INACTIVATION SUSCEPTIBILITY OF ENTERIC VIRUSES TREATED WITH IRON, PLANT, AND BACTERIAL CONSTITUENTS

by

Adrienne E.H. Shearer

A dissertation submitted to the Faculty of the University of Delaware
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Animal and Food Sciences

Summer 2018

INACTIVATION SUSCEPTIBILITY OF ENTERIC VIRUSES TREATED WITH IRON, PLANT, AND BACTERIAL CONSTITUENTS

by

Adrienne E.H. Shearer

Approved: _____
Limin Kung, Jr., Ph.D.
Chair of the Department of Animal and Food Science

Approved: _____
Mark W. Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Douglas J. Doren, Ph.D.
Interim Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Kalmia E. Kniel, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Dallas G. Hoover, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Rolf Joerger, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Efstathia Papafragkou, Ph.D.
Member of dissertation committee

ACKNOWLEDGEMENTS

I am deeply grateful for the wisdom, enthusiasm, encouragement, and support of my advisor, Dr. Kalmia Kniel, and committee members Dr. Dallas Hoover, Dr. Rolf Joerger, and Dr. Efstathia Papafragkou. I am very fortunate to have been guided by such an esteemed team of educators, dedicated scientists, and genuinely kind people. I am honored by their faith in me as a student and colleague.

I am also grateful for colleagues who were integral to the decision and support necessary to pursue this opportunity, especially Drs. Mark Parcells, Alejandro Mazzotta, Michael Kelley, Dilip Chandarana, and Manan Sharma.

I would also like to thank the University of Delaware faculty, staff, and students, past and present, for excellence in instruction, professional support, and friendship. I would like to recognize Dr. Pei Chui, Rafael Castañeda Saldaña, and Pauline Pantuliano for support with the ZVI research. I extend special thanks to Diane Venninger, Elizabeth Appleby, Marcy Murphy, Phaedra Tavlarides-Hontz, Brian Ladman, Kirsten Hirneisen, Jie Wei, Qing Wang, Brienna Anderson, and Shani Craighead for guidance, support, and friendship.

I would especially like to express my gratitude to dear friends Irene Soucy, Cheryl Bush, Maria Pautler, and Susan Olson for uplifting conversations when needed most.

Finally, there are no words adequate to express my love, admiration, and gratitude to my family who have given unconditional love, compassion, inspiration, patience, humor, opportunity, and wisdom through this and everything: most especially, my parents, Brenda and Walter Hagelstein, Jr.; husband, Andrew Shearer; children, Aubrey Shearer and Benjamin Shearer; and sister, Jeanine Heath. I am also blessed with dear family, Geoff, Geoffrey, Abigail, Cathy, Amanda, Richard, Mary Ann, Heidi, and growing family, Sara, and Liz, and my sweet Sally.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Human norovirus (HuNov) is the leading global cause of acute gastroenteritis. An estimated 20 million cases of norovirus illness occur annually in the United States, of which more than 56,000 require hospitalization and up to 800 result in death. Norovirus is transmitted via the fecal-oral route through direct contact with an infected individual, consumption of contaminated food or water, and contact with contaminated surfaces. The number of domestically-acquired norovirus illnesses in the United States attributed to foodborne transmission is estimated at 5.5 million. Foods that require considerable handling and which do not receive treatment to inactivate virus prior to consumption are particularly vulnerable to serve as norovirus transmission vehicles.

Human norovirus prevalence is likely due in part to its ability to adhere to, persist on, and transfer among a variety of food and food contact surfaces. Norovirus directly interacts with cell surface glycans of host cells as well as similar molecules found on some plant tissues and fecal bacteria. The impact of these interactions for norovirus transmission and persistence has not been fully elucidated. Methods to remove or inactivate norovirus from fresh produce without comprising sensory attributes have limited efficacy. Strategies to mitigate contamination are needed to enhance the safety of foods.

In the absence of an efficient cell-culture model for HuNov, research was undertaken to evaluate the resistance of HuNov surrogates, Tulane virus (TV) and murine norovirus (MNV), to inactivation strategies and ecological pressures. Novel viral inactivation strategies evaluated included zero-valent iron (ZVI) and plant-derived proteases for potential to enhance safety of water and ready-to-eat produce, respectively. Viral and bacterial interactions were evaluated for direct association, antiviral bacterial metabolic products, and bacterial impact on viral thermal and chlorine resistance.

Inactivation strategies demonstrated effectiveness of ZVