	1.0 – 3.2		Anellis et al., 1972
<i>Listeria innocua</i>	lettuce	15	air	0.19		Trigo et al., 2009
<i>Listeria monocytogenes</i>	Shrimp paste	Not stated	air	0.25		Rashid et al., 1992
	Minced chicken	Not stated	air	0.417 – 0.553		Patterson, 1989
	Roast beef	Not stated	air	0.644 ± 0.061		Grant and Patterson, 1992
	Cauliflower (cooked, crushed)	Not stated	air	0.564 ± 0.055		Grant and Patterson, 1992
	Potato	Not stated	air	0.532 ± 0.047		Grant and Patterson, 1992

*low protein stocks

**radiation dose (kGy) only

Table 1.5: Gamma Irradiation Inactivation of Foodborne Microorganisms (continued)

Microorganism	Product	Temp (°C)	Atmosphere	D ₁₀ (kGy)	Reduction (log ₁₀)	Reference
Gram negative bacteria						
<i>Escherichia coli</i>	Minced chicken	4	air	0.351 – 0.388		Patterson, 1988
	Minced pork	10	air	0.339		Grant and Patterson, 1991
<i>Escherichia coli</i> O157:H7	Ground beef (low fat)	-16 ± 1	air	0.307		Clavero et al., 1994
	lettuce	15	air	0.14		Trigo et al., 2009
<i>Salmonella typhimurium</i>	Ground beef	18-20	air	0.55		Tarkowski et al., 1984
	Cauliflower (cooked, crushed)	Not stated	air	0.590 ± 0.075		Grant and Patterson, 1992
	potato	Not stated	air	0.464 ± 0.080		Grant and Patterson, 1992
	Minced chicken	4	air	0.436 – 0.502		Patterson, 1988
Viruses						
Canine calicivirus*	tap water	25		0.30**	3	De Roda et al., Husman 2004
Coxsackie virus	Ground beef (raw)	-30	air	7.5		Sullivan et al., 1973
		-90	Air	6.8		Sullivan et al., 1973
	Ground beef (cooked)	-30	Air	6.8		Sullivan et al., 1973
		-90	Air	8.1		Sullivan et al., 1973
F-coliphage	Hard-shelled clams	4		13.5		Harewood 1994

*low protein stocks

**radiation dose (kGy) only

Table 1.5: Gamma Irradiation Inactivation of Foodborne Microorganisms (continued)

Microorganism	Product	Temp (°C)	Atmosphere	D ₁₀ (kGy)	Reduction (log ₁₀)	Reference
Feline calicivirus*	Tap water	25		0.5**	3	De Roda Husman et al., 2004
Hepatitis A virus	Live oysters	Not stated	Air	2.02		Mallett et al., 1991
	strawberries	Not stated	air	2.97		Bidawid et al., 2000
MS2	Tap water	25		0.120**	3	De Roda Husman et al., 2004
Poliovirus	Hard-shelled clams	Not stated	Air	3.1		Mallett et al., 1991
Rotavirus		Not stated	Air	2.4**	1	Mallett et al., 1991

*low protein stocks

**radiation dose (kGy) only

Table 1.6: Pulsed Electric Field Inactivation of Foodborne Microorganisms

Microorganism	Medium	Treatment Parameters*	Log Reduction	Reference
Gram negative bacteria				
<i>E. coli</i> NCTC 9534	Citric-phosphate buffer	2, 4.0-7.0, 25, 50-300, 300-1800, < 35	2.5-6.2	Garcia, Gomez, Raso et al., 2005
<i>E. coli</i> NCTC 8739	Orange-juice-milk beverage	2.91, 4.05, 15-40, 2.5, 0-700, NR, < 55	3.8	Rivas et al., 2006
<i>E. coli</i>	Carrot juice	5.1, 6.05, 20, 1.5, 310-2176, 400, < 40	3.8	Zhong, Chen, Wu, Wang, Liao, Hu, and Zhang, 2005
<i>E. coli</i>	Watermelon juice	3.66, 5.46, 35, 4, 2000, 7541, < 40	4.0	Mosqueda-Melgar et al., 2007
<i>E. coli</i> O157:H7	Apple juice	2.3, 3.7, 31, 4, 202, 705, 29	2.6	Evrendilek & Zhang 2005
<i>E. coli</i> O157:H7	Skim milk	6.2, 6.7, 24, 2.8, 141, 548, 30	1.3-2.0	Evrendilek & Zhang 2005
<i>Salm. Enteritidis</i>	Skim milk	4.5-6.8, 6.5, 35-55, 0.25-3, 2.1-3.5, 30-90, 62	1.4	Floury, Grosset, Leconte, Pasco, Madec, & Jeantet 2006
<i>Salm. Typhimurium</i> ATCC 13311	Citric-phosphate buffer	2, 3.0-7.0, 12-25, 2, 20-400, NR, < 35	1.0-3.0	Garcia, Gomez, Manas, et al., 2005
<i>Pseudomonas fluorescens</i>	Skim milk	5.1, 6.7, 24-31, 2, 20, 65-139, < 15-55	>5	Craven et al., 2008
<i>Yersinia enterocolitica</i> ATCC 9610	Citric-phosphate buffer	2, 3.0-7.0, 12-25, 2, 20-400, NR, <35	4.0-6.0	Garcia, Gomez, Manas, et al., 2005
Gram positive bacteria				
<i>Lactobacillus brevis</i>	Orange juice	4.24, 3.56, 5-35, 4, 50-100, 5194, 32	5.8	Elez-Martinez et al., 2005
<i>Lact. Plantarum</i>	Orange juice-milk beverage	2.91, 4.05, 15-40, 2.5, 0-700, NR, < 55	2.5	Sampedro et al., 2006

*Parameters: conductivity pH (mS cm⁻¹), PEF intensity (kV cm⁻¹), pulse width (μs), total pulse time (μs), total energy (kJ L⁻¹), temperature (°C), respectively.

*NR = Not Reported

*adapted from Wan et al., 2009

Table 1.6: Pulsed Electric Field Inactivation of Foodborne Microorganisms (continued)

Microorganism	Medium	Treatment Parameters*	Log Reduction	Reference
<i>Listeria innocua</i>	Citric-phosphate buffer	4.53, 7.0, 35-45, 2, 10-70, 50-300, 15-65	1-6	San Martin et al., 2007
<i>L. monocytogenes</i> ATCC 15313	Citric-phosphate buffer	2, 3.0-7.0, 12-25, 2, 20-400, NR*, < 35	1.5-6.0	Garcia, Gomez, Manas, et al., 2005, Gomez et al., 2005
<i>L. monocytogenes</i> ATCC 51742	Watermelon juice	3.66, 5.46, 35, 4, 2000, 7541, < 40	3.77	Mosqueda-Melgar et al., 2007
<i>Staphylococcus aureus</i>	Citric-phosphate buffer	2, 7.0, 15-28, 2, 50-400, NR, < 35	1.5-4	Rodriguez-Calleja et al., 2006

Table 1.7: Effect of High Intensity Pulsed Light Treatment on Foodborne Microorganisms

Microorganism	Medium	Energy (J)	No. of pulses	Initial Population (log)	Reduction (log)	Reference
Bacteria						
Aerobic mesophiles	Bulk tank milk	25	110	3	> 2	Smith et al., 2002
Aerobic mesophiles	White cabbage	7	675	3.64	0.64	Gomez-Lopez et al., 2005
Aerobic mesophiles	Iceberg lettuce	7	675	6.5	1.24	Gomez-Lopez et al., 2005
<i>Pseudomonas aeruginosa</i>	Agar	3	200	8.7	6.8	Rowan et al., 1999
<i>Pseudomonas fluorescens</i>	Agar	7	50	5.6	4.2	Gomez-Lopez et al., 2005
<i>Salmonella enteritidis</i>	Shelled eggs	0.5	8	7.92	7.92	Dunn 1995
<i>Salmonella enteritidis</i>	Agar	3	200	9.7	5.6	Rowan et al., 1999
<i>Salmonella typhimurium</i>	Agar	7	50	5.4	3.2	Gomez-Lopez et al., 2005
<i>Eschericia coli</i>	Alfalfa seeds	5.6	135	8	0.94-1.82	Shamara and Demicri 2003
<i>Eschericia coli</i>	Salmon fillets	5.6	135	8.7	0.24-0.91	Ozer and Demicri 2005
<i>Eschericia coli</i>	Agar	7	50	5.3	4.7	Gomez-Lopez et al., 2005
<i>Eschericia coli</i>	Agar	3	512	8.3	6.82	MacGregor et al., 1998
<i>Listeria monocytogenes</i>	Salmon fillets	5.6	135	8.7	0.72-.08	Ozer and Demicri 2005
<i>Listeria monocytogenes</i>	Agar	7	50	5	2.8	Gomez-Lopez et al., 2005
<i>Listeria monocytogenes</i>	Agar	3	200	9.4	4.4	Rowan et al., 1999

*Fetal calf serum

*adapted from Elmnasser et al., 2007 and Roberts and Hope, 2003.

Table 1.7: Effect of High Intensity Pulsed Light Treatment on Foodborne Microorganisms (continued)

Microorganism	Medium	Energy (J)	No. of pulses	Initial Population (log)	Reduction (log)	Reference
<i>Staphylococcus aureus</i>	Agar	3	200	9.4	5.1	Rowan et al., 1999
<i>Staphylococcus aureus</i>	agar	7	50	5.5	> 5.1	Gomez-Lopez et al., 2005
<i>Clostridium perfringens</i>	Agar	7	50	3.3	> 2.9	Gomez-Lopez et al., 2005
Fungi						
<i>Monilia fructigena</i>	Agar	7	1500	6	4	Marquenie et al., 2003
<i>Aspergillus niger</i>	Saline solution	1	5	6	4.8	Wekhof et al., 2001
Yeasts						
<i>Saccharomyces cerevisiae</i>	Agar	3	100	8.4	3.7	Rowan et al., 1999
<i>Candida lambica</i>	Agar	7	50	3.4	2.8	Gomez-Lopez et al., 2005
Viruses						
Herpes simplex virus type 1 (enveloped)	PBS+5%v/v *FCS	1	1	NG	2.2	Roberts and Hope, 2003
Sindbis (enveloped)	PBS+5%v/v *FCS	1	1	NG	3.1	Roberts and Hope, 2003
Encephalomyocarditis	PBS+5%v/v *FCS	1	1	NG	3.6	Roberts and Hope, 2003
Polio virus type 1	PBS+5%v/v *FCS	1	1	NG	3.2	Roberts and Hope, 2003
Hepatitis A virus	PBS+5%v/v *FCS	1	1	NG	4.1	Roberts and Hope, 2003
Bovine parvovirus	PBS+5%v/v *FCS	1	1	NG	1.5	Roberts and Hope, 2003
Canine parvovirus	PBS+5%v/v *FCS	1	1	NG	> 6.4	Roberts and Hope, 2003

*Fetal calf serum

*adapted from Elmnasser et al., 2007 and Roberts and Hope, 2003.

Table 1.8: Effect of High Hydrostatic Pressures on Viruses

Virus	Product	Pressure (MPa)	Time (min)	Temp (°C)	Reduction (Log₁₀)	Reference
HAV	Oysters	400	1	9	3	Calci et al., 2005
	Strawberries	375	5	21	4.3	Kingsley et al., 2005
	Green Onions	375	5	21	4.7	Kingsley et al., 2005
	Sausages	500	5	4	3.23	Sharma et al., 2008
	Oyster slurry	500	10	20	ND	Black et al., 2010
	Cell culture medium	450	5	Ambient	>6	Kingsley et al., 2002
	Cell culture medium	400	10	Ambient	>2	Grove et al., 2008
Rotavirus	Cell culture medium	300	2	25	8	Khadre and Yousef., 2002
Poliovirus	Cell culture medium	600	5	Ambient	No red	Kingsley et al., 2004
	Cell culture medium	600	60	20	No red	Wilkinson et al., 2001
	Cell culture medium	600	5	Ambient	No red	Grove et al., 2008
Aichivirus	Cell culture medium	600	5	Ambient	No red	Kingsley et al., 2004
Coxsackievirus B5	Cell culture medium	600	5	Ambient	No red	Kingsley et al., 2004
Coxsackievirus A9	Cell culture medium	600	5	Ambient	7.6	Kingsley et al., 2004
FCV	Cell culture medium	275	5	Ambient	>6	Kingsley et al., 2002
	Cell culture medium	200	4	-10	5	Chen et al., 2005
	Cell culture medium	200	4	20	0.3	Chen et al., 2005
	Sausages	300	3	Ambient	5	Grove et al., 2008

*adapted from Baert et al., 2009

Table 1.8: Effect of High Hydrostatic Pressures on Viruses (continued)

Virus	Product	Pressure (MPa)	Time (min)	Temp (°C)	Reduction (Log₁₀)	Reference
FCV	Oyster tissue	500	5	4	2.89	Sharma et al., 2008
	Mussels	250	5	20	2.84	Murchie et al., 2006
MS2	Cell culture medium	600	10	21	3.5	Guan et al., 2006
	Sausages	500	5	4	1.47	Sharma et al., 2008
MNV-1	Cell culture medium	450	5	20	6.85	Kingsley et al., 2007
	Oysters	400	5	5	4	Kingsley et al., 2007
ΦX 174	Cell culture medium	600	60	21	1	Guan et al., 2006
Λ imm434	Cell culture medium	400	5	21	7.5	Guan et al., 2006
T4	Cell culture medium	450	5	21	6	Guan et al., 2006
T4	Oyster slurry	500	10	20	5.29	Black et al., 2010
f2	PBS	550	5	21	2	Guan et al., 2006
GA	PBS	550	5	21	2	Guan et al., 2006
Qβ	PBS	400	5	21	1	Guan et al., 2006
SP	PBS	400	5	21	1	Guan et al., 2006

*adapted from Baert et al., 2009

Table 1.9: Effect of High Pressure Homogenization on Foodborne Microorganisms

Microorganism	Medium	Pressure (MPa)	Temp (°C)	Log ₁₀ Reduction	Reference
Gram-positive bacteria					
<i>Bacillus subtilis</i>		100		3	Popper and Knorr, 1990
<i>Enterococcus faecalis</i>	PBS	300	25	< 2	Wuytack et al., 2002
<i>Enterococci</i>	Whole caprine milk	100	NG	3	Guerzoni et al., 1999
<i>Lactobacillus plantarum</i>	PBS	300	25	< 2	Wuytack et al., 2002
<i>Leuconostoc dextranicum</i>	PBS	300	25	< 2	Wuytack et al., 2002
<i>Listeria innocua</i>	PBS	300	25	< 2	Wuytack et al., 2002
<i>Listeria innocua</i>	Carrot juice	350	< 10	5	Pathanibul et al., 2009
<i>Staphylococcus aureus</i>	PBS	300	25	< 2	Wuytack et al., 2002
<i>Streptococcus lactis</i>		100		1	Popper and Knorr, 1990
Gram-negative bacteria					
<i>E. coli</i> MG1655	PBS	200	25	~2.6	Wuytack et al., 2002
<i>E. coli</i> LMM1010	PBS	200	25	~2.9	Wuytack et al., 2002
<i>E. coli</i> K-12	Apple juice	200	2	4.11	Kumar et al., 2009
<i>E. coli</i> K-12	0.9% NaCl water	225	~60	7	Taylor et al., 2007
<i>Escherichia coli</i>	Apple juice	> 250	< 10	> 5	Pathanibul et al., 2009
<i>Escherichia coli</i>		100		3	Popper and Knorr, 1990

Table 1.9: Effect of High Pressure Homogenization on Foodborne Microorganisms (continued)

Microorganism	Medium	Pressure (MPa)	Temp (°C)	Log ₁₀ Reduction	Reference
<i>Pseudomonas fluorescens</i>	PBS	200	25	4.6	Wuytack et al., 2002
<i>Salmonella typhimurium</i>	PBS	200	25	~2	Wuytack et al., 2002
<i>Shigella flexneri</i>	PBS	200	25	~3.3	Wuytack et al., 2002
<i>Yersinia enterocolitica</i>	PBS	200	25	~2	Wuytack et al., 2002
Viruses					
MS2 Coliphage	PBS	300	75	3	D'Souza et al., 2009
Murine norovirus-1	PBS	300	75	0.8	D'Souza et al., 2009

Table 1.10: The Efficacy of Decontamination Procedures of Fresh Produce to Reduce the Level of Viruses

Virus	Matrix	Decontamination Procedure	Log ₁₀ Reduction	Reference
Hepatitis A virus	10 g lettuce/fennel/carrot/100ml	Water 5 min	0.1, 1, 0.9	Croci et al., 2002
	15 g strawberries/200 ml	NaOCl 200 ppm, 0.5 min	1.0 ^b	Butot et al., 2008
	1.2 g lettuce/30 ml	20 ppm chlorine 10 min	>1.7	Casteel et al., 2008
Rotavirus	15 g strawberries/200ml	Water 0.5 min	1.5	Butot et al., 2008
	15 g strawberries/200ml	NaOCl 200 ppm, 0.5 min	>1.5 ^b	Butot et al., 2008
Canine calicivirus	Cell culture medium	NaOCl 300 ppm, 10 min	> 3	Duizer et al., 2004
Feline calicivirus	100 g strawberries/100 ml	PAA ^c 300 ppm; 150 ppm 10 min	3 ^b ;1 ^b	Gulati et al., 2001
	10 g lettuce/100 ml	PAA ^c 300 ppm; 150 ppm 10 min	3 ^b , 2 ^b	Gulati et al., 2001
	100 g strawberries/100 ml; 10 g lettuce/100 ml	Water 10 min	2;2	Gulati et al., 2001
	100 g strawberries/100 ml	NaOCl 200 ppm; 800 ppm 10 min	0 ^b ;1 ^b	Gulati et al., 2001
	10 g lettuce/100 ml	NaOCl 200 ppm; 800 ppm 10 min	0 ^b ;1.5 ^b	Gulati et al., 2001
	3 cm ² disks of lettuce in 5 ml sanitizer solution, 2 min	Bleach 50 ppm; 100 ppm; 200 ppm; PAA 80 ppm, 3 % H ₂ O ₂	2.2; 2.6; 2.9; 2.9; 2.8	Allwood et al., 2004
	15 g strawberries/200 ml	NaOCl 200 ppm, 0.5 min	>1.6 ^b	Butot et al., 2008
	Cell culture medium	NaOCl 300 ppm, 10 min	< 2	Duizer et al., 2004
Murine norovirus 1	2 onion bulbs/100 ml	Water 0.42 min	0.4	Baert et al., 2008
	10 g spinach leaves/350 ml	Water 2 min	1.0	Baert et al., 2008

^b Compared to water

* adapted from Baert et al., 2009

Table 1.10: The Efficacy of Decontamination Procedures of Fresh Produce to Reduce the Level of Viruses (continued)

Virus	Matrix	Decontamination Procedure	Log₁₀ Reduction	Reference
Murine norovirus 1	50 g lettuce/500 ml	Water 5 min	1.1	Baert et al., in press
	50 g lettuce/500 ml	NaOCl 200 ppm, 5 min	1.0 ^b	Baert et al., in press
	50 g lettuce/500 ml	PAA 80 ppm; 250 ppm, 5 min	0.8 ^b ; 1.4 ^b	Baert et al., in press
MS2	100 g lettuce/1 L	Chlorine 100 ppm, 5 min	0.7	Dawson et al., 2005
	1.2 g lettuce/30 ml	Chlorine 20 ppm, 10 min	> 1.8	Casteel et al., 2008
	3 cm ² disks of lettuce in 5 ml sanitizer solution, 2 min	Bleach 50 ppm; 100 ppm; 200 ppm; PAA 80 ppm; 3% H ₂ O ₂	1.9; 2.7; 2.9; 2.8; 2.6	Allwood et al., 2004
	5 cm ² sections of lettuce	10 s H ₂ O ₂ (2%) followed by 30 s UV (0.63 mW s/cm ²), 50°C	4.1	Xie et al., 2008
	Cut lettuce (5 cm ²) 200 ppm 3 min	Ca(ClO) ₂ 200 ppm 3 min	1.7	Xie et al., 2008

^b Compared to water

* adapted from Baert et al., 2009

Table 1.11: Dietary Sources of Plant Phenolics

Phenolic compounds	Dietary sources
<i>Phenolic acids</i>	
Hydroxybenzoic acids	Blueberries, cereals, cranberries, oilseeds
Hydroxycinnamic acids	Apricots, blueberries, carrots, cereals, pears, cherries, citrus fruits, oilseeds, peaches, plums, spinach, tomatoes, eggplants
<i>Flavonoids</i>	
Anthocyanins	Bilberries, black and red currants, blueberries, cherries, chokecherries, grapes, strawberries
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, cranberries, endive, leeks, lettuce, onions, olive, pepper, tomatoes
Chalcones	Apples
Flavones	Citrus fruits, celery, parsley, spinach, rutin
Isoflavones	Soybeans
Xanthones	Mango, mangosteen
<i>Tannins</i>	
Condensed	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolysable	Pomegranate, raspberries

*adapted from Naczk and Shahidi, 2006

Table 1.12: Natural Plant Components, Health Benefits, and Antimicrobial Effects

Plant Source	Active Component	Health Benefit	Antimicrobial Effect	Reference
Elderberry extract (<i>Sambucus nigra</i>)	Anthocyanins, tannins	Antioxidant, anti-inflammatory	Influenza virus A,B, Herpes Type 1	Zakay-Rones et al. (2004), Serkedjieva et al. (1990)
Olive leaf extract (<i>Olea europaea</i>)	Polyphenolic compounds oleuropein and hydroxytyrosol	Antioxidant, anti-inflammatory	HIV-1, <i>Bacillus cereus</i> , <i>E. coli</i> , <i>Candida albicans</i>	Lee-Huang et al. (2003), Pereira et al. (2007)
Pomegranate (<i>Punica granatum</i>)	Anthocyanins, proanthocyanidins (PAC), ellagitannins	Antioxidant, anti-inflammatory, antidiabetic	<i>Staphylococcus aureus</i> , <i>Klebsiella pneumonia</i> , human influenza A/Hong Kong (H3N2), HIV-1	Viuda-Martos et al. (2010), Haidari et al. (2009), Neurath et al. (2005)
American Cranberry (<i>Vaccinium macrocarpum</i>)	Anthocyanins, proanthocyanidins (PAC)	Antitumor, antiulcer, antioxidant, anti-inflammatory, UTI prevention	(<i>Escherichia coli</i> , <i>Listeria monocytogenes</i> , FCV-F9, MNV-1, MS2, reovirus, influenza A	Viskelis et al. (2009), Su et al. (2010a), Lipson et al. (2007), Weiss et al. (2005)
Blueberry (<i>Vaccinium corymbosum</i>)	Anthocyanins, proanthocyanidins (PAC), quercetin	Antioxidant, cardiovascular health	(<i>Citrobacter freundii</i> , <i>Enterococcus faecalis</i> , hepatitis C virus	Burdulis et al. (2009), Takeshita et al. (2009)
Orange (<i>Citrus sinensis</i>)	flavonoids, phenolic acids, terpenes, carotenoids	Antioxidant	<i>Staphylococcus aureus</i> , <i>Escherichia coli</i>	Benelli et al. (2010)
Grape seed (<i>Vitis vinifera</i>)	anthocyanins, proanthocyanidins (PAC), flavan-3-ols, flavonols	Antioxidant, cardiovascular health	<i>Bacillus cereus</i> , <i>Bacillus subtilis</i> , FCV-F9, coxsackievirus A7, reovirus	Butkhup et al. (2010), Iwasawa et al. (2009), Lipson et al. (2007)
Grapefruit (<i>Citrus paradise</i>)	Flavanones naringin and hesperidin	Antioxidant, anti-inflammatory	<i>Staphylococcus xylosus</i> , <i>Lactobacillus. Sakei</i> , <i>L. curvatus</i> , <i>Salmonella enteritidis</i> , hepatitis C virus	Viuda-Martos et al. (2008), Cvetnic and Vladimir-Knezevic (2004), Nahmias et al. (2007)

CHAPTER II

Survival of Human Norovirus Surrogates in Milk, Orange and Pomegranate Juice, and Juice Blends at Refrigeration (4°C)

Human Norovirus Surrogate Survival in Milk and Orange Juice

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Abstract

Fresh fruits, juices and beverages have been implicated in human noroviral and hepatitis A virus outbreaks. The purpose of this study was to determine the survival of human norovirus surrogates (murine norovirus, MNV-1; feline calicivirus, FCV-F9; and bacteriophage MS2) in juices (orange and pomegranate juices), juice blends (pomegranate and orange juice) and milk over 0, 1, 2, 7, 14, and 21 days at refrigeration (4°C). Juices, juice blends, and milk were inoculated with each virus over 21 days, serially diluted in cell culture media, and plaque assayed. MNV-1 showed no reduction in titer after 21 days in orange juice and milk, but moderate reduction (1.4 log) in pomegranate juice from a titer of 5 log₁₀ PFU/ml. However, MNV-1 was completely inactivated after 7 days in the orange and pomegranate juice blend. FCV-F9 from a titer of 6 log₁₀ PFU/ml was completely reduced after 14 days in orange as well as pomegranate juice and by ~ 3 logs after 21 days in milk at 4°C. Interestingly, FCV-F9 was completely inactivated after 1 day in the orange and pomegranate juice blend at 4°C. MS2 was reduced by ~1.28 log after 21 days in orange juice from a titer of 6 log₁₀ PFU/ml, and < 1 log after 21 days in milk or pomegranate juice, with juice blends showing minimal reduction (< 1 log) after 21 days at 4°C. These results show the survival pattern of noroviruses that aid in the transmission of foodborne viral outbreaks. The data obtained can be used in quantitative viral risk assessment studies and to develop improved measures to prevent virus survival towards controlling outbreaks.

Introduction

Foodborne outbreaks that cause gastroenteritis and death worldwide are increasingly recognized as being viral in origin. Some of the epidemiologically significant human enteric viruses of public health concern include the human noroviruses (NoV), hepatitis A virus (HAV), rotaviruses, astroviruses, adenoviruses, Aichi viruses, human enteroviruses such as coxsackieviruses and echoviruses, parvoviruses, and other small round viruses (Sair et al., 2002). Noroviruses, in particular, are responsible for 96% of all gastroenteritis cases, according to the Centers for Disease Control, with over 23 million annual viral gastroenteritis cases in the United States alone (Donaldson et al, 2008; Mead et al., 1999; 2000). Several human NoV and HAV outbreaks have been associated with fruits (Calder et al., 2003) and juices, including a 2004 HAV outbreak in Egypt that sickened 351 persons due to contaminated orange juice (Fiore, 2004; Frank et al., 2007). Also, in November 2008, a outbreak at a university dining hall in Michigan sickened 451 persons, where an ill food service worker worked briefly before being sent home (CDC, 2010a). These outbreaks show the need to have better hygienic practices and control measures to prevent foodborne viral outbreaks in the food industry. It is therefore important to better understand viral survival and transmission in the environment.

Enteric viruses are transmitted primarily via the fecal-oral route by contaminated water and food. Foods, particularly fruits and vegetables, can become contaminated during harvest by irrigation waters or poor hygienic systems surrounding the farm. However, an added complication is the secondary spread from person-to person via vomitus, aerosols, and fomites (Greening, 2006). Ill persons preparing foods, especially ready-to eat (RTE) foods, are a major source of human NoV infection as described above. Persons that are ill commonly have symptoms of diarrhea and vomiting; vomitus can become aerosolized and further spread the

illness. Foods at risk include shellfish, fresh produce, and berry fruits and juices, and other RTE foods that do not undergo further processing (Sair et al., 2002).

Human NoV and HAV show varying resistance to environmental stresses such as acid, heat, drying, pressure, disinfectants and UV, but are generally environmentally resilient (Greening, 2006). Currently, human NoV remain uncultivable in the lab, therefore cultivable noroviruses (such as murine norovirus and feline calicivirus) are used as surrogates to examine their behavior, survival, and infectivity (Doultree et al., 1999; Wobus et al., 2006). Feline calicivirus (FCV-F9) belongs to the *Caliciviridae* family and is similar to the human NoV in many aspects such as shape, size, and genome organization. Murine norovirus (MNV-1) also belongs to the *Caliciviridae* family but is more closely related to the human NoV as it is within the *Norovirus* genus. MS2 bacteriophage infects its host, *Escherichia coli* 15597B and is a single-stranded 27-34 nm RNA virus with an icosahedral symmetry within the *Leviviridae* family (Calender, 1988; Dawson et al., 2005). MS2, along with other animal norovirus surrogates has been previously studied for inactivation by chemical and processing means, as well as to determine survivability and as an indicator of fecal contamination (Grove et al., 2008; Kingsley et al., 2007; Sharma et al., 2008; Bae and Schwab 2008; D'Souza and Su 2010).

Foods and beverages may become virally contaminated after processing. It is important to study virus survival over time in juices to develop proper control strategies, determine risk associated with contaminated juices, and prevent outbreaks. Pomegranate juice and its associated polyphenols are reported to possess antibacterial and antiviral properties (Haidari et al., 2009; Al-Zoreky 2009) and have recently shown to reduce the infectivity of human NoV surrogates (Su et al., 2010b). It is therefore also remains intriguing to study the effect of pomegranate juice and its polyphenols blended with orange juice on the survival of human NoVs in comparison to

survival in the individual juices alone, and to determine if these blends will help in preventing viral survival after contamination. The objective of this study was to determine the survival of three human norovirus surrogates, murine norovirus (MNV-1), feline calicivirus (FCV-F9), and MS2 bacteriophage, over 21 days at refrigeration temperature (4°C) in milk, orange and pomegranate juices, as well as blends of orange and pomegranate juice, and orange juice and pomegranate polyphenols (PP). The data obtained from this study can be used to develop improved control strategies to prevent foodborne viral outbreaks.

Materials and Methods

Viruses and Cell Lines

Feline calicivirus (FCV-F9) and its host Crandell Reese Feline Kidney (CRFK) cells as well as bacteriophage MS2 and its host *E. coli* B-15597 were obtained from ATCC (Manassas, VA). Murine norovirus (MNV-1) was obtained as a gift from Dr. Skip Virgin of Washington University in St. Louis, Missouri. Murine norovirus host cells (RAW 264.7) were obtained from the cell culture collection at the University of Tennessee.

Virus Propagation and Preparation

FCV-F9 and MNV-1 stock was prepared by inoculating FCV-F9 or MNV-1 onto confluent CRFK or RAW 264.7 cells in 175 cm² flasks and incubating at 37°C and 5% CO₂ until 90-100% cell lysis was observed as described before (D'Souza and Su, 2010; Su et al., 2010a). Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY) was used for cell culture medium. The inoculated flasks were

freeze-thawed and centrifuged at 5000 x *g* for 10 min at room temperature. The supernatant was filtered through a 0.2 micron filter, aseptically aliquoted, and stored in a -80°C freezer. The recovered FCV-F9 and MNV-1 viruses were plaque assayed as described below to determine the titer and used as viral stocks for the entire study.

Bacteriophage MS2 was propagated in its host *E. coli* B-15597 at 37°C for ~18 h in Trypticase Soy Broth (TSA) containing 0.1% glucose, 2mM CaCl₂, and 10µg/ml Thiamine. Viruses were recovered as described above for FCV-F9 and MNV-1 after centrifugation and plaque assayed as described below.

Infectious Plaque Assays

CRFK and RAW 264.7 cells were cultivated and used for FCV-F9 and MNV-1 plaque assays, respectively. Briefly, the cell suspension was added to six-well plates at 4 x 10⁵ cells per well and incubated with 5% CO₂ at 37°C until ~90% confluency. Cells were then infected with 0.5 ml of treated and untreated virus that were serially diluted in cell culture medium after aspirating the media. After incubation for 2 h at 37°C and 5% CO₂, the virus suspension was aspirated and the cells were overlaid with 2 ml DMEM containing 2% FBS, 0.75% agarose, and HyClone ® 1% penicillin-streptomycin (Logan, Utah). After further incubation for 48 h, 1 ml of a secondary overlay medium containing 0.01% neutral red was added to stain the plates and plaques were counted after incubation for 24 h at 37°C.

MS2 Plaque Assay

The double agar layer method of Bae and Schwab (2008) was used for the MS2 infectivity plaque assay. Briefly, *E. coli* B-15597 culture host was allowed to grow for 6-h in TSA containing 0.1% glucose, 2mM CaCl₂, and 10 µg/ml Thiamine at 37°C, and 0.3 ml of this

host was combined with 0.7 ml of serially diluted MS2 phage. These were mixed with top agar (0.6%) and overlaid/poured on to TSA bottom agar plates. Once solidified, plates were incubated overnight at 37°C and plaques were counted.

Survival Study

Survival of MNV-1 (initial titer of 6-log PFU/ml) and FCV-9 (initial titer of 7-log PFU/ml) and bacteriophage MS2 (initial titer of 7-log PFU/ml) was determined in pulp-free orange juice (OJ) (Tropicana®, Bradenton, FL), skim milk (Kroger®, Cincinnati, OH) and pomegranate juice (PJ) (PomWonderful®, Los Angeles, CA) or juice blends (1:1 v/v orange juice: pomegranate juice and 1:1 v/v orange juice: pomegranate polyphenol (4 mg/ml); POM Wonderful®) or phosphate buffered saline (PBS; control). Each virus (0.2 ml) was aseptically added to 1.8 ml juice, juice blend, or milk (reducing the titer by 1 log₁₀ PFU/ml) and stored for 0, 1, 2, 7, 14, and 21 days at refrigeration temperature (4°C). At each time point, the virus was serially diluted in DMEM containing 10% FBS and plaque assayed as described above. Survival of each virus was also determined in half strength juice and pomegranate polyphenol (PP) controls. Each virus (0.2 ml) was added to 0.9 ml pulp-free orange juice (Tropicana®) with 0.9 ml PBS, 0.9 ml pomegranate juice (PomWonderful®) and 0.9 ml PBS, and 0.9 ml pomegranate polyphenols at 4 mg/ml (PomWonderful®) and 0.9 ml PBS, and stored for 0, 1, 2, 7, 14, and 21 days to serve as half-juice and polyphenol controls. At each time point, the virus was serially diluted and plaque assayed as described above.

Statistical Analysis

Statistical analysis on the FCV-F9, MNV-1, and MS2 infectivity assay data was done using ANOVA and Tukey's test on a completely randomized design with four replications using

the SAS system for windows version 9.2 (SAS Institute Inc., Cary, NC). Data were analyzed for differences in survivability over time and with each medium (milk, orange juice, or pomegranate juice) for duplicate treatments of each virus.

Results

Survival Characteristics of FCV-F9 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C)

FCV-F9 in PBS showed minimal reduction after 2 days ($\sim 1 \log_{10}$ PFU/ml), or 7 days ($\sim 1.1 \log_{10}$ PFU/ml), and significant reduction after 14 and 21 days (2.6 and $2.8 \log_{10}$ PFU/ml respectively, $p < 0.05$) storage at refrigeration temperatures. Feline calicivirus (FCV-F9) at ~ 6 - $\log$ PFU/ml in skim milk showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 0, 1, 2, and 7 days at 4°C. However, significant reductions in FCV titers were obtained after 14 and 21 days resulting in reductions of 2.6 and $2.8 \log_{10}$ PFU/ml, respectively ($p < 0.05$). FCV-F9 in OJ showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 0, 1, and 2 days at 4°C. Our results showed that the survival decreased slightly after 7 days by $1.48 \log_{10}$ PFU/ml and with no detectable virus (by plaque assays) in OJ after 14 and 21 days at 4°C. FCV-F9 in PJ alone had slight reduction after 2 days ($1 \log_{10}$ PFU/ml) and 7 days ($1.3 \log_{10}$ PFU/ml) and significant reduction after 14 days ($2.35 \log_{10}$ PFU/ml) ($p < 0.05$). FCV-F9 was completely inactivated after 21 days in PJ (Table 2.1).

Survival Characteristics of MNV-1 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C)

MNV-1 in PBS showed minimal reduction after 14 ($<1 \log_{10}$ PFU/ml), and 21 days ($\sim 1.08 \log_{10}$ PFU/ml) at 4°C. For murine norovirus (MNV-1) at a titer of $6 \log_{10}$ PFU/ml in skim milk, titers did not decrease after 0, 1, 2, 7, 14, and 21 days at 4°C. Similarly, MNV-1 titer in OJ did not decrease after 0, 1, 2, 7, 14, and 21 days at 4°C. However, significant decreases in titer occurred after 14 and 21 days in PJ showing 1.8 and $2.02 \log_{10}$ PFU/ml reduction, respectively ($p < 0.05$) (Table 2.2).

Survival Characteristics of MS2 in Milk, Orange Juice, and Pomegranate Juice over 21 Days at Refrigeration Temperature (4°C)

MS2 in PBS alone (1:1 v/v) showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 21 days at 4°C and appeared to be the most resilient of all three viruses tested at refrigeration temperature. For bacteriophage MS2 (at a titer of $6 \log_{10}$ PFU/ml) in skim milk and PJ, titers decreased slightly ($<1 \log_{10}$ PFU/ml) after 0, 1, 2, 7, 14, and 21 days at 4°C. MS2 titer in OJ also decreased slightly after 14 ($1.02 \log_{10}$ PFU/ml) and 21 days ($1.28 \log_{10}$ PFU/ml) at 4°C (Table 2.3).

Survival Characteristics of FCV-F9, MNV-1, and MS2 in Pomegranate Juice and Pomegranate Polyphenol Diluted in PBS over Time at 4°C

For FCV-F9 in PJ diluted in PBS, minimal reduction after 2 days ($< 1 \log_{10}$ PFU/ml), some reduction after 7 days ($\sim 2 \log_{10}$ PFU/ml), and complete reduction after 14 days at 4°C was obtained. FCV-F9 titers in PP with PBS (final concentration 1.8 mg/ml) were completely reduced ($\sim 6.2 \log_{10}$ PFU/ml) after 1 day at 4°C (Table 4). For MNV-1 in PJ with PBS, minimal reduction after 7 days ($< 1 \log_{10}$ PFU/ml) and some reduction after 14 days and 21 days (~ 1.4

$\log_{10}$ PFU/ml) at 4°C was obtained. MNV-1 in PP with PBS showed minimal reduction after 7 days ($< 1 \log_{10}$ PFU/ml), some reduction after 14 days ($\sim 1.5 \log_{10}$ PFU/ml) and significant reduction after 21 days ($\sim 2.39 \log_{10}$ PFU/ml, $p < 0.05$) at 4°C (Table 5). MS2 in PJ with PBS showed slight reduction after 7 days ($0.78 \log_{10}$ PFU/ml) and 14 days ($0.82 \log_{10}$ PFU/ml) and a total reduction of only $1.84 \log_{10}$ PFU/ml after 21 days at refrigeration. MS2 in PP with PBS showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 21 days at 4°C (Table 2.6). Among the three tested viruses, MS2 was the most persistent in pomegranate juice or PP at the concentrations and temperature used in this study.

Survival Characteristics of FCV-F9, MNV-1, and MS2 in Blends of Orange Juice and PBS, Orange Juice and Pomegranate Juice or Orange Juice and Pomegranate Polyphenols

FCV-F9 showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 1 day at 4°C in the OJ and PBS (1:1 v/v). However, FCV-F9 showed significant reductions after 2 and 7 days, ~ 2.61 and $\sim 3.18 \log_{10}$ PFU/ml, respectively, and complete reduction in titer after 14 days at 4°C in OJ diluted in PBS (Table 1, 4). FCV-F9 (titer 6-log PFU/ml) was completely inactivated after 1 day at 4°C in the blend of OJ and PJ. PJ contains 3.6 mg/ml polyphenols (Aviram et al., 2008) and upon mixing with virus the final concentration is 1.8 mg/ml. FCV-F9 showed some reduction after 1 day ($\sim 1.5 \log_{10}$ PFU/ml) and complete reduction in titer after 2 days at 4°C in the OJ and PP combination (PP final concentration of 1.8 mg/ml) (Table 2.4).

MNV-1 showed minimal reduction ($< 1 \log_{10}$ PFU/ml) even after 21 days at 4°C in OJ with PBS (1:1 v/v) (Table 2, 5). MNV-1 at titers of 5-log PFU/ml in the OJ and PJ blend showed some reduction after 1 day ($\sim 1.67 \log_{10}$ PFU/ml), significant reduction in titer after 2 days ($\sim 3.14 \log_{10}$ PFU/ml) and complete reduction after 7 days at 4°C ($p < 0.05$). However, MNV-1 showed

somewhat lower reduction in the OJ and PP blend than the OJ and PJ blend with minimal reduction in titer after 1 day ($\sim 0.79 \log_{10}$ PFU/ml) and significant reduction after 2 days ($\sim 1.88 \log_{10}$ PFU/ml; $p < 0.05$). However, similar to the results obtained with the OJ and PJ blend after 7 days, complete reduction in titer was observed after 7 days at 4°C in the combination of OJ and pomegranate polyphenols (final concentration 1.8 mg/ml) (Table 2.5).

MS2 in OJ combined with PBS (1:1 v/v) showed minimal reduction ($< 1 \log_{10}$ PFU/ml) after 21 days at 4°C and appeared to be the most resilient of all three viruses tested at refrigeration temperature. For MS2 at titers of 6-log PFU/ml in OJ combined with PJ or OJ combined with PP (final concentration 1.8 mg/ml), minimal reduction ($< 1 \log_{10}$ PFU/ml) after 21 days at 4°C was observed (Table 2.6).

Discussion

Human NoV are of particular concern due to their ease of transmission and numerous endemic outbreaks associated with produce and RTE foods. Atmar et al. (2008) estimated that from 10^6 to 10^9 NoV particles/gram can be excreted from the feces of infected individuals, with an infectious dose of 10 virus particles or less (CDC, 2010b). The growing number of human NoV outbreaks associated with fresh produce, fruits, and juices drives our needs to understand survival and persistence of these viruses in juices and milk.

MNV-1 is the currently available culturable model system that provides the opportunity to understand the relationship between basic mechanisms of NoV replication in tissue culture and pathogenesis in a natural host (Wobus et al., 2006). MNV-1 is more resistant than FCV-F9 to high hydrostatic pressures and low pH values (Cannon et al., 2006; Kingsley et al., 2007).

When exposed to a pH of 2 at 37°C for 30 minutes, MNV-1 titers were found to be reduced by <1 log (Cannon et al., 2006). This resistance to acidic environments correlates with the reported enteric virus outbreaks from fruits and fruit juices. Hence, MNV-1 was used in this study as a surrogate to determine persistence in juices and milk at refrigeration over 21 days, along with FCV-F9, the previously studied surrogate of human NoV.

Novel approaches to control foodborne viral spread from juices as well as data to determine risks associated with survival of viruses in juices are needed. This study to understand survival of foodborne virus surrogates in milk and juices over storage at refrigeration showed that MNV-1 was more persistent than FCV-F9 in both milk and orange juice even after 21 days at 4°C. However, bacteriophage MS2 was found to be the most persistent of the three tested viruses, as the titers of MS2 was minimally reduced by the tested juices and juice combinations after 21 days at 4°C. The pH of PJ was found to be 3.34; OJ was 3.83, the OJ-PJ blend was 3.49, and OJ-PP blend was 3.72. Even so, it appears that the viruses could survive these pH conditions of individual juices in a manner similar to surviving the acidic conditions of the gastrointestinal tract to cause infection.

Due to produce items being a common source of contamination, Dawson et al. (2005) studied the survival of MS2 bacteriophage on fresh iceberg lettuce, baton carrot, cabbage, spring onion, curly leaf parsley, capsicum pepper, tomato, cucumber, raspberries, and strawberries. MS2 was found to survive for a very long time over extended shelf life of the produce, where <1 log₁₀ reduction was observed after 50 days at 4 and 8°C (Dawson et al., 2005). Additionally, Croci et al. (2002) showed that hepatitis A virus survived after storage at 4°C for 9 days on spiked lettuce with a very slight decrease in titer, but complete reduction was observed after 4 days on carrot and after 8 days on fennel. In a study by Kurdziel et al. (2001) on produce stored

at refrigeration for 2 weeks, the decimal reduction times (*D*-values) for lettuce was reported as 11.6 days; white cabbage, 14.2 days; frozen strawberries, 8.4 days with no decline in titers of fresh raspberries and green onion. The results obtained in this study showing survival of MS2 in juices over 21 days at refrigeration are similar to these findings. Even though, the survival of human enteric viruses on environmental surfaces, water, soil and food has been reviewed and reported (Rzezutka and Cook, 2004), not much research has been carried out on foodborne virus survival in juices.

Additionally, our results that show survival of the human NoV surrogate, MNV-1 at refrigeration temperatures is also in agreement with other findings and studies using refrigeration temperatures (4°C). Cannon et al. (2006) observed minimal reduction in titer (<1 log) of MNV-1 in wet and dry fecal suspensions on stainless steel after 7 days at 4°C. Along with being stable in the environment and under various storage conditions, MNV-1 has also been found to be relatively resistant to many processing methods such as thermal pasteurization, high hydrostatic pressure, and high pressure homogenization (Baert et al., 2008b; Kingsley et al, 2007; D'Souza et al., 2009).

Regarding FCV-F9, one study observed a 2 log₁₀ reduction in FCV-F9 on lettuce after 7 days and more than 2.5 log₁₀ reduction on strawberries after 6 days at refrigeration temperature (4°C) (Mattison et al., 2007). Our results in this study showed moderate reduction in titer (2.28 log₁₀ PFU/ml) of FCV-F9 with a titer of 6-log PFU/ml after 21 days of storage in PBS in refrigeration temperature (4°C) that is in keeping with these findings. Bae and Schwab (2008) found that FCV-F9 infectivity reduction rates in contaminated surface and ground water were significantly higher (0.18 and 0.09 log₁₀/day) at 25°C than poliovirus or other norovirus surrogates such as MNV-1, and MS2. Surprisingly, FCV-F9 seemed stable at pH values of 6

(Duizer et al., 2004; Hewitt and Greening, 2004). However, the virus was inactivated by 5 logs (Duizer et al., 2004) when incubated at a pH of 2 or lower for 30 minutes at 37°C. FCV-F9 was also found to be relatively stable at freezing temperatures; however infectivity was reduced by 0.44 log after 5 cycles of freeze-thawing (Duizer et al., 2004). Frozen storage was also shown not to significantly reduce the viability of hepatitis A virus, rotavirus or FCV in produce and herbs, except the infectivity of FCV in strawberries and raspberries associated with acid pH (Butot et al., 2008). Mbithi et al. (1991) showed that relative humidity and temperature play a role in the survival of enteric viruses on environmental surfaces, where hepatitis A virus survived longer at low RH (which is different than other enteroviruses) and low temperature (5°C) than at higher humidity and higher temperatures (35°C).

Our results show that bacteriophage MS2 is the sturdiest of three tested viruses and most persistent virus over time in all tested juices and juice combinations. Our findings are consistent with other research reports on MS2, where Guan et al. (2006) found that MS2 was very resistant to pressure, in comparison to other coliphages, and was inactivated by $<1 \log_{10}$ PFU/ml at pressures of 500 MPa for 5 minutes at 20°C in phosphate-buffered saline (Guan et al., 2006). These researchers suggested that MS2 was a suitable surrogate for hepatitis A virus (HAV) (Guan et al., 2006).

The human norovirus, although not culturable, can be detected on surfaces using polymerase chain reaction assays. Barker and others (2004) found that NoV was still detectable on contaminated surfaces (door handles and telephone receivers) that were cleaned with a detergent-based cleanser or a combined hypochlorite/detergent cleanser with 5000 ppm chlorine Barker et al., 2004). Another study found that human NoV persists in an infective state for long periods of time on various surfaces. Lamhoujeb and others (2009) detected infective NoV on polyvinyl

chloride and stainless steel for 56 and 49 days, respectively, at 7° C (Lamhoujeb et al., 2009). In general, from our findings and other studies, the FCV-F9, MNV-1, and MS2 have similarities and differences in survival characteristics in comparison to human Norovirus. Our findings that show the least persistence of FCV-F9 in juices over time at refrigeration is in agreement with these reports on FCV-F9. FCV-F9 is found to be relatively easy to inactivate by heat, processing conditions, and chemical treatments. Cannon et al. (2006) showed that 56°C, 63°C, and 72°C, a 1- log₁₀ reduction was seen after 6.7, 0.4 and 0.1 minutes, respectively for FCV-F9 (Cannon et al., 2006). Though genetically similar to NoV, studies suggest that FCV-F9's instability and sensitivities to treatments and environmental conditions make it the least suitable non-enveloped surrogate. MNV-1 is more closely related to NoV and our studies show that it is more suitable surrogate for NoV than FCV-F9. Bae and Schwab (2008) revealed that MNV-1 was one of the most persistent viruses in environmental waters (compared to FCV-F9, MS2, and Poliovirus) and is potentially the superior surrogate virus (Bae and Schwab, 2008). Our studies revealed that MS2 is the sturdiest virus compared to MNV-1 and FCV. MS2, although not genetically related to human NoV, is considered a good NoV surrogate. MS2 was found to be a norovirus surrogate in environmental waters, for MS2 nucleic acid reductions closely resembled Poliovirus and norovirus reductions (Bae and Schwab, 2007). The three viruses studied are suitable human norovirus surrogates.

Although viruses and other microorganisms are controlled by processing methods and chemical treatments, post-processing contamination could occur that needs to be controlled. Many fruits and their extracts reportedly have natural antimicrobial properties that need to be explored to enhance food safety. Corbo et al. (2009) studied the antimicrobial properties of thymol, lemon extract, and grape fruit seed extract (GFSE) that was found to slow down the

growth of spoilage microorganisms within fresh fish burger fillets, including *Photobacterium phosphoreum*, *Shewanella putrefaciens*, and *Pseudomonas fluorescens*. Luther et al. (2007) found that Chardonnay and black raspberry seed flour extracts (using 3.3 and 3.2 mg seed flour equivalent/ml, respectively) both contained phenolic acid and had bacteriocidal effects on *E. coli* with zero survival after incubation at 35°C for 24 hours.

Many studies have documented the health benefits of the pomegranates, and pomegranate extracts have also been shown to be used as a natural antibacterial and antiviral compound (Wolfe et al., 2008; Al-Zoreky, 2009; Haidari et al., 2009). Kanatt et al. (2010) found that pomegranate peel extract at low concentrations (0.01%) inhibited the growth of some Gram-positive bacteria, including *Staphylococcus aureus*. Haidari et al., (2009) evaluated ellagic acid, caffeic acid, luteolin, and punicalagin, the four major polyphenols in pomegranate extract and found that punicalagin proved to be effective against influenza virus infectivity. They suggested that punicalagin acts by blocking RNA replication of the virus and inhibiting chicken red blood cell agglutination by the virus (Haidari et al., 2009). Recently, Su et al. (2010b) showed that pomegranate juice (PJ) and pomegranate polyphenols (PP) could reduce the viral titers of MS2, FCV-F9 and MNV-1. After one hour incubation at room temperature (25°C) in pomegranate juice, low titer ($\sim 10^5 \log_{10}$ PFU/ml) MS2, FCV-F9 and MNV-1 were reduced by 0.32, 2.56, and 1.32 log PFU/ml, respectively (Su et al., 2010b). In the same study, MS2, FCV-F9 and MNV-1 were reduced by 0.41, 4.54, and 1.30, respectively, after a 1 hour incubation at 25°C in pomegranate polyphenol (4 mg/ml), with MS2 being the most resistant to treatment and FCV the most susceptible (Su et al., 2010b).

This study also showed synergistic effects of the blend of orange and pomegranate juice compared to orange juice or pomegranate juice alone against the survival of FCV-F9 and MNV-

1. The OJ-PJ blend decreased the survival of FCV-F9 and MNV-1 at a faster rate when compared to each individual juice alone. FCV-F9 titers (6-log_{10} PFU/ml) were completely reduced after 1 day in the OJ-PJ blend at 4°C , whereas in orange juice alone FCV-F9 titer was completely reduced after 14 days and only after 21 days in pomegranate juice alone. Also, MNV-1 titers were completely reduced after 7 days in the blend of OJ and PJ. However, MNV-1 titers dropped by 0.12 log after 21 days at 4°C in orange juice and 2.02 log after 21 days in pomegranate juice alone. Thus, the combination of orange juice and pomegranate juice may potentially have a synergistic detrimental effect on these viruses.

PJ reportedly contains 3.6 mg/ml polyphenols (Aviram et al., 2008) and upon mixing with virus the final concentration is 1.8 mg/ml. Therefore, OJ-PP blends containing final concentration of PP at 1.8 mg/ml were used to determine the effects of PP alone without the effects of other components of PJ. Both, FCV-F9 and MNV-1 survived longer in the OJ-PP blend than the OJ-PJ blend, showing that the pH and some other components of PJ that work together with OJ may play a role in decreasing survival.

Bacteriophage MS2, the toughest virus among the three tested viruses, showed minimal reduction ($<1\text{ log}_{10}$ PFU/ml) in all tested media (milk, juices, and juice combinations) except for a 1.18 and 1.84 log_{10} PFU/ml reduction in OJ combined with PBS and PJ combined with PBS, respectively, after 21 days at 4°C . All virus treatments were compared to PBS controls, which resulted in a 2.28, 1.08, and 0.56 log_{10} PFU/ml reduction for FCV-F9, MNV-1, and MS2, respectively, after 21 days at 4°C .

Although the exact antiviral mechanisms of pomegranate juice towards the tested norovirus surrogates are currently unknown, for the enveloped influenza virus, it is theorized that

pomegranate juice can possibly reduce the amount of virus particles released into media, inhibit virus proliferation, or block the replication of the virus RNA (Haidari et al., 2009).

Bacteriophage MS2 was found to survive the longest in all tested media over refrigeration for 21 days and is reported to be very resistant to relatively low pH and high pressures (Langlet et al., 2007; D'Souza et al., 2009). This could be due to the sturdy capsid structure of the bacteriophage, making it resistant to processing treatments or due to the pI of the virus that makes it resistant to pomegranate juice and pomegranate polyphenol treatments. FCV-F9 appeared to be the most sensitive of the tested surrogates, where sensitivity to low pH could possibly cause structural damage to the viral capsid or potentially block binding to host cells, thus reducing infectivity.

Also, the juices were combined with PBS, making them half of their original strength in order to determine the individual effect of juices on the viruses to be used as controls for the study with blends as the strengths of each juice would be reduced to half upon mixing. The concentration of pomegranate polyphenols was diluted the same concentration as that found in PJ in order to be used as controls. However, the PP combined with PBS (half strength control) had a final concentration of 1.8 mg/ml pomegranate polyphenol.

Overall, milk was found to be the most protective medium among the tested media for virus survival and pomegranate juice combined with orange was found to be the least protective. These findings on survival indicate that even after 21 days, some human NoV surrogates remain detectable in milk and juices, under common household storage conditions. Thus, if the juices are contaminated either before or after purchase, the risk of foodborne viral illness exists under normal refrigeration storage conditions of juices. However, blending of nutritious healthy and tasty juices can provide a lethal/detrimental effect on the infectivity and survival of the tested

viruses. Combination of juices with natural antimicrobial properties could potentially inactivate microorganisms and viruses over time if the juice is contaminated post-processing, and thus lower the risk of persons becoming ill.

Although human NoV surrogates remained viable over time in juices alone, our results show that post-processing contamination can be prevented using a combination of juice blends such as orange juice and pomegranate juice or orange juice and pomegranate polyphenols that can potentially inactivate the foodborne viral contamination after contact and storage at refrigerated temperatures. When processing technologies, proper hygiene, proper sanitary conditions, appropriate control strategies remain in place and low levels of contamination still occur, blends of nutritive and healthy juices with antiviral properties may prove to be an attractive control alternative for the beverage industry. While increasing the antioxidant potential of the juices using blends, along with increased shelf-life and extended storage of juices, a unique method to control survival of foodborne viruses and prevent foodborne outbreaks may also be obtained.

The reported data on virus survival in juices over three weeks (21 days) at refrigeration temperature (4°C) provides the information needed for future quantitative viral risk assessment studies. Future research will focus on combining different fruit juices (such as cranberry or orange and cranberry and blueberry juices) to determine antiviral effects and virus survival over time.

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Appendix

Table 2.1: Reduction in Titers of FCV-F9 (~6 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS). All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in FCV-F9 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)				
Storage Time (Days)	OJ	Milk	PJ	PBS
0	0.0 ± 0.0D	0.0 ± 0.0C	0 ± 0F	0.0 ± 0.0E
1	0.46 ± 0.1C	0.14 ± 0.05C	0.11 ± 0.03E	0.04 ± 0.04E
2	0.66 ± 0.29C	0.3 ± 0.13C	1.0 ± 0.04D	0.97 ± 0.02D
7	1.48 ± 0.19B	0.92 ± 0.02BC	1.30 ± 0.03C	1.11 ± 0.04C
14	6 ± 0.0A	2.59 ± 2.41AB	2.35 ± 0.05B	2.1 ± 0.04B
21	6 ± 0.0A	2.79 ± 2.28A	5.53 ± 0A	2.28 ± 0.05A

Table 2.2: Reduction in Titters of MNV-1 (~5 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS). All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in MNV-1 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)				
Days of Refrigeration	OJ	Milk	PJ	PBS
0	0.0 ± 0.0B	0.0 ± 0.0B	0±0E	0.0 ± 0.0D
1	0 ± 0.0B	0 ± 0.0B	0±0.05E	0.05 ± 0.06CD
2	0 ± 0.03B	0.06 ± 0.06A	0.33±0.21D	0.12 ± 0.09C
7	0 ± 0.0B	0.01 ± 0.02AB	0.85±0.07C	0.96 ± 0.08B
14	0 ± 0.0B	0.02 ± 0.05AB	1.8±0.05B	0.98 ± 0.08AB
21	0.12 ± 0.07A	0.04 ± 0.06AB	2.02±0.05A	1.08 ± 0.08A

Table 2.3: Reduction in Titters of MS2 (~6 log PFU/ml) at 4°C over 21 Days in Orange Juice (OJ), Milk, and Pomegranate Juice (PJ), and Phosphate Buffered Saline (PBS). All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in MS2 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)				
Days of Refrigeration	OJ	Milk	PJ	PBS
0	0.0 ± 0.0B	0.0 ± 0.0B	0±0B	0±0C
1	0 ± 0.0B	0 ± 0.0B	0.04±0.04B	0.42±0.01B
2	0 ± 0.03B	0.06 ± 0.06A	0.07±0.02AB	0.36±0.02B
7	0 ± 0.0B	0.01 ± 0.02AB	0.08±0.01AB	0.53±0.02A
14	0 ± 0.0B	0.02 ± 0.05AB	0.06±0.02AB	0.56±0.02A
21	0.12 ± 0.07A	0.04 ± 0.06AB	0.14±0.05A	0.56±0.01A

Table 2.4: Reduction in Titers of FCV-F9 (~6 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP). PBS with OJ, PJ, and PP were used as control for comparison. All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in FCV-F9 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)						
Days of Refrigeration	PBS	OJ/PBS	PP/PBS	PJ/PBS	OJ/PJ	PP/OJ
0	0.0 ± 0.0E	0.0 ± 0.0D	0.0 ± 0.0B	0.0 ± 0.0E	0.0 ± 0.0B	0.0 ± 0.0C
1	0.04 ± 0.04E	0.03 ± 0.02D	3.81 ± 0.0A	0.4 ± 0.1D	5.39 ± 0.0A	1.47 ± 0.15B
2	0.97 ± 0.02D	2.61 ± 0.15C	3.81 ± 0.0A	0.92 ± 0.07C	5.39 ± 0.0A	5.38 ± 0.0A
7	1.11 ± 0.04C	3.18 ± 0.09B	3.81 ± 0.0A	1.97 ± 0.24B	5.39 ± 0.0A	5.38 ± 0.0A
14	2.1 ± 0.04B	6.55 ± 0.0A	3.81 ± 0.0A	6.22 ± 0.0A	5.39 ± 0.0A	5.38 ± 0.0A
21	2.28 ± 0.05A	6.55 ± 0.0A	3.81 ± 0.0A	6.22 ± 0.0A	5.39 ± 0.0A	5.38 ± 0.0A

Table 2.5: Reduction in Titers of MNV-1 (~5 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP). PBS with OJ, PJ, and PP were used as control for comparison. All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in MNV-1 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)						
Days of Refrigeration	PBS	OJ/PBS	PP/PBS	PJ/PBS	OJ/PJ	PP/OJ
0	0.0 ± 0.0D	0.0 ± 0.0B	0.0 ± 0.0D	0.0 ± 0.0D	0.0 ± 0.0D	0.0 ± 0.0D
1	0.05 ± 0.06CD	0.3 ± 0.07B	0.49 ± 0.21C	0.02 ± 0.03D	1.67 ± 0.03C	0.79 ± 0.03C
2	0.12 ± 0.09C	0.62 ± 0.13A	0.53 ± 0.03C	0.38 ± 0.05C	3.14 ± 0.21B	1.88 ± 0.08B
7	0.96 ± 0.08B	0.61 ± 0.11A	0.6 ± 0.1C	0.75 ± 0.06B	5.63 ± 0.0A	5.69 ± 0.0A
14	0.98 ± 0.08AB	0.58 ± 0.08A	1.51 ± 0.07B	1.37 ± 0.04A	5.63 ± 0.0A	5.69 ± 0.0A
21	1.08 ± 0.08A	0.61 ± 0.13A	2.39 ± 0.12A	1.4 ± 0.03A	5.63 ± 0.0A	5.69 ± 0.0A

Table 2.6: Reduction in Titters of MS2 (~6 log PFU/ml) at 4°C over 21 Days in Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or OJ and Polyphenol (PP). PBS with OJ, PJ, and PP were used as control for comparison. All experiments were carried out in duplicate and repeated at least twice. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in MS2 titers (Log₁₀ PFU/mL) at refrigeration temperature (4°C)						
Days of Refrigeration	PBS	OJ/PBS	PP/PBS	PJ/PBS	OJ/PJ	PP/OJ
0	0±0C	0±0C	0±0D	0±0C	0±0D	0±0C
1	0.42±0.01B	0.13±0.11C	0.03±0.05D	0±0C	0.01±0.02D	0.03±0.05C
2	0.36±0.02B	0.16±0.07C	0.07±0.05D	0±0C	0±0D	0.04±0.04C
7	0.53±0.02A	0.24±0.07C	0.23±0.02C	0.78±0.01B	0.14±0.04C	0.05±0.03C
14	0.56±0.02A	0.68±0.09B	0.42±0.06B	0.82±0.04B	0.30±0.05B	0.58±0.07B
21	0.56±0.01A	1.18±0.14A	0.68±0.01A	1.84±0.02A	0.64±0.06A	0.80±0.02A

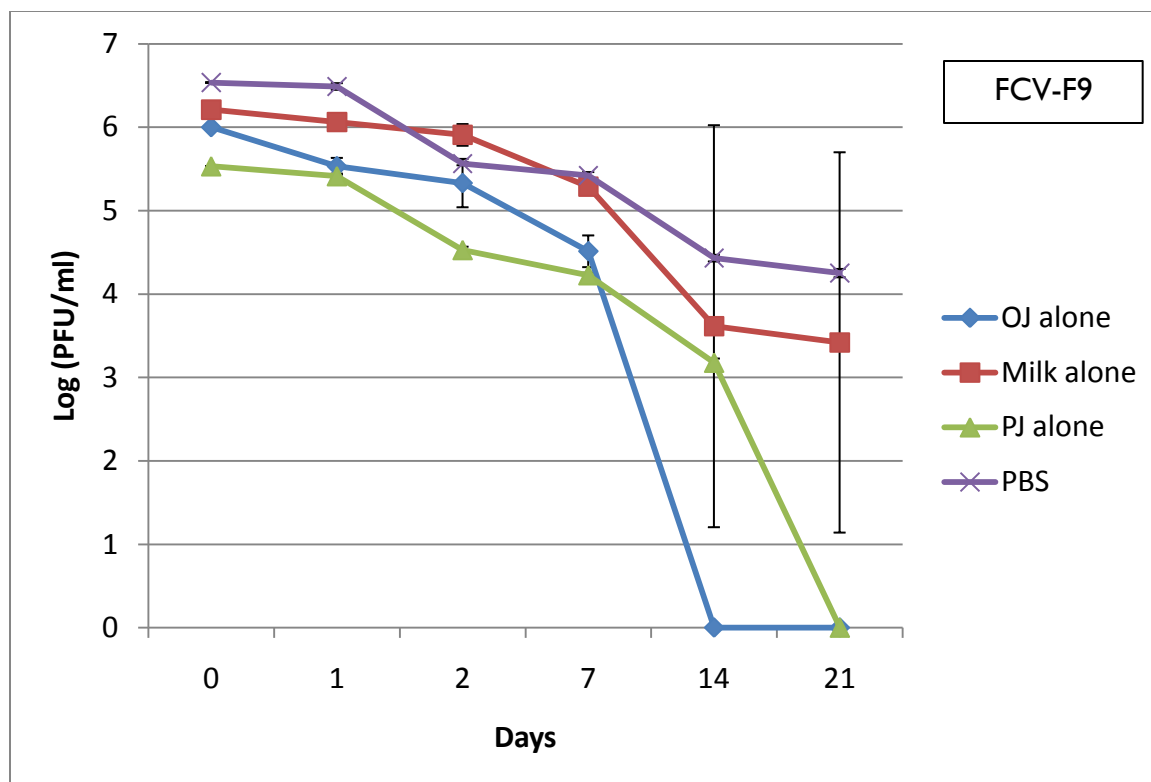


Figure 2.1: Recovery of FCV-F9 from Titer of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days.

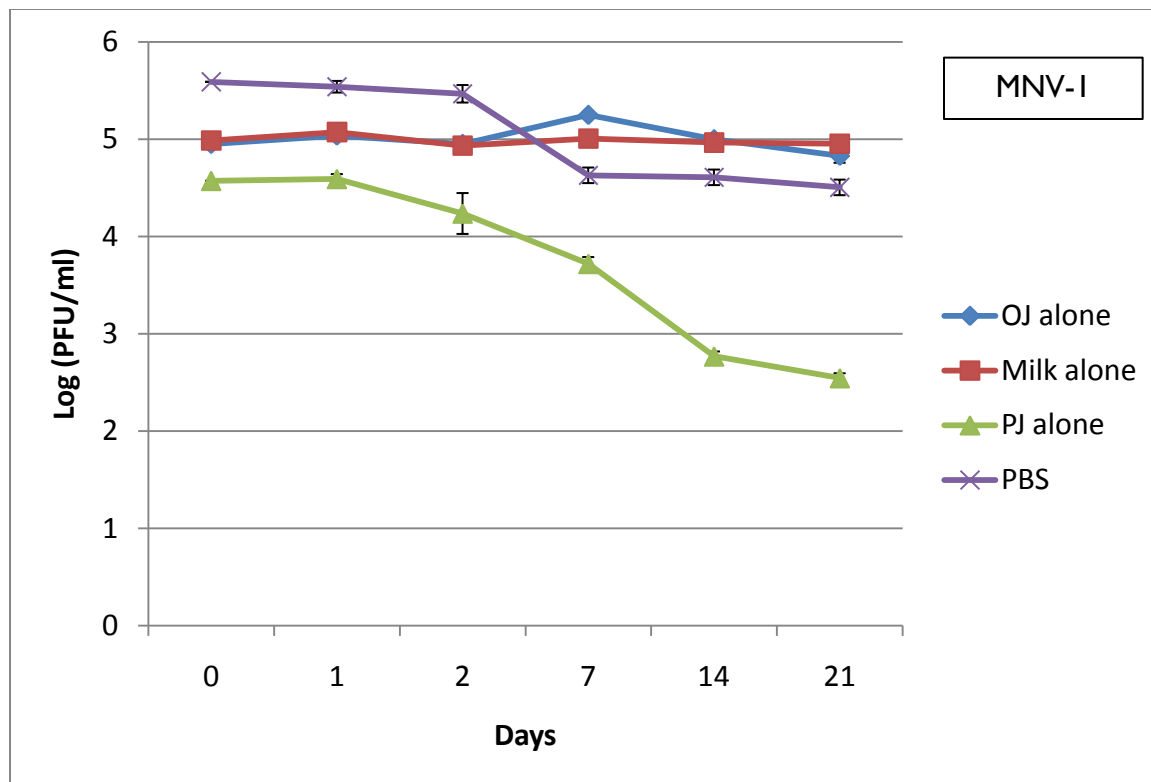


Figure 2.2: Recovery of MNV-1 from Titer of $\sim 5 \log_{10}$ PFU/ml (10^5 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days.

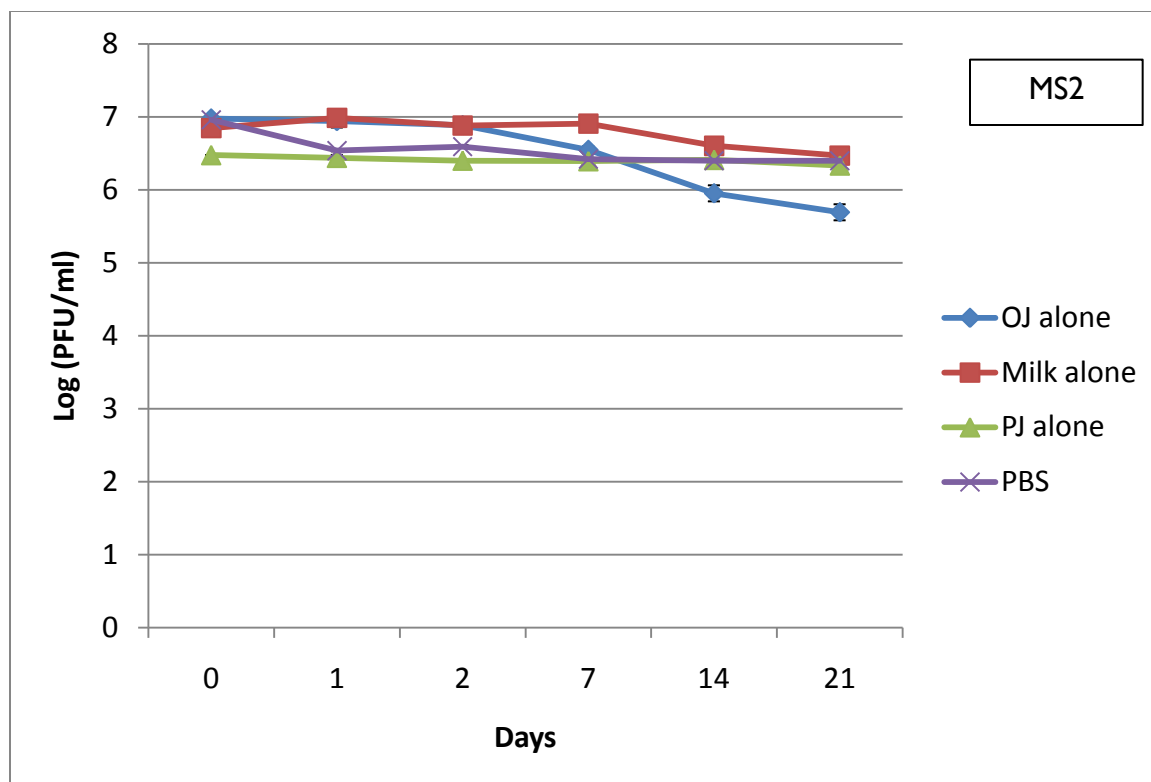


Figure 2.3: Recovery of MS2 from Titer of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) in Orange Juice (OJ), Milk, Pomegranate Juice (PJ) or Phosphate Buffered Saline (PBS) (control) at Refrigeration Temperature (4°C) over 21 Days.

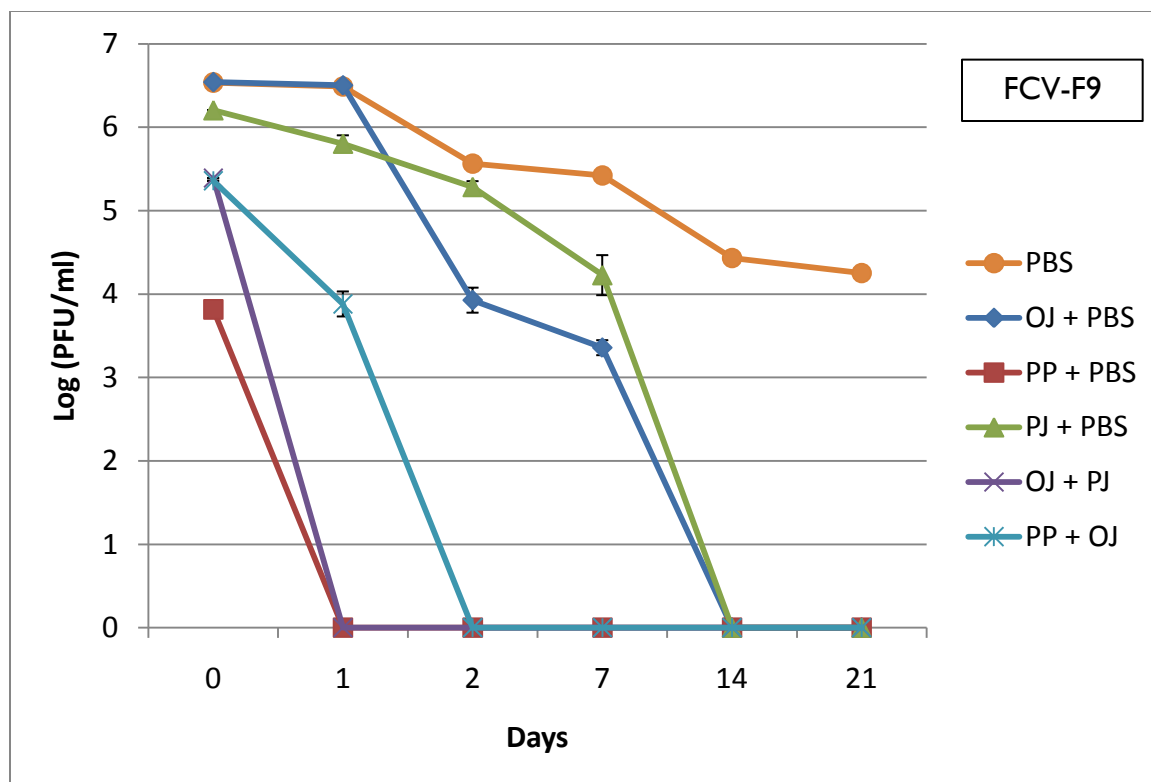


Figure 2.4: Recovery of FCV-F9 at Titers of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days. Phosphate buffered saline (PBS) with orange juice, pomegranate juice, and pomegranate polyphenol were used as controls for comparison purposes.

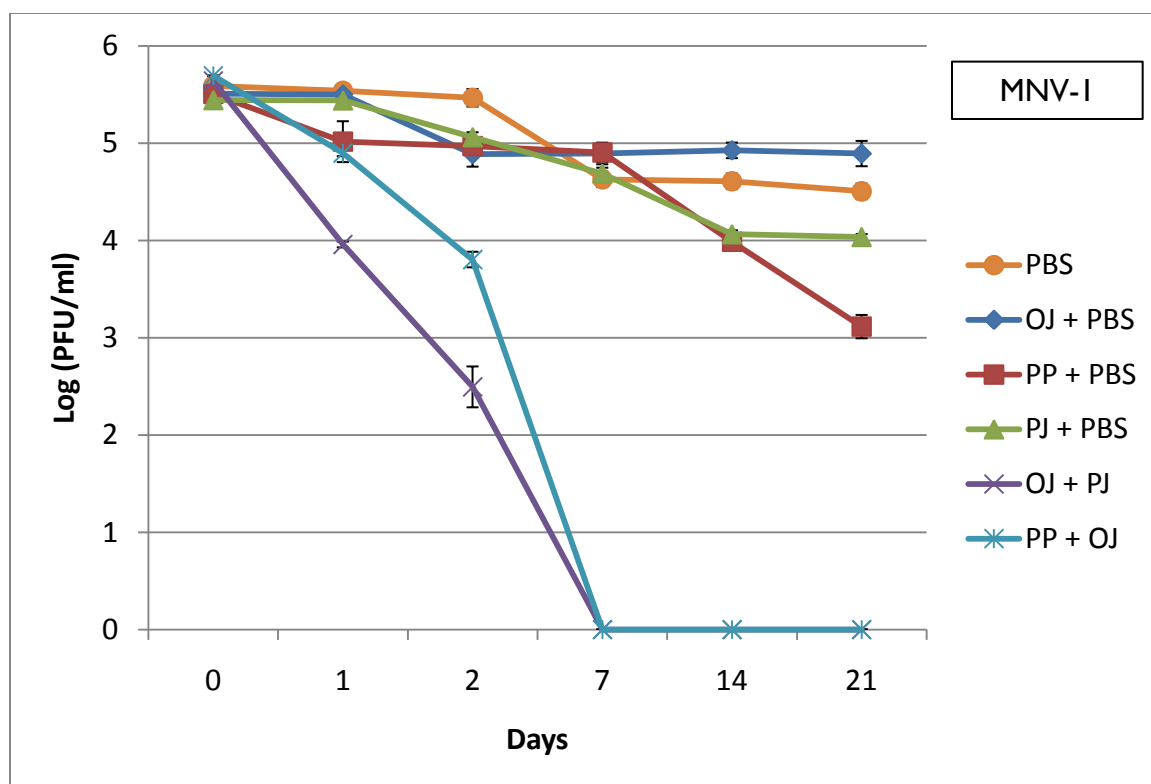


Figure 2.5: Recovery of MNV-1 at Titters of $\sim 5 \log_{10}$ PFU/ml (10^5 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days. Phosphate buffered saline (PBS) with orange juice, pomegranate juice, and pomegranate polyphenol were used as controls for comparison purposes.

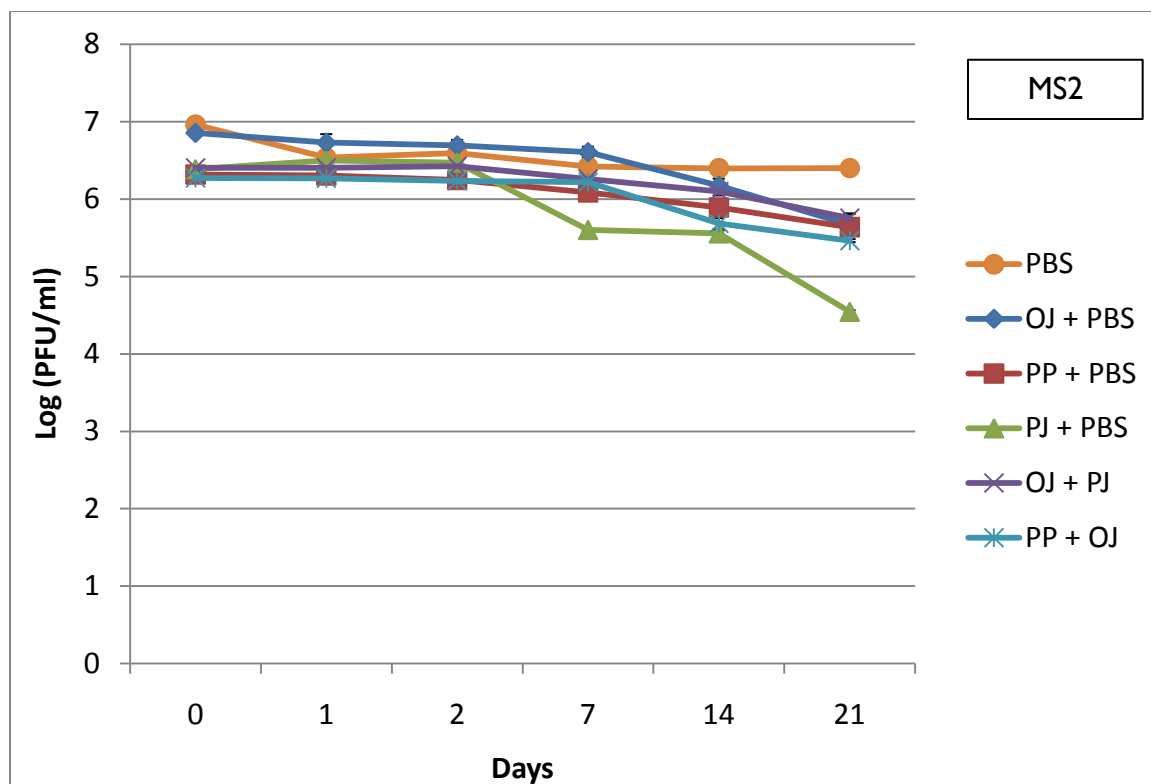


Figure 2.6: Recovery of MS2 at Titers of $\sim 6 \log_{10}$ PFU/ml (10^6 PFU/ml) from Blends of Orange Juice (OJ) and Pomegranate Juice (PJ) or Orange Juice and Pomegranate Polyphenols (PP) at Refrigeration Temperature (4°C) over 21 Days. Phosphate buffered saline (PBS) with orange juice, pomegranate juice, and pomegranate polyphenol were used as controls for comparison purposes.

CHAPTER III

High Pressure Homogenization and Human Norovirus Surrogates in Milk, Orange Juice, and Pomegranate Juice Blends

Application of High Pressure Homogenization for the Inactivation of Human Norovirus
Surrogates in Milk, Orange Juice, and Pomegranate Juice Blends

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Abstract

Novel processing technologies such as high pressure homogenization (HPH) that inactivate viruses in fluids and yet retain nutritional attributes remain in high demand. In the absence of culturable human noroviruses, cultivable murine norovirus (MNV-1) and feline calicivirus (FCV-F9) surrogates are currently used. The objectives of this research were (1) to determine the effects of HPH alone and with an emulsifier (lecithin) on MNV-1 and FCV-F9 in skim milk and orange juice (OJ) and (2) to determine HPH effects on FCV-F9, MNV-1, and bacteriophage MS2 in juice blends such as OJ blended with pomegranate juice (PJ). Experiments were conducted in duplicate using pressures of 0, 100, 200, 250, and 300 MPa for <2 s and plaque assayed in duplicate. For FCV-F9 in milk, reductions of $\geq 4 \log_{10}$ PFU/ml at 300 MPa, ~ 1.3 log at 250 MPa, and with no observed reduction at ≤ 200 MPa were obtained. In OJ, FCV-F9 was reduced by ≥ 4 and $\sim 1 \log_{10}$ PFU/ml only at 300 and 250 MPa, respectively. MNV-1 in milk showed reductions of ~ 1.3 and insignificant $\sim 0.1 \log_{10}$ PFU/ml only at 300 and 250 MPa, respectively. MNV-1 in OJ was reduced by ~ 0.8 and $\sim 0.4 \log_{10}$ PFU/ml at 300 and 250 MPa, respectively. MNV-1 in milk or OJ containing lecithin showed 1.32 or 2.5 log reduction, respectively at 300 MPa. FCV-F9 showed complete reduction in OJ or milk combined with lecithin at 300 MPa, and showed reductions of 1.77 and 0.78 log at 250 MPa, respectively. In the PJ-OJ combination, FCV-F9 was completely reduced, and MNV-1 and MS2 were significantly reduced in titer at 250 MPa (1.04 and 1.49 $\log_{10}$ PFU/ml, respectively). At 300 MPa in PJ-OJ, MNV-1 had a 1.78 $\log_{10}$ PFU/ml reduction and MS2 had a 4.1 $\log_{10}$ PFU/ml reduction. These results show that HPH has potential for commercial use to inactivate foodborne viruses in juices.

Introduction

Diseases spread through foods are frequently of bacterial, viral, or parasitic origin. In the United States, foodborne diseases have been estimated to cause around 9.4 million illnesses and 1,351 deaths each year (Scallan et al., 2011). Human viral food-borne illnesses are increasing in numbers at a global level. Even in the developed world, viruses in foods are the major cause of gastroenteritis, which can be detrimental to human health. Among the human enteric viruses causing distress to public health are the human noroviruses (NoVs), hepatitis A virus, rotaviruses, astroviruses, human enteroviruses such as coxsackieviruses and echoviruses, Aichiviruses, sapoviruses, and other small round viruses (Sair et al., 2002a). Human NoVs are now recognized as the most common cause of all foodborne disease in the United States, estimated to be responsible for 5.5 million (58%) cases annually (Grove et al., 2006; Scallan et al., 2011). Noroviruses are nonenveloped single-stranded RNA viruses belonging to the *Caliciviridae* family approximately 30 to 35 nm in diameter (Bok et al., 2009). Gastroenteritis from foods is often thought to be from a bacterial pathogen, but it is becoming increasingly apparent that this is a common misconception. Bacterial pathogens in foods usually require a high infectious dose to make one sick, whereas as few as 10 viral particles can cause health problems (CDC, 2010). The number of foodborne viral cases is more than likely underreported due to the mild onset of symptoms and relatively short illness time. Human NoVs, in particular, were recently estimated to be responsible for more than 90% of nonbacterial gastroenteritis endemics worldwide (Patel et al., 2008).

Human enteric viruses are spread by the fecal-oral route via transmission through contaminated environmental surfaces and food, person-to-person contact, person-to-fomite contact, infectious vomitus, and aerosolization (Patel et al., 2009). Foods that are at highest risk

include ready to eat foods (RTE), such as salads, fruits, and vegetables that are not further processed (Sair et al., 2002b). There is a growing risk of foodborne viral transmission associated with food handlers, who when infected and do not practice proper hygiene are potential sources of contamination in food processing and preparation facilities. Fruit juices have also been the source of outbreaks, including a major outbreak of hepatitis A among tourists returning from Egypt in 2004 that involved 351 infected individuals from 9 European countries and orange juice was most likely the infection vehicle (Frank et al., 2007). In February 2009, 38 persons were sickened by human norovirus genotype II.4 during two Dutch airline coach trips who visited the same hotel in Germany caused by the consumption of juice in large self-service containers with a tap (Visser et al., 2010). In August of 1991, an alarming number of 3000 individuals were sickened with human norovirus on domestic airline flights who had consumed orange juice served to passengers on the flights (Fleet et al., 2000).

Surprisingly, viruses have not been routinely tested in foods in the past due to costly extraction and culture- based methods. Human noroviruses are not yet culturable in the laboratory, but can be detected via reverse-transcriptase (RT-PCR), a labor-intensive and costly procedure. Therefore cultivable surrogates are used that include feline calicivirus (FCV-F9) and murine norovirus (MNV-1). Feline calicivirus has been used as a human norovirus surrogate in several studies and, although a respiratory pathogen, appears to be a better surrogate than enteric caliciviruses and has the same resistance to desiccation as the human noroviruses (Clay et al., 2006; Duizer et al., 2004). However, MNV-1 shares closer biological and molecular properties with other human noroviruses, as it belongs to Norovirus genus under genogroup V (Wobus et al., 2006).

Human enteric viruses can persist for weeks to months in the environment and in foods, and are minimally inactivated by some thermal and/or chemical means (Sair et al., 2002b). Undesirable sensory and texture attributes of foods are commonly produced after they are processed by heat. Irradiation, pulsed electric fields, ultraviolet laser treatments, and ultra high pressure, have been introduced as novel methods that are safe for consumers, to non-thermally preserve foods, while retaining nutritional and sensory attributes of the food product (Cardello, 2003; D'Souza et al. 2009). One novel processing method for fluid foods is high pressure homogenization (HPH). HPH uses many combined factors such as pressure, mild heat, shear stress, cavitation, and turbulence to inactivate viruses (Taylor et al, 2007). Recently, HPH treatment of MNV-1 and MS2 bacteriophage in phosphate buffered saline (PBS) showed a 3 log PFU/ml reduction of MS2 and a minimal ~ 0.8 log PFU/ml reduction in MNV-1 at 300 MPa (D'Souza et al., 2009). Pomegranate polyphenols (PP) were also shown to reduce viral titers of human enteric viral surrogates, FCV-F9, MNV-1, and MS2 after 1 h at room temperature where FCV-F9 was found to be completely reduced, MNV-1 had a $1.3 \log_{10}$ PFU/ml reduction, and MS2 was reduced by 0.41 log PFU/ml with 4 mg/ml PP (Su et al., 2010a). The addition of pomegranate polyphenols to juices could potentially decrease the energy requirements associated with high pressures and result in cost savings. Our hypothesis was that the non-enveloped viral capsids could possibly be destroyed using a combination of food grade and generally recognized as safe substances such as pomegranate polyphenols and pressure; or lecithin and pressure; or alternatively these combinations could prevent binding of the virus to food matrices or food particles allowing direct exposure for inactivation by HPH.

Based on these hypotheses and earlier findings, the objectives of this research were (1) to determine the feasibility of using HPH to inactivate human norovirus surrogates, FCV-F9 and

MNV-1 in skim milk and orange juice for industrial applications; and (2) to determine if the pressure requirements to inactivate these viruses could be lowered by using emulsifiers such as lecithin, or by using a combination/blend of orange juice-pomegranate juice (associated with the anti-viral properties of pomegranate polyphenols), that could potentially act as a hurdle approach to save energy and cost for sustaining the environment.

Materials and Methods

Viruses and Cell Lines

FCV-F9 and Crandall Reese Feline Kidney (CRFK) cells, and bacteriophage MS2 and its host *E. coli* B-15597, were obtained from ATCC (Manassas, VA). MNV-1 was obtained from Dr. Skip Virgin of Washington University in St. Louis, Missouri, and its host, RAW 264.7 cells were obtained from the cell culture collection at the University of Tennessee (as described in our earlier studies, Horm and D'Souza, 2011).

FCV-F9 stock was prepared by inoculating FCV-F9 onto confluent CRFK cells in 175 cm² flasks, incubating at 37°C and 5% -CO₂ for 24 hours until 90-100% cell lysis was observed as described before (D'Souza et al., 2009; Su et al., 2010a). The inoculated flask was freeze-thawed and centrifuged at 5000x *g* for 10 min at room temperature. The supernatant was filtered, aliquoted, and kept in a -80°C freezer. The FCV-F9 was plaque assayed to determine the titer and used as viral stock for the entire experiment.

MNV-1 stock was prepared by inoculating MNV-1 onto confluent RAW cells in 175 cm² flasks, followed by incubation at 37°C and 5% CO₂ for 4-6 days until 90-100% cell lysis was observed following the standard procedures described earlier (D'Souza et al., 2009; Su et al.,

2010a). The inoculated flask was freeze-thawed and centrifuged at 5000x *g* for 10 minutes at room temperature. The supernatant was filtered, aliquoted, and kept in a -80°C freezer. Recovered MNV-1 was plaque assayed to determine the titer and used as viral stock for the entire experiment.

Bacteriophage MS2 was propagated in its host *E. coli* B-15597 at 37°C for ~18 h in Trypticase Soy Broth (TSA) (3%) containing 0.1% glucose, 2mM CaCl₂, and 10 µg/ml Thiamine, and following the procedure of Bae and Schwab (2008). Viruses were recovered as described above for FCV-F9 after centrifugation and plaque assayed as described below.

FCV-F9 Plaque Assay

CRFK cells were cultivated using Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 2% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY) as described before (D'Souza et al., 2009; Horm and D'Souza, 2011). The cell suspension was next added to six-well plates and incubated under 5% CO₂ at 37°C until ~90% confluency. After aspirating the media, CRFK cells were then infected with 0.5 ml of treated and untreated FCV-F9 that were serially diluted in DMEM containing 2% FBS. After 2 h incubation at 37°C and 5% CO₂, the virus suspension was aspirated and the cells were overlaid with 2 ml DMEM containing 2% FBS, 0.75% agarose, and HyClone® 1% penicillin-streptomycin (Logan, Utah). After further incubation for 48 h, one-ml of a secondary overlay medium containing 0.01% neutral red was added to stain the plates, and plaques were counted after incubation for 24 hours at 37°C.

MNV-1 Plaque Assay

RAW 264.7 cells were cultivated using Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY) as described before (Su et al., 2010a; Horm and D'Souza, 2011). The cell suspension was added to six-well plates and incubated under 5% CO₂ at 37°C until 80-90% confluency. Once confluent, RAW 264.7 cells were infected with 0.5 ml of ten-fold serial viral dilutions after aspirating the media. Ten-fold serial dilutions were prepared by adding 167 microliters of virus treatments to 1.5 ml of Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY). After 72- h incubation, 2 ml of secondary overlay medium containing 0.02% neutral red was added to stain the plates and plaques were counted after 2-h incubation at 37°C.

MS2 Plaque Assay

The double agar layer method of Bae and Schwab (2008) was used for MS2 infectivity plaque assay (Bae and Schwab, 2008). *E. coli* B-15597 culture host was allowed to grow for 6-h in TSA (3%) containing 0.1% glucose, 2mM CaCl₂, and 10 µg/ml Thiamine at 37°C, and 0.3 ml of this host was combined with 0.7 ml of serially diluted MS2 phage. These were mixed with top agar (0.6%) and overlaid/poured on to TSA bottom agar plates. Once solidified, plates were incubated overnight at 37°C and plaques were counted.

Preparation of Lecithin

One hundred and twenty-five micrograms (0.125 grams) of lecithin was added to 500 ml of both phosphate buffered saline (PBS) and skim milk (Kroger®, Cincinnati, OH) and dissolved by bringing to a boil. The frozen-concentrate pulp-free orange juice (Tropicana®, Bradenton,

FL) was reconstituted accordingly, substituting with a lecithin stock solution to obtain a final concentration of 0.025% lecithin.

HPH Unit and Treatments

All processes were carried out in a FPG 12500 bench top high-pressure homogenizer (Stansed Fluid Power, Ltd, Essex, UK) with the regulator temperature held at 2°C using a water bath. A computer program (Lookout, V 5.1, National Instruments, Austin, TX) was connected to the homogenizer to continuously record pressure and temperature during treatments. Five-hundred ml of each treatment were run through the homogenizer. Fifteen ml of each treatment sample were collected at 300, 250, 200, 100, and 0 MPa, placed on ice, then aliquoted and kept in the -80°C freezer prior to diluting and assaying.

The treatments using HPH alone including FCV-F9, MNV-1, or MS2 in PBS (control); FCV-F9, MNV-1, or MS2 in skim milk (Kroger®, Cincinnati, OH); and FCV-F9, MNV-1, or MS2 in pulp-free orange juice (OJ) (Tropicana®, Bradenton, FL). One ml of each virus was added to 500 ml of each solution; PBS, milk and OJ. Each treatment was run through the high pressure homogenizer and pressures were recorded at 0, 100, 200, 250, and 300 MPa. After each pressure treatment, two samples were collected and the entire experiment was replicated twice.

The treatments with lecithin and HPH were FCV-F9 or MNV-1 in PBS with 0.025% lecithin (control), FCV-F9 or MNV-1 in milk with 0.025% lecithin, and FCV-F9 or MNV-1 in OJ with 0.025% lecithin. Similarly as described above, 1 ml of each virus was added to 500 ml of each solution; PBS with lecithin, milk with lecithin, or OJ with lecithin and sampled twice through the high pressure homogenizer at 0, 100, 200, 250, and 300 MPa and the experiment was replicated.

The HPH treatments with juice combinations were FCV-F9, MNV-1, or MS2 in OJ combined with pomegranate juice (PJ) (PomWonderful®, Los Angeles, CA) or OJ combined with pomegranate polyphenols (PP) (PomWonderful®) (2 mg/ml). One ml of each virus was added to 500 ml of each solution; OJ combined with PJ or OJ combined with PP and run through the high pressure homogenizer and pressures at 0, 100, 200, 250, and 300 MPa twice using duplicate samples.

Statistical Analysis

Statistical analysis on the FCV-F9, MNV-1, and MS2 infectivity assay data was carried out using the SAS system for windows version 9.2 (Statistical Analysis Institute (SAS), Cary, NC). Data were analyzed for differences in pressure with each medium (milk, orange juice, pomegranate juice, lecithin combinations, and juice combinations) using two replications on duplicate treatments of each virus.

Results

Effect of HPH on FCV-F9 in PBS, Milk, Orange Juice, and Lecithin Combinations

Feline calicivirus (FCV-F9) showed minimal reduction ($<1 \log_{10}$ PFU/ml) in all tested media at pressures 0, 100 and 200 MPa. FCV-F9 in PBS (control) and PBS with 0.025% lecithin showed 3.10 and 2.02 $\log_{10}$ PFU/ml reduction, respectively ($p < 0.05$) at 250 MPa. However, complete reduction in titer was achieved in PBS and PBS with lecithin (0.025%) at 300 MPa from an initial titer of $\sim 6 \log_{10}$ PFU/ml. FCV-F9 in milk and milk with lecithin showed a 1.22 and 0.78 $\log_{10}$ PFU/ml reduction, respectively ($p < 0.05$) at 250 MPa. At 300 MPa, FCV-F9 titers were completely reduced in milk ($\sim 4.66 \log_{10}$ PFU/ml) and significantly reduced in milk with

0.025% lecithin ($\sim 3.95 \log_{10}$ PFU/ml). FCV-F9 in OJ and OJ with 0.025% lecithin showed slight titer reduction at 250 MPa with 1.31 and 1.76 $\log_{10}$ PFU/ml reduction, respectively, while complete reduction was achieved at 300 MPa. Addition of lecithin appeared to have no additional effect on FCV-F9 and this virus was easily inactivated at pressure above 250 MPa (Figure 3.1).

Effect of HPH on MNV-1 in PBS, Milk, Orange Juice, and Lecithin Combinations

In all tested media, murine norovirus (MNV-1) was minimally reduced ($<1 \log_{10}$ PFU/ml) at pressures of 0, 100 and 200 MPa. MNV-1 PBS and PBS with 0.025% lecithin showed minimal reduction ($<1 \log_{10}$ PFU/ml) at pressures up to 250 MPa and moderate reduction at 300 MPa (~ 1.98 and $1.46 \log_{10}$ PFU/ml), respectively. In milk or milk with lecithin, MNV-1 was minimally reduced in titer at 250 MPa (~ 0.47 and $0.23 \log_{10}$ PFU/ml), respectively, and was slightly reduced at 300 MPa (~ 0.91 and $1.32 \log_{10}$ PFU/ml), respectively. MNV-1 in OJ was minimally reduced at 250 MPa ($\sim 0.16 \log_{10}$ PFU/ml) and slightly reduced at 300 MPa ($\sim 1.34 \log_{10}$ PFU/ml). In OJ with lecithin, MNV-1 was minimally reduced at 250 MPa ($\sim 0.83 \log_{10}$ PFU/ml) and significantly reduced at 300 MPa ($\sim 2.58 \log_{10}$ PFU/ml) (Figure 3.2).

Effect of HPH on FCV-F9 and MNV-1 in Juice Combinations

FCV-F9 was minimally reduced at 200 MPa ($<1 \log_{10}$ PFU/ml) and completely reduced in a combination of OJ and PJ at 250MPa. Similarly, FCV-F9 titer was minimally reduced at 200 MPa ($\sim 0.14 \log_{10}$ PFU/ml) in the combination of OJ and PJ, and completely reduced at 250 MPa. MNV-1, in the combination of OJ and PJ, was slightly reduced at 250 MPa ($\sim 1 \log_{10}$ PFU/ml) and significantly reduced at 300 MPa ($\sim 1.78 \log_{10}$ PFU/ml). In the combination of OJ and PP,

MNV-1 was minimally reduced in titer at 250 MPa ($\sim 1 \log_{10}$ PFU/ml) and significantly reduced ($\sim 2.31 \log_{10}$ PFU/ml) at 300 MPa (Figure 3.3).

Effect of HPH on MS2 in PBS, Milk Orange Juice, and Pomegranate Juice Combinations

MS2 was minimally reduced ($< 1 \log_{10}$ PFU/ml) in PBS (control), milk, and OJ at pressure up to 250 MPa, and significantly reduced in titer in PBS and milk (2.37 and $2.21 \log_{10}$ PFU/ml, respectively) at 300 MPa. In the combination of OJ and PJ, MS2 was significantly reduced in titer at 250 MPa ($\sim 1.48 \log_{10}$ PFU/ml) and almost completely reduced at 300 MPa, with a $4.1 \log_{10}$ PFU/ml reduction. MS2 in OJ combined with PP was reduced by $< 1 \log_{10}$ PFU/ml at pressures up to 250 MPa, but was significantly reduced in titer ($\sim 3.03 \log_{10}$ PFU/ml) at 300 MPa (Table 3.1).

Discussion

Foodborne viruses have been subjected to numerous traditional and novel processes in order to inactivate them. Even though, it is well known how these foodborne viruses are affected and inactivated by heat in cell culture and some food matrices, only few studies on novel methods that need further research and standardization for routine application have been reported. Guan et al. (2006) found that MS2 was very resistant to high hydrostatic pressure, in comparison to other coliphages, and was inactivated by $< 1 \log_{10}$ PFU/ml at pressures of 500 MPa for 5 minutes at 20°C in phosphate-buffered saline (Guan et al., 2006). These researchers suggested that MS2 was a suitable surrogate for hepatitis A virus (HAV) (Guan et al., 2006).

The primary advantages of a non-thermal process such as hydrostatic pressure processing (HPP) over thermal processing are minimal chemical and physical effects exerted on most foods

while imparting a microbial kill step (Grove et al., 2006). Most human and animal viruses are inactivated at hydrostatic pressures of 450 MPa or less, while other viruses are resistant at pressure up to 920 MPa (Grove et al., 2006). Many authors suggest that HPP denatures the virus capsid proteins, therefore preventing host cell binding and infection (Kovac et al., 2010; Hogan et al., 2005; Khadre and Yousef, 2002; Kingsley et al., 2002; Buckow and Heinz, 2008; Li et al., 2009; Tang et al., 2010). Homogenization is a method utilized primarily in the food industry to disperse particles to a uniform size to, for most cases, prevent separation. High pressure has recently been added to this technology as a means to prolong fluid food shelf life, while still maintaining nutritional and sensory characteristics. The development of applications at increasingly higher pressures has also created an interest in using it as a benefit for inactivation of microorganisms that occurs during this process and even to use high pressure homogenization as a preservation process (Diels et al., 2004). This process, particularly at higher pressures, disrupts the bacterial cell where Gram-positive bacteria are reported to be more resistant to HPH than Gram-negative bacteria (Diels and Michiels, 2006; Wuytack et al., 2002). It is thought that Gram-positive bacteria are more resistant due to their thick peptidoglycan cell wall layer. Middleburg and O'Neill (1993) also believed that peptidoglycan structure has an important role in the resistance to high pressure homogenization in which one of the parameters, the mean effective strength, is correlated with the peptidoglycan cross-linkage (Middleburg and O'Neill, 1993; Wuytack et al., 2002).

Inactivation of *E. coli* and *Listeria innocua* by high pressure homogenization were previously tested in apple and carrot juices. Pathanibul et al. (2009) found that *E. coli* was reduced by greater than 5 logs at pressures exceeding 250 MPa, while *L. innocua* required pressures greater than 350 MPa to achieve the same 5 log reduction (Pathanibul et al., 2009).

Kinglsey et al. (2002) subjected hepatitis A and feline calicivirus to high pressures using high hydrostatic pressure and found that a 7 log reduction (complete inactivation) of HAV was achieved after 5 minutes at 450 MPa or more and FCV-F9 was completely inactivated after 5 minutes of 275 MPa or more (Kinglsey et al., 2002). Recently, the effect of HPH against murine norovirus and MS2 bacteriophage in phosphate buffered saline was studied and found that at 300 MPa there was approximately a 3 log reduction for MS2 and about 0.8 log reduction in MNV-1 (D'Souza et al., 2009). In this study, human norovirus surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) were spiked in juices and HPH effects were studied. D'Souza and others (2009) demonstrated that MNV-1 is fairly resistant to pressure for it showed no significant reduction in titer when subjected to homogenization pressures of 100 and 200 MPa, with slight reduction in titers at 250 MPa (0.22 log PFU) and 300 MPa (0.80 log PFU) (D'Souza et al., 2009). FCV-F9 is more sensitive in to high pressures, for Grove (2006) stated that there was complete inactivation of a 7-log culture of FCV-F9 after exposure to 275 MPa or more (Grove et al., 2006). The inactivation mechanisms are still unclear as to how the high pressure homogenizer inactivates the viruses, but D'Souza and others (2009) theorized that the process causes changes or disruption in viral capsid structure and different viruses may have differing resistance levels due to dissimilarity in their coat proteins as well as morphological differences.

The media in which the viruses were processed had varying effects, with some being protective. FCV-F9 in PBS was significantly reduced at 250 MPa ($\sim 3.10 \log_{10}$ PFU/ml) and completely reduced at 300 MPa. Interestingly, FCV-F9 in milk had less reduction at 250 and 300 MPa, with 1.22 and 4.66 $\log_{10}$ PFU/ml reductions, respectively. Similarly, MNV-1 in PBS had 0.39 and 1.98 $\log_{10}$ PFU/ml reductions at 250 and 300 MPa, respectively. In milk, MNV-1 had a similar reduction at 250 MPa ($\sim 0.47 \log_{10}$ PFU/ml) and a lesser reduction at 300 MPa (~ 0.91).

Milk as a medium appears to have a protective effect on FCV-F9 and MNV-1. Although more acidic than milk, OJ appeared to have a protective effect on the viruses during HPH processing, when compared to PBS. As stated previously, FCV-F9 in PBS was significantly reduced at 250 MPa ($\sim 3.10 \log_{10}$ PFU/ml) and completely reduced at 300 MPa. In OJ, FCV-F9 had a $1.31 \log_{10}$ PFU/ml reduction at 250 MPa, and was completely reduced at 300 MPa. As mentioned above, MNV-1 in PBS had 0.39 and $1.98 \log_{10}$ PFU/ml reductions at 250 and 300 MPa, respectively. In OJ, MNV-1 showed 0.16 and $1.34 \log_{10}$ PFU/ml reductions at 250 and 300 MPa, respectively. Similarly, MS2 showed 0.59 and $2.37 \log_{10}$ PFU/ml reduction at 250 and 300 MPa, respectively, compared to 0.07 and $0.71 \log_{10}$ PFU/ml log reductions in OJ at the same pressures. As mentioned above, Bidawid and others (2000) found that milk, especially with higher fat content, had a protective effect on hepatitis A virus that underwent thermal treatment (Bidawid et al., 2000). Su and others (2010b) subjected the human norovirus surrogates FCV-F9, MNV-1, and bacteriophage MS2 to high intensity ultrasound (HIUS) and found that the viral inactivation was greatly reduced when suspended in OJ compared to PBS (Su et al., 2010b). It was noted that the food matrix may reduce the virus exposure to ultrasonic waves. This correlates with our findings in this study and the juice matrix could be protecting the viruses from the detrimental effects of HPH (Su et al., 2010b).

In addition, FCV-F9 is sensitive to pH and inactivated at pH values < 3 and > 9 (Cannon et al, 2006). MNV-1 was found to be stable across the pH range of 2-10, with less than 1 log reduction in infectivity at pH 2 (Cannon et al, 2006). Langlet et al. (2007) studied the effects of pH on plaque forming unit counts and found that in a potassium chloride (KCl) solution adjusted to pH values of 6.7, 3.9 and 2.5 there was a 0, 1.11 and 3 $\log_{10}$ reduction, respectively on bacteriophage MS2 (Langlet et al., 2007). In this experiment, it was hypothesized that milk

would have a protective effect on the viruses. It is also hypothesized that low pH juices, in combination with HPH would have additional effects on the viruses to inactivate them at lower pressures.

FCV-F9 was clearly the most sensitive virus to HPH. In PBS and OJ, FCV-F9 was completely inactivated at 300MPa. For FCV-F9, milk as a food matrix was the most protective, for it was not completely reduced ($\sim 4.66 \log_{10}$ PFU/ml) at 300 MPa. MNV-1 and bacteriophage MS2 were both sturdy viruses and fairly resistant to high pressures. Milk and OJ appeared protective of MNV-1, for at 300 MPa, a 0.91 and 1.34 $\log_{10}$ PFU/ml reduction occurred, respectively, compared to a 1.98 $\log_{10}$ PFU/ml reduction in PBS. MS2 on the other hand was most protected by the OJ food matrix. At 300 MPa, minimal reduction ($\sim 0.71 \log_{10}$ PFU/ml) was seen in OJ, while 2.37 and 2.21 $\log_{10}$ PFU/ml reductions were seen in PBS and milk, respectively.

D'Souza and others (2009) found that MS2 and MNV-1 in PBS subjected to HPH were reduced by 3 and 0.8 $\log_{10}$ PFU/ml, respectively at 300 MPa. This manuscript describes new findings; including fluid food matrices, the use of lecithin and the use of juice blends (discussed below). Our study found that the fluid food matrices of milk and OJ had a protective effect on the viruses. These results reveal the importance of using hurdle approaches, such as lecithin, juice blends, and/or other GRAS substances.

Due to the process of HPH being costly, and therefore a major drawback to its commercialization, hurdle approaches appear to be promising alternatives together with HPH. Adding compounds, such as emulsifiers, to the fluid food being treated for viral or microbial inactivation should theoretically inactivate the virus at a lower pressure, saving energy and cost.

The food industry has long recognized the importance of lecithin as a critical component in processing. Johnson and others (1984) studied the ability of isoelectric casein supplemented with lecithin to recover enteric viruses from estuarine sediments composed of sand, silt, and clay. It was suggested that the release of virus by lecithin-containing eluents may be associated with a decrease in hydrophobic bonding and a lowering of the surface tension energy (Johnson et al., 1984). Hydrophobic bonding to the clay surface was suggested to be disrupted by the surfactant effect of lecithin allowing greater contact with water molecules and virus release into suspending medium (Johnson et al., 1984, Dizer et al., 1984). These findings suggest that the addition of lecithin to foods may enable the release of virus from food particles). In this current study, the emulsifier lecithin was added to both tested animal viral surrogates in PBS, orange juice, and milk prior to processing.

Lecithin is naturally occurring in plant and animal tissues and is extracted from egg yolks and soy beans. It is composed of phosphoric acid, choline, fatty acids, glycerol, glycolipids, triglycerides, and phospholipids. Lecithin is a Food and Drug Administration (FDA) Generally Recognized as Safe (GRAS) food substance (ID code 8002-43-5; 21 CFR sec. 184.1400) (FDA, 2009). Lecithin helps to smooth food textures and serves as an emulsifying agent in margarines and chocolates (controls viscosity and crystallization) and chewing gum (as a softening and plasticizing agent) (Ramadan and Asker, 2009). Ramadan and Asker (2009) found that soy lecithin alone had minimal inactivation effect on bacteria and viruses (Ramadan and Asker, 2009). The goal for using lecithin in this experiment was to capitalize on the use of lecithin to help further breakdown the proteins of the viral capsid, therefore reducing the amount of pressure required to inactivate the virus. However, the results of our study show that lecithin had little to no additional effect on reducing the titers of either FCV-F9 or MNV-1.

As shown in Figures 1 and 2, even though the addition of the emulsifier lecithin had little to no effect on FCV-F9, it surprisingly reduced MNV-1 in milk and OJ. MNV-1 in OJ showed 0.16 and 1.34 log₁₀ PFU/ml reductions at 250 and 300 MPa, respectively. In comparison, MNV-1 in OJ combined with lecithin at 250 and 300 MPa showed a 0.83 and 2.58 log₁₀ PFU/ml, respectively. MNV-1 at 300 MPa showed a 0.91 log₁₀ PFU/ml reduction in milk, and a greater reduction (1.32 log₁₀ PFU/ml) in milk combined with lecithin. Although MNV-1 is the sturdier of the two viruses when exposed to high pressure, the addition of lecithin helped reduced its titer. Further research to understand the interaction between lecithin and the viral capsid during HPH inactivation needs to be undertaken.

Therefore, another hurdle approach was the use of juice combinations/blends with known anti-microbial properties subjected to HPH. The natural antimicrobial components of fruits and their extracts against foodborne viruses, as a way to enhance food safety, needs to be studied. The antimicrobial properties of thymol, lemon extract, and grape fruit seed extract (GFSE at 20, 40, and 80 ppm) has been shown to be effective against spoilage microorganisms within fresh fish burger fillets has been studied (Corbo et al., 2009). The health benefits of pomegranates and pomegranate extracts include its natural antibacterial and antiviral components (Wolfe et al., 2008; Al-Zoreky, 2009; Haidari et al., 2009). *Staphylococcus aureus* growth was found to be inhibited by low concentrations (0.01%) of pomegranate peel extract (Kanatt et al., 2010). Haidari et al., (2009) evaluated major polyphenols in pomegranate extract and found that punicalagin proved to be effective against influenza by possibly blocking RNA replication of the virus (Haidari et al., 2009). Recently, Su et al. (2010a) showed that pomegranate juice could reduce the viral titers of MS2, FCV-F9 and MNV-1 after 1 hour incubation at room temperature (25°C), where low titer (~10⁵ log₁₀ PFU/ml) MS2, FCV-F9 and MNV-1 were reduced by 0.32,

2.56, and 1.32 log PFU/ml, respectively (Su et al., 2010a). In the same study, MS2, FCV-F9 and MNV-1 were reduced by 0.41, 4.54, and 1.30, respectively, after a 1 hour incubation at 25°C in pomegranate polyphenol (4 mg/ml), with MS2 being the least effected (Su et al., 2010a). Haidari et al. (2009) suggested that, for the enveloped influenza virus, pomegranate juice could possibly reduce the amount of virus particles released into media, inhibit virus proliferation, or block the replication of the virus RNA (Haidari et al., 2009).

Our study also found that the use of tasty juice blends with known antimicrobial properties, have a combined effect on the viruses. MNV-1 in PBS and PJ combined with OJ showed similar reductions at 300 MPa ($\sim 1.8 \log_{10}$ PFU/ml), but a slightly greater reduction ($2.31 \log_{10}$ PFU/ml) in PP combined with OJ. FCV-F9 showed a $3 \log_{10}$ PFU/ml reduction in PBS at 250 MPa, but was completely inactivated in PJ and PP combined with OJ. OJ combined with PJ had a greater effect on MS2. At 300 MPa, MS2 in PBS, PP combined with OJ, and PJ combined with OJ, showed 2.37, 3.04, and $4.10 \log_{10}$ PFU/ml reductions, respectively. Overall, the direct pomegranate polyphenol had a great effect on the viruses than the pomegranate juice, possibly due to a more direct interaction with the viruses. The addition using juice blends could be used to reduce the pressure requirements by 50 MPa in some cases.

Therefore, hurdle approaches using natural antimicrobials as in juice blends may help enhance the safety of juices while simultaneously providing additional antioxidants to increase the nutritive value of juice products and extend shelf-life. High pressure homogenization looks promising as an alternative technology for commercial use to inactivate human norovirus surrogates in fluid foods at pressures of 300 MPa and higher. Although lecithin had minimal additional effect on the viruses by HPH treatment that was used to lower the required inactivation pressures, the combination of orange juice-pomegranate juice blends did have an

effect on the tested viruses, resulting in lowered pressure requirements for inactivation. This suggests that GRAS (generally recognized as safe) antimicrobials could potentially be added to juices for viral reduction and need to be explored in future *in vitro* and *in vivo* experiments.

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Appendix

Table 3.1: Effect of High Pressure Homogenization on Bacteriophage MS2 in PBS (control), Milk, Orange Juice (OJ), OJ Combined with Pomegrante Juice (PJ), OJ Combined with Pomegranta Polyphenols (PP). Values between treatment pressures with different letters denote significant differences ($p < 0.05$).

Reduction in MS2 titers (Log_{10} PFU/mL) after HPH processing					
Pressure (Mpa)	PBS	Milk	OJ	OJ/PJ	OJ/PP
0	0±0C	0±0C	0±0B	0±0C	0±0B
100	0.04±0.04C	0.07±0.12BC	0.01±0.03B	0.06±0.11C	0.03±0.02B
200	0.04±0.03C	0.27±0.15BC	0.09±0.06B	0.48±0.22C	0.18±0.03B
250	0.59±0.01B	0.33±0.08B	0.07±0.03B	1.48±0.12B	0.59±0.14B
300	2.37±0.09A	2.21±0.11A	0.71±0.34A	4.1±0.45A	3.03±0.64A

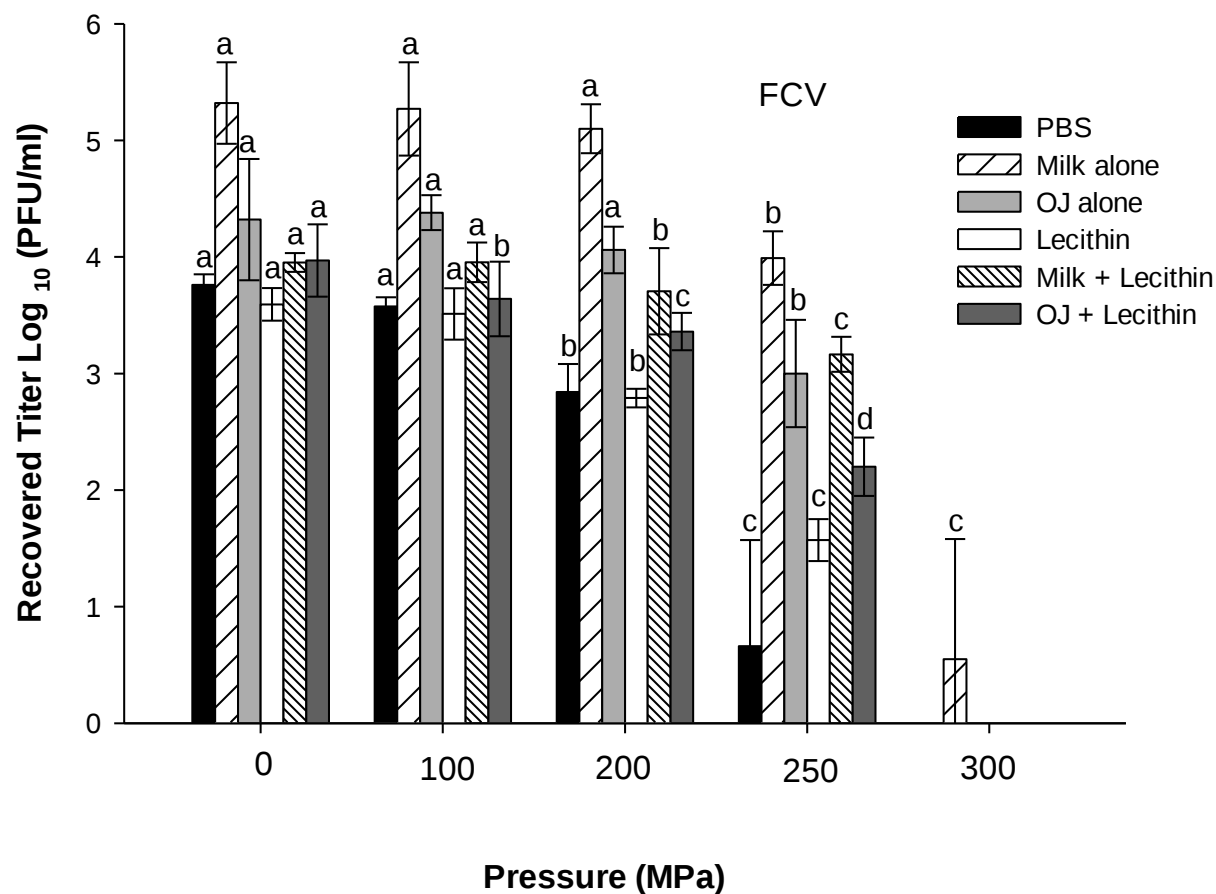


FIGURE 3.1: Recovery of FCV-F9 in PBS (control), Milk, Orange Juice (OJ), PBS with Lecithin, Milk with Lecithin, and OJ with Lecithin using High Pressure Homogenization (HPH) Treatments of 0, 100, 200, 250, and 300 MPa. Values between treatment pressures with different letters denote significant differences ($p < 0.05$).

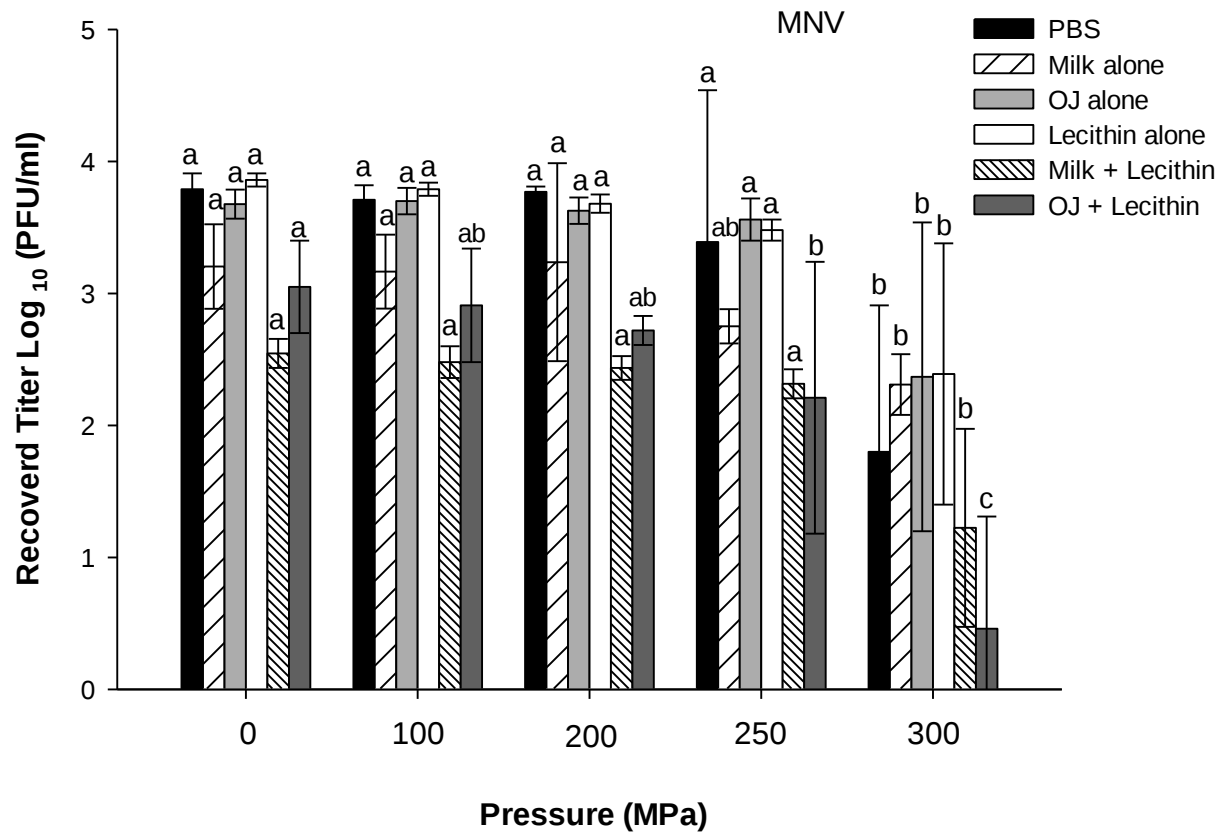


FIGURE 3.2: Recovery of MNV-1 in PBS (control), Milk, Orange Juice (OJ), PBS with Lecithin, Milk with Lecithin, and OJ with Lecithin at 0, 100, 200, 250, and 300 MPa using High Pressure Homogenization (HPH). Values between treatment pressures with different letters denote significant differences ($p < 0.05$).

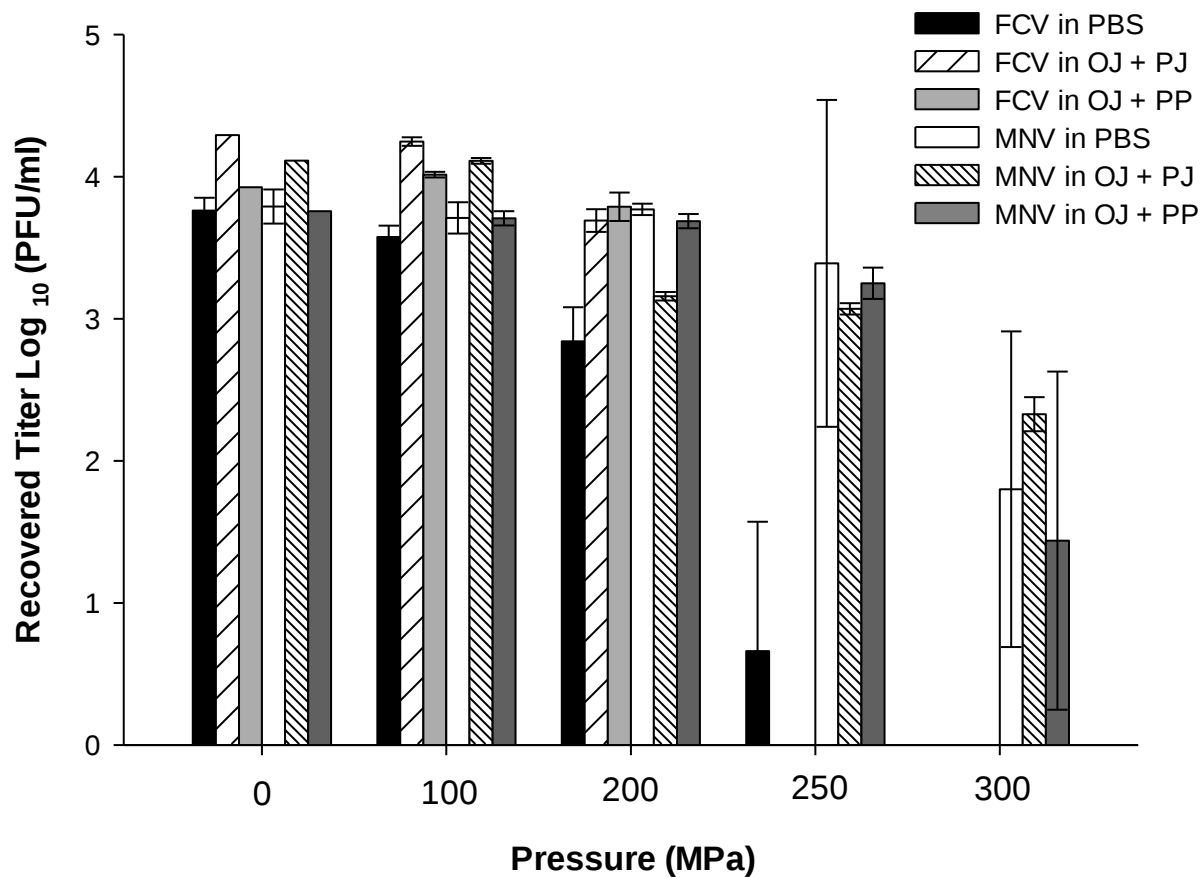


FIGURE 3.3: Recovery of MNV-1 and FCV-F9 in PBS (control), Orange Juice (OJ) Combined with Pomegranate Juice (PJ) and OJ Combined with Pomegranate Polyphenols (PP) using High Pressure Homogenization. Values between treatment pressures with different letters denote significant differences ($p < 0.05$).

CHAPTER IV

Survival of Human Norovirus Surrogates in Blueberry Juice at Refrigeration (4°C) and their Inactivation in Blueberry Juice using High Pressure Homogenization

Survival and Inactivation of Human Norovirus Surrogates in Blueberry Juice

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Key words: Norovirus, Survival, FCV-F9, MNV-1, MS2, Blueberry Juice, High Pressure Homogenization

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Abstract

Human noroviruses have been implicated in gastrointestinal outbreaks associated with fresh fruits, juices, and ready-to-eat foods. In order to determine transmission risk, survival characteristics of cultivable human norovirus surrogates (murine norovirus, MNV-1; feline calicivirus, FCV-F9; and bacteriophage MS2) in blueberry juice (BJ) (pH = 2.77) after 0, 1, 2, 7, 14, and 21 days at refrigeration temperatures (4°C) were studied. High pressure homogenization (HPH), a novel processing method for viral inactivation BJ was also studied. Samples of BJ or phosphate buffered saline (PBS; pH 7.2 as controls) were inoculated with each surrogate virus, stored over 21 days at 4°C, and assayed using standard plaque assays. FCV-F9 (~7 log₁₀PFU/ml) was found to be undetectable after 1 day in BJ at refrigeration (4°C). MNV-1 (~6 log₁₀PFU/ml) showed minimal reduction (~1 log₁₀PFU/ml) after 14 days and higher reduction (1.95 log₁₀PFU/ml; p<0.05) after 21 days in BJ at 4°C. Bacteriophage MS2 (~7 log₁₀PFU/ml) showed significant reduction (1.93 log₁₀PFU/ml; p<0.05) after 2 days and was undetectable after 7 days in BJ at 4°C. In comparison to PBS controls, FCV-F9 remained viable for up to 21 days (2.28 log₁₀PFU/ml reduction); similarly, MNV-1 and MS2 survived after 21 days with only 1.08 and 0.56 log₁₀PFU/ml reduction, respectively. Intriguingly, when HPH was used, FCV-F9 and bacteriophage MS2 showed complete reduction after minimal homogenization pressures at 2°C in BJ (pH = 2.77), possibly due to combination of juice pH, juice components, and mechanical effects. MNV-1 titers in BJ were slightly reduced at pressures of 250 (0.33 log₁₀PFU/ml) and 300 MPa (0.71 log₁₀PFU/ml). Viral survival characteristics in BJ at 4°C correlate well with the ease of human norovirus transmission via juices that can be prevented using HPH for viral inactivation in juices.

Introduction

Foodborne viruses are recognized as the major causes of foodborne illness and death worldwide, with higher incidences reported possibly due to improved surveillance as well as increased international travel and trade. Viruses alone reportedly cause an estimated 5.5 million foodborne illnesses (59%), 27% of hospitalizations, and 12% of deaths in the United States alone (Scallan et al., 2011). The human noroviruses (NoV), hepatitis A virus (HAV), rotaviruses, astroviruses, adenoviruses, Aichiviruses, hepatitis E virus, and human enteroviruses such as coxsackieviruses and echoviruses, remain a concern to public health (Sair et al., 2002; D'Souza et al., 2007). In addition, the emerging recombinant human norovirus strains such as GII.4b capable of causing death in the elderly and immunocompromised, drives the need to understand and curb transmission and prevalence of these virulent viruses (Siebenga et al., 2009; Donaldson et al., 2008).

It is well recognized that human foodborne viruses are transmitted primarily via the fecal-oral route through contaminated water and food (especially ready-to-eat foods that do not undergo further processing) infectious vomitus, environmental surfaces, aerosolization, and infected individuals through person-to-person contact or person-to-fomite contact (Patel et al., 2009; Sair et al., 2002). Several human NoV and HAV outbreaks have been reported, including in January of 2009, where 36 persons became ill after eating a norovirus-contaminated salad on a German military base (Wadl et al., 2010). Juice beverages are a common vehicle of foodborne illness, but studies have shown that some juices naturally contain antimicrobial components. Even so, notable viral outbreaks have been traced to fruit juices as sources, including an outbreak in February 2009 that sickened 38 persons from two Dutch airline coach trips who visited the same hotel in Germany, caused by the consumption of juice in large self-service containers with

a tap (Visser et al., 2010). A major outbreak of hepatitis A among tourists returning from Egypt in 2004 involved 351 infected individuals from 9 European countries and orange juice was most likely the infection vehicle (Frank et al., 2007). One of the largest norovirus outbreaks occurred on domestic airline flights during August of 1991, involving 3000 individuals who had consumed orange juice served to passengers on the flights (Fleet et al., 2000). These outbreaks show the need to have better hygienic practices and control measures to prevent foodborne viral outbreaks in the fruit juice industry.

Blueberries, both the lowbush “wild” type (*Vaccinium angustifolium* Aiton) and the highbush “cultivated” type (*Vaccinium corymbosum* L.), are flowering plants native to North America and are commercially cultivated and sold fresh or processed for juices, jams, and use in baked goods (Kalt et al., 2001). Blueberries contain structurally related polyphenols, including anthocyanins and flavonoids. Anthocyanins are water-soluble pigments that appear red, purple, or blue in plants, producing the blue color in blueberries, and are associated with a wide range of biological activities that may positively affect health (Burdulis et al., 2009). Studies have shown that anthocyanins may reduce the risk of heart disease, protect arterial endothelial cells, decrease the risk of cancer, and modulate immune response (Burdulis et al., 2009). Along with health benefits, anthocyanins from berry extracts have been studied for their antimicrobial properties. Burdulis et al. (2009) tested eight different bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, and, *Salmonella Typhimurium*, and found that *Citrobacter freundii* and *Enterococcus faecalis* were the most sensitive to anthocyanin extracts of blueberry (*Vaccinium corymbosum* L.) and bilberry (*Vaccinium myrtillus* L.) fruits. In addition, blueberries contain proanthocyanidins (PAC), also referred to as condensed tannins, that are considered to be active against carcinogenesis and bacterial adherence during urinary tract infections (Smith et al., 2000;

Bomser et al., 1996; Howell et al., 1998; Ofek et al., 1991). Recently, Takeshita et al. (2009) found that blueberry PAC inhibits the expression and replication of hepatitis C virus (Takeshita et al., 2009). Additionally, pomegranate juice (PJ) and its coupled/associated polyphenols are reported to have antibacterial and antiviral properties (Haidari et al., 2009; Al-Zoreky 2009). Su and others (2010b) and have recently shown that PJ reduces the infectivity of human NoV surrogates. This research leads us to believe that blueberries may also possess components with a range of antiviral properties, yet, the survival of foodborne human noroviruses in blueberry juice is currently unknown.

In the absence of an infectivity assays and cell culture host systems for human NoV, cultivable (+) single-stranded RNA viral surrogates such as murine norovirus (MNV-1); feline calicivirus (FCV-F9) and bacteriophage MS2 are used instead (Doultree et al., 1999; Wobus et al., 2006; Calender, 1988; Dawson et al., 2005). These viruses have been previously studied to determine enteric virus survival in various environments and under various conditions (Grove et al., 2008; Kingsley et al., 2007; Sharma et al., 2008; Bae and Schwab 2008; D'Souza and Su 2010).

Human enteric viruses can persist in the environment and foods for weeks to months, and are quite resistant to some standard traditional thermal and/or chemical inactivation methods (Sair et al., 2002). Besides, thermal processing of foods can produce undesirable sensory and texture properties unsuitable to consumers. Therefore, novel non-thermal processing technologies, such as irradiation, pulsed electric fields, ultraviolet laser treatments, and ultra high pressure, have been introduced as ways to non-thermally preserve foods, while maintaining nutritional and sensory attributes of the food product, that are safe and desirable for consumers (Cardello, 2003). High pressure homogenization (HPH) is a novel process for fluid food

inactivation based on many combined factors such as pressure, mild heat, shear stress, cavitation, and turbulence to inactivate microorganisms (Taylor et al., 2007). Previously, our laboratory reported on the inactivation of MS2 and MNV-1 by HPH in phosphate buffered saline, where homogenization pressures of 300 MPa showed a ~3 log PFU and ~0.8 log PFU reduction MS2, and MNV-1, respectively from initial titers of ~6 log PFU (D'Souza et al., 2009).

In this study, blueberry juice (BJ) was used as the medium to determine survival characteristics of human NoV surrogates (FCV-F9, MNV-1, and MS2) at refrigeration temperatures over 21 days (to simulate household storage conditions of juices). Another aim of this study was to determine the suitability of using HPH as a novel alternative approach for human NoV inactivation in BJ for application in industrial settings.

Materials and Methods

Viruses and Cell Lines

As described in our earlier studies, feline calicivirus (FCV-F9) and its host Crandell Reese Feline Kidney (CRFK) cells, and bacteriophage MS2 and its host *E. coli* B-15597 were obtained from ATCC (Manassas, VA), while Murine norovirus (MNV-1) was kindly provided as a gift by Dr. Skip Virgin of Washington University in St. Louis, Missouri (Horm and D'Souza, 2011; Su and D'Souza, 2011). MNV-1 host cells (RAW 264.7) were obtained from the University of Tennessee (UTIA) cell culture collection.

FCV-F9 stocks were prepared as previously described (D'Souza et al., 2009; D'Souza and Su, 2010; Su et al., 2010a). Briefly, FCV-F9 was infected onto confluent CRFK cells in 175 cm² flasks and incubated at 37°C and 5% CO₂ for 24 h until 90-100% cell lysis was observed (D'Souza and Su, 2010; Su et al., 2010a). This flask was freeze-thawed and centrifuged at room

temperature at 5000 x *g* for 10 min. The supernatant was then filtered, aliquoted, and stored at -80°C. Lastly, the FCV-F9 viruses were plaque assayed to determine the titer and used as viral stocks for the entire study.

Similarly, MNV-1 stock was prepared using confluent RAW cells in 175 cm² flasks and infecting with MNV-1, followed by incubation at 37°C and 5% CO₂ for 4-6 days until 90-100% cell lysis was observed and procedures described above were followed for viral stocks and assays (Su et al., 2010a).

Following previously described procedures, bacteriophage MS2 was propagated in its host *E. coli* B-15597 at 37°C for ~18 h in Trypticase Soy Broth (TSA) (3%) containing 0.1% glucose, 2mM CaCl₂, and 10 µg/ml Thiamine (D'Souza et al., 2009). Viruses were recovered as described above for FCV-F9 and plaque assayed as described below.

FCV-F9 Plaque Assay

Using Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 2% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY), CRFK cells were cultivated as described earlier (D'Souza and Su, 2010). Briefly, confluent CRFK cells were infected with 0.5 ml of treated or untreated FCV-F9 that were serially diluted in DMEM containing 2% FBS after aspirating the media. The infected cells were incubated for 2 h at 37°C and 5% CO₂ and the virus suspension was aspirated. The cells were then overlaid with 2 ml DMEM containing 2% FBS, 0.75% agarose, and HyClone® 1% penicillin-streptomycin (Logan, Utah). After 48 h incubation, 1 ml of a secondary overlay medium containing 0.01% neutral red was added to stain the plates. Lastly, the plaques were counted after 24 h incubation at 37°C.

MNV-1 Plaque Assay

Using Biowhittaker® Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic (Invitrogen™, Grand Island, NY), RAW 264.7 cells were cultivated as described earlier (Su et al., 2010a). After adding the cell suspension to six-well plates, they were incubated under 5% CO₂ at 37°C until 80-90% confluency. After aspirating the media, confluent RAW 264.7 cells were infected with 0.5 ml of ten-fold serial viral dilutions. The cells were overlaid with 2 ml DMEM containing 10% FBS, 0.75% agarose, and HyClone® 1% penicillin-streptomycin (Logan, UT) after 2.5-h incubation at 37°C with 5% CO₂. Lastly, 2ml of secondary overlay medium containing 0.02% neutral red was added to stain the plates after 72-h incubation, and plaques were counted after 2-h incubation.

MS2 Plaque Assay

The double agar layer method of Bae and Schwab (2008) was used for the MS2 infectivity plaque assay (Bae and Schwab, 2008). As described before, 0.3 ml of 6-h cultures of *E. coli* B-15597 host in TSA containing 0.1% glucose, 2mM CaCl₂, and 10 µg/ml thiamine was combined with 0.7 ml of serially diluted MS2 phage, mixed with top agar (0.6%) and poured on to TSA plates (D'Souza et al., 2009). Plaques were counted after incubation at 37°C overnight.

Survival Study

Survival of MNV-1 (6-log₁₀ PFU/ml), FCV-F9 (7-log₁₀ PFU/ml), and bacteriophage MS2 (7-log₁₀ PFU/ml) was determined in blueberry juice (BJ) (Oceanspray® Diet Blueberry Juice, Massachusetts USA 02349) or phosphate buffered saline (PBS; pH=7.2, control). Two-hundred microliters (0.2 ml) of each virus was added to 1.8 ml juice or PBS and stored for 0, 1, 2, 7, 14,

and 21 days at refrigeration temperature (4°C). The virus was serially diluted in DMEM containing 10% FBS and plaque assayed, as described above, at each time point. Each experiment was replicated four times and assayed twice. In addition, to determine the effects of juice components alone, the survival study of virus in PBS at a pH similar to BJ of 2.77 over 21 days was also undertaken. Oxalic acid was added to PBS until a pH of 2.77 was attained, and each virus was added to the solution, serially diluted, and plaque assayed either immediately or over 21 day storage at 4°C, as described above.

HPH Unit and Treatments

The treatments for HPH included FCV-F9 (7-log₁₀PFU/ml), MNV-1 (6-log₁₀PFU/ml), or MS2 (7-log₁₀PFU/ml) in PBS (control) and FCV-F9, MNV-1, or MS2 in BJ (Oceanspray® Diet Blueberry Juice, Massachusetts USA 02349) at pressures from 0 to 300 MPa. One ml of each virus was added to 500 ml of PBS or BJ. Each treatment (500 ml) was run through the high pressure homogenizer as described earlier (D'Souza et al., 2009) and pressures were recorded at 0, 100, 200, 250, and 300 MPa and 15 mls of duplicate samples were collected at each pressure. Each experiment was performed thrice. These samples were immediately placed on ice, then aliquoted and stored at -80°C prior to diluting and assaying.

Briefly, all processes were carried out in a FPG 12500 bench top high-pressure homogenizer (Stansed Fluid Power, Ltd, Essex, UK), with the regulator temperature held at 2°C using a water bath under ice. A computer program that continuously recorded pressure and temperature during treatments (Lookout, V 5.1, National Instruments, Austin, TX) was connected to the homogenizer.

Statistical Analysis

Statistical analysis on the FCV-F9, MNV-1, and MS2 infectivity assay data was done using ANOVA and Tukey's test on a completely randomized design using the SAS system for windows version 9.2 (Statistical Analysis Institute (SAS), Cary, NC). Data were analyzed for differences in survivability over time in PBS and blueberry juice with four duplicate treatments of each virus. Data were also analyzed for differences in pressure inactivation with blueberry juice for three replication treatments of each virus.

Results

Survival Trends of FCV-F9, MNV-1, and MS2 in PBS (pH=7.2 and pH=2.77) and Blueberry Juice over 21 Days at Refrigeration Temperature (4°C)

FCV-F9 at $\sim 7\text{-log}_{10}\text{PFU/ml}$ in blueberry juice (BJ) showed complete reduction after 1 day at refrigeration temperature (4°C). MNV-1 at $\sim 6\text{-log}_{10}\text{PFU/ml}$ showed minimal reduction ($\sim 1\text{ log}_{10}\text{PFU/ml}$) after 14 days in BJ and significant reduction ($1.95\text{ log}_{10}\text{ PFU/ml}$) after 21 days in BJ at 4°C. Bacteriophage MS2 at $\sim 7\text{-log}_{10}\text{PFU/ml}$, showed significant reduction ($1.93\text{ log}_{10}\text{PFU/ml}$) after 2 days and complete reduction in titer after 7 days in BJ at 4°C (Table 4.1).

In comparison, FCV-F9 in PBS at pH 7.2 at refrigeration temperature showed minimal reduction in titer after 7 days ($\sim 1\text{ log}_{10}\text{ PFU/ml}$) and significant reduction ($2.28\text{ log}_{10}\text{ PFU/ml}$) after 21 days of storage, but survived longer in PBS than in BJ. MNV-1 in PBS at 4°C showed slight to minimal reduction ($\sim 1\text{ log}_{10}\text{ PFU/ml}$) after 21 days, which was lower in reduction than compared to MNV-1 in BJ over 21 days ($1.95\text{ log}_{10}\text{ PFU/ml}$). Similarly, MS2 in PBS showed minimal reduction ($<1\text{ log}_{10}\text{ PFU/ml}$) after 21 days of storage at refrigeration temperature (4°C), compared to complete reduction after 2 days in BJ. Interestingly, MS2 survived the longest in PBS, but MNV-1 survived the longest in BJ at 4°C (Table 4.1).

Survival of FCV in PBS at pH 2.77 (simulating conditions of pH similar to BJ but without the effect of juice) showed significant reduction ($2.27 \log_{10}$ PFU/ml) after 2 days and complete reduction after 7 days at 4°C. MNV-1 showed minimal reduction ($0.44 \log_{10}$ PFU/ml) after 21 days in PBS (pH=2.77) at refrigeration temperature. MS2 showed significant reduction after 7 and 14 days (2.74 and $3.37 \log_{10}$ PFU/ml, respectively) and complete reduction after 21 days at 4°C (Table 4.1).

Effect of HPH on FCV-F9, MNV-1, and MS2 in Blueberry Juice

FCV-F9 and surprisingly MS2 were completely inactivated after minimal homogenization processing pressures in BJ. MNV-1 was slightly reduced in titer at 250 MPa ($0.33 \log_{10}$ PFU/ml) and 300 MPa ($0.71 \log_{10}$ PFU/ml). In comparison, MNV-1 in PBS was minimally reduced in titer in pressures up to 250 MPa ($0.39 \log_{10}$ PFU/ml) and significantly reduced in titer ($1.98 \log_{10}$ PFU/ml) at 300 MPa from an initial titer of $\sim 5 \log_{10}$ PFU/ml (Table 4.2).

Discussion

Fruit juices are of particular concern in food safety, for major foodborne viral outbreaks have been documented. As mentioned in the introduction, 351 tourists returning from Egypt were sickened by hepatitis A and orange juice was found to be the probable infectious source (Frank et al., 2007). While FCV-F9 and MNV-1 are used for human NoV studies, Guan et al. (2006) suggested that MS2 was a suitable surrogate for hepatitis A virus (HAV) and also perhaps enteroviruses, due to its persistence in adverse environmental conditions (Guan et al., 2006). Therefore, understanding the survival, persistence and transmission of human enteric viruses are key factors to prevent and control outbreaks.

Our control experiments in PBS (pH = 7.2) showed survival of FCV-F9, MNV-1, and MS2 over 21 days at 4°C that resulted in 2.28, 1.08, and 0.56 log₁₀ PFU/ml reductions, respectively, with MS2 being the most persistent in PBS. When PBS at pH 2.77 similar to the pH of BJ was used, survival of FCV-F9 showed complete reduction after 7 days at 4°C. Similarly, MS2 was completely reduced after 21 days. MNV-1 showed minimal reduction (<1 log₁₀ PFU/ml) after 21 days in PBS (pH=2.77) at 4°C.

Viruses have been shown to survive on various foods and surfaces for extended periods of time and to be relatively resistant to low pH values. Regarding studies with FCV-F9 on environmental surfaces, D'Souza et al. (2006) found that FCV-F9 was detectable on a number of environmental surfaces up to 7 days at room temperature, with an initial drop in titer of 2-3 log₁₀ after 1 h followed by an additional 2-4 log inactivation occurring between 24 and 48 hours (D'Souza et al., 2006). On brass and computer keyboards at room temperature (25°C), FCV-F9 infectivity was found to be reduced by 90% after less than 4 h (Clay et al., 2006). Another study observed a 2 log₁₀ PFU/ml reduction, from an initial titer of 5 log₁₀ PFU/ml, in FCV-F9 on lettuce after 7 days and more than 2.5 log₁₀ reduction on strawberries after 6 days at refrigeration temperature (4°C) (Mattison et al., 2007). Dawson et al. (2005) showed that MS2 bacteriophage survived on fresh iceberg lettuce, baton carrot, cabbage, spring onion, curly leaf parsley, capsicum pepper, tomato, cucumber, raspberries, and strawberries for a very long time over extended shelf life of the produce, with minimal reduction (<1 log₁₀ PFU/ml) after 50 days at 4 and 8°C (Dawson et al., 2005). MNV-1 is known to be more resistant than FCV-F9 to low pH values (Cannon et al., 2006). When exposed to a pH of 2 at 37°C for 30 minutes, MNV-1 titers were reported to be reduced by <1 log (Cannon et al., 2006). FCV-F9, on the other hand, seemed surprisingly stable at pH values of 6 (Duizer et al., 2004; Hewitt and Greening, 2004). However,

FCV-F9 was reduced by 5 logs (Duizer et al., 2004) when incubated at a pH of 2 or lower for 30 minutes at 37°C.

In our study, when determining the survival characteristics of FCV-F9, MNV-1, and MS2 in BJ, we found that MNV-1 was the sturdiest virus among the tested three viruses. MNV-1 persisted in BJ over 21 days with only a 1.95 log₁₀PFU/ml reduction. FCV-F9 was the most sensitive among the tested viruses, as expected based on previous studies (Cannon et al., 2006), being completely inactivated after 1 day in BJ at 4°C. Bacteriophage MS2 was found to persist slightly in BJ, and was completely reduced after 7 days at 4°C. This appears to be in agreement with the finding of others, for the low pH and antimicrobial compounds in BJ showed the greatest effect on the sensitive FCV-F9.

Although heat treatments and chemical disinfectants are commonly used to control microorganisms, they have many drawbacks associated with food texture, flavor and nutritive value that make it increasingly important to use alternative “green” technologies such as natural, plant-derived antimicrobials. Many plants, especially berry fruits, contain biologically active compounds that have shown to benefit health and inhibit microbial growth. Pomegranates (*Punica granatum* L.) contain a wide range of phytochemicals, including tannins, anthocyanins and other polyphenols that have antioxidant, antidiabetic, antibacterial, antiviral, and anticarcinogenic activities, among others (Viuda-Martos et al., 2010). Haidari et al. (2009) evaluated four major polyphenols in pomegranate extract and found that punicalagin proved to be effective against influenza (Haidari et al., 2009). Recently, the viral titers of MS2, FCV-F9 and MNV-1 were reduced by pomegranate juice and pomegranate polyphenols (Su et al., 2010b). Su et al. (2010a) also studied the effect of cranberry juice (CJ) and cranberry proanthocyanidins (PAC) on the infectivity of human enteric virus surrogates, after 1 hour at room temperature and

found that FCV-F9 was undetectable, and MNV-1, MS2, and phiX-174 were reduced by 2.75, 0.8, and 3.67 log₁₀ PFU/ml, respectively (Su et al., 2010a). This leads us to the importance of investigating blueberries, which also contain phytochemicals, as having natural antimicrobial constituents and could possibly have antiviral effects on fruit juice that is contaminated post-processing.

Blueberries contain vitamins, minerals, a number of flavonoid phytochemicals, and condensed tannins known as proanthocyanidins (PAC), that contribute to organoleptic properties such as stability, astringency, and bitterness (Takeshita et al., 2009; Arnold and Noble, 1978; Brossaud et al., 2001). The flavonol quercetin found in blueberries also seems to be the compound in red wines related to protecting the heart (Smith et al. 2000; Formica and Regelson, 1995). The recent findings of Takeshita et al. (2009) showing that PAC extracted from blueberry leaves inhibit the expression of subgenomic hepatitis C virus (HCV) RNA (Takeshita et al., 2009), suggests the need to explore blueberry PACs as an anti-viral compound. Our data reported in this study show that FCV-F9 and MS2 did not survive more than 7 days in BJ at 4°C, potentially linking components of BJ with antiviral activity.

Hence, in comparison to controls using PBS, it is not surprising that the viruses did persist for shorter periods of time in BJ, but still long enough to transmit norovirus infections during contamination. Based on the results with pH at 2.7 alone, it appears that additional factors associated with BJ play a role in the decreased survival of the tested surrogates over refrigeration. The shorter survival in BJ, can be attributed to low pH, and natural polyphenols found in BJ, along with the other “unidentified/uncharacterized” components of the BJ juice. Hence, further studies to determine if BJ contains substances that have natural antiviral properties to prevent foodborne gastroenteritis and to help enhance food safety need to be

explored/ investigated. However, the ability of the tested viruses to survive in BJ albeit over a relatively shorter period of time at refrigeration compared to PBS control, enhances our need to improve and maintain safety of juices to prevent foodborne disease outbreaks.

Based on previous MNV-1 studies (D'Souza et al., 2009), predictably the experiments using high pressure homogenizer (HPH) showed that MNV-1 was the most resilient among the tested viruses to pressure treatment. MNV-1 was resistant to higher pressures, being reduced by only 0.33 log₁₀ PFU/ml at pressures up to 250 MPa in BJ. At 300 MPa, MNV-1 had only a 0.71 log₁₀ PFU/ml reduction. This correlates with a study by D'Souza et al. (2009), demonstrating that MNV-1 is fairly resistant to pressure for it showed insignificant reduction in titer when subjected to homogenization pressures of 100 and 200 MPa, with slight reduction in titers at 250 MPa (0.22 log PFU) and 300 MPa (0.80 log PFU) (D'Souza et al., 2009). In this study, FCV-F9 and MS2 were not detectable after processing at the lowest recordable pressures, perhaps due to the fluctuation in pressures using new valves and technical difficulties associated with the HPH machine, that need to be stated and addressed. However reductions for FCV-F9 are not surprising, for FCV-F9 is reported to be sensitive to high pressure treatments. Grove et al. (2006) stated that there was complete inactivation of a 7-log FCV after exposure to 275 MPa or more (Grove et al., 2006). Kinglsey et al. (2002 and 2007) subjected hepatitis A and feline calicivirus to high pressures using high hydrostatic pressure and found that a 7 log reduction (complete inactivation) of HAV was achieved after 5 minutes at 450 MPa or more and FCV was completely inactivated after 5 minutes of 275 MPa or more (Kingsley et al., 2002; Kingsley et al., 2007). Recently, the effect of HPH against murine norovirus and MS2 bacteriophage in phosphate buffered saline was studied and found that at 300 MPa there was approximately a 3 log reduction for MS2 and about 0.8 log reduction in MNV-1 (D'Souza et al., 2009).

The means/mechanisms are still unclear as to how the high pressure homogenizer inactivates foodborne viruses. D'Souza et al. (2009) and other researchers (Moroni et al., 2002) theorized that the HPH process causes changes or disruption in viral capsid structure and different viruses may have differing resistance levels due to dissimilarity in their coat proteins as well as morphological differences (D'Souza et al., 2009; Moroni et al., 2002). This study shows that HPH along with hurdle approaches may provide better inactivation of foodborne viruses in fluid foods. The use of GRAS (generally recognized as safe) substances with known antimicrobial/antiviral properties, in combination with processing technologies may be beneficial and should be further explored.

In summary, our data suggest that MNV-1 is the most resilient and resistant virus among the three tested surrogates in blueberry juice and after HPH treatment. MNV-1 survived the longest period of time (up to 21 days) at 4°C in the naturally low pH BJ. Expectedly, FCV-F9 was the most sensitive virus, not detectable after 1 day in BJ in refrigeration temperatures. MNV-1 was also the most resistant virus after HPH processing, being detectable after treatment pressures of 300 MPa. BJ, with its antimicrobial components, in combination with HPH, were the most probable reason that FCV-F9 and MS2 were not detectable after treatment. These studies show the survivability of human norovirus surrogates in BJ that could account for NoV transmission and foodborne outbreaks and the application of a novel-processing method to possibly reduce foodborne viral risks and outbreaks via juices.

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Appendix

Table 4.1: Reduction of FCV-F9 (~7 log₁₀PFU/ml), MNV-1(~6 log₁₀PFU/ml), and MS2 (~7 log₁₀PFU/ml) at 4°C over 21 Days in Blueberry Juice (BJ) and Phosphate Buffered Saline (PBS at pH=7.2 and pH=2.77). All experiments were carried out four times in duplicates. Different letters denote significant differences when compared within each column alone (p <0.05).

Reduction in virus titers (Log ₁₀ PFU/mL) at refrigeration temperature (4°C)									
Days of Refrigeration	FCV/BJ	FCV/PBS (pH 7.2)	FCV/PBS (pH 2.77)	MNV/BJ	MNV/PBS (pH 7.2)	MNV/PBS (pH 2.77)	MS2/BJ	MS2/PBS (pH 7.2)	MS2/PBS (pH 2.77)
0	0 ± 0B	0.0 ± 0.0E	0±0D	0 ± 0D	0.0 ± 0.0D	0±0C	0 ± 0D	0 ± 0C	0±0E
1	5.06 ± 0.05A	0.04 ± 0.04E	1.06±0.05C	0.04 ± 0.06D	0.05 ± 0.06CD	0.09±0.03BC	0.84 ± 0.04C	0.42 ± 0.01B	0±0.01E
2	5.06 ± 0.05A	0.97 ± 0.02D	2.27±0.04B	0.28 ± 0.09C	0.12 ± 0.09C	0.09±0.04BC	1.93 ± 0.02B	0.36 ± 0.02B	0.92±0.10D
7	5.06 ± 0.05A	1.11 ± 0.04C	5.09±0A	1 ± 0.03B	0.96 ± 0.08B	0.13±0.04BC	6.32 ± 0.02A	0.53 ± 0.02A	2.74±0.02C
14	5.06 ± 0.05A	2.1 ± 0.04B	5.09±0A	1.03 ± 0.08B	0.98 ± 0.08AB	0.17±0.04B	6.32 ± 0.02A	0.56 ± 0.02A	3.37±0.13B
21	5.06 ± 0.05A	2.28 ± 0.05A	5.09±0A	1.95 ± 0.04A	1.08 ± 0.08A	0.44±0.04A	6.32 ± 0.02A	0.56 ± 0.01A	5.76±0A

Table 4.2: Reduction of FCV-F9 (initial titer of $\sim 6 \log_{10}$ PFU/ml), MNV-1 ($\sim 5 \log_{10}$ PFU/ml), and MS2 ($\sim 6 \log_{10}$ PFU/ml) after High Pressure Homogenization Processing in Blueberry Juice (BJ) and Phosphate Buffered Saline (PBS). All experiments were replicated three times. Different letters denote significant differences when compared within each column alone ($p < 0.05$).

Reduction in virus titers (Log_{10} PFU/mL) after high pressure homogenization						
Pressure (MPa)	FCV/BJ	FCV/PBS	MNV/BJ	MNV/PBS	MS2/BJ	MS2/PBS
100	$3.76 \pm 0.0A$	$0.18 \pm 0.08D$	$0.04 \pm 0.06C$	$0 \pm 0.11B$	$5.2 \pm 0.0A$	$0.04 \pm 0.04C$
200	$3.76 \pm 0.0A$	$0.91 \pm 0.24C$	$0.08 \pm 0.05C$	$0 \pm 0.04B$	$5.2 \pm 0.0A$	$0.04 \pm 0.03C$
250	$3.76 \pm 0.0A$	$3.1 \pm 0.91B$	$0.33 \pm 0.10B$	$0.39 \pm 1.15B$	$5.2 \pm 0.0A$	$0.59 \pm 0.01B$
300	$3.76 \pm 0.0A$	$3.76 \pm 0.0A$	$0.71 \pm 0.17A$	$1.98 \pm 1.11A$	$5.2 \pm 0.0A$	$2.37 \pm 0.09A$

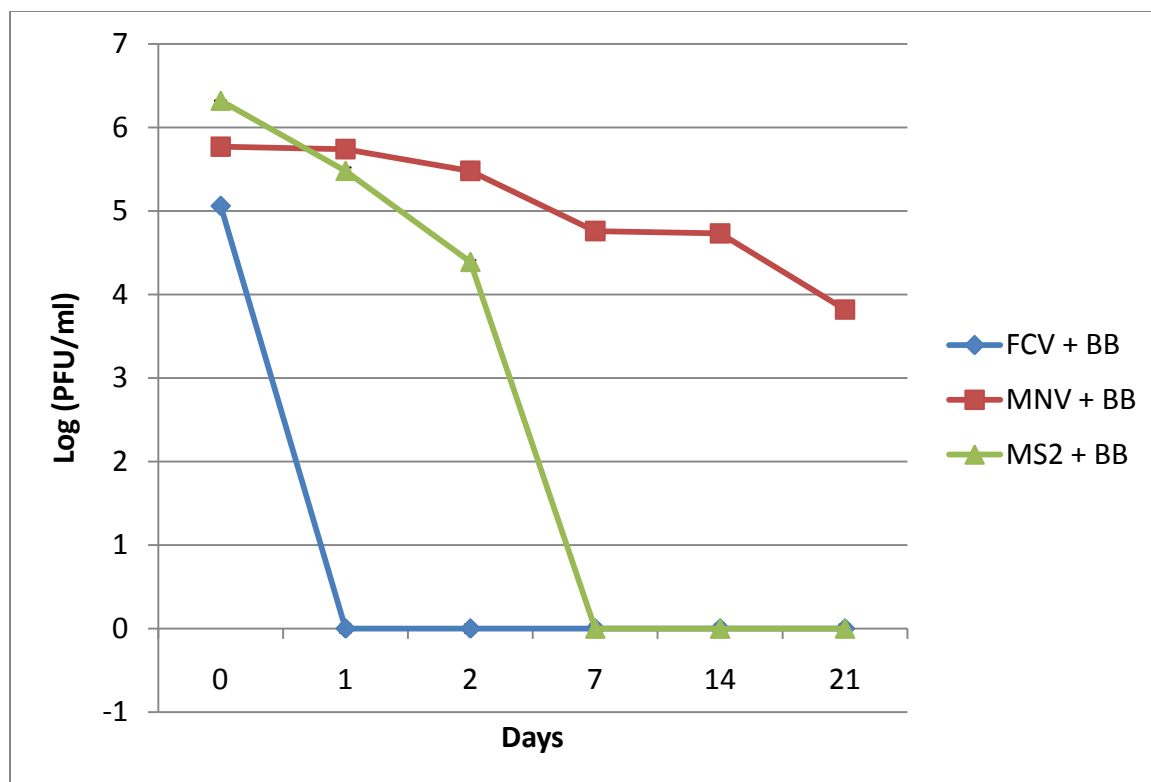


FIGURE 4.1: Recovery of FCV-F9 (from initial titers of $\sim 7 \log_{10}$ PFU/ml), MNV-1($\sim 6 \log_{10}$ PFU/ml), and MS2 ($\sim 7 \log_{10}$ PFU/ml) from Blueberry Juice (BJ) at Refrigeration Temperature (4°C) over 21 Days.

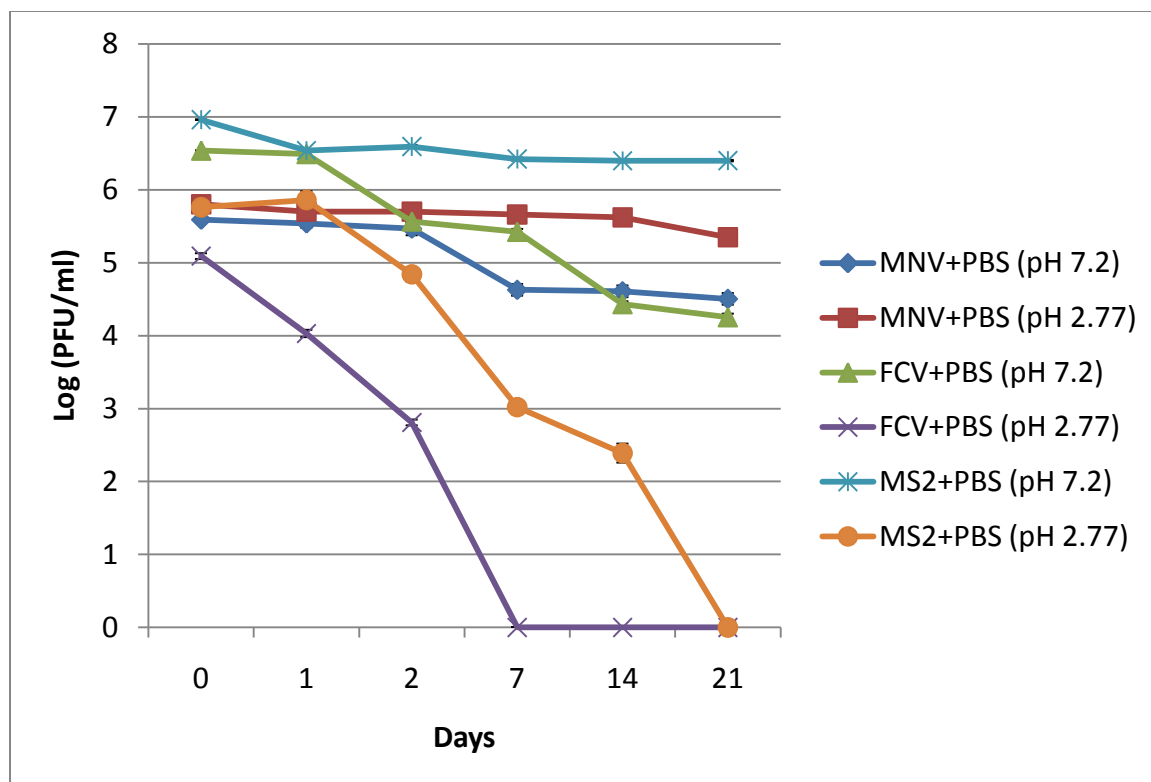


FIGURE 4.2: Recovery of FCV-F9 (from initial titers of $\sim 7 \log_{10}$ PFU/ml), MNV-1($\sim 6 \log_{10}$ PFU/ml), and MS2 ($\sim 7 \log_{10}$ PFU/ml) from phosphate buffered saline (PBS; pH 7.2 and 2.77) at refrigeration temperature (4°C) over 21 days.

Vita

Katie Marie Horm was born in Anderson, South Carolina on October 21, 1985. She grew up in Anderson County and graduated from Westside High School in 2004. She continued her education at the Clemson University where she earned a B.S. degree majoring in Food Science 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. Katie will pursue a career in the food industry concentrating in Microbiology and Quality Control.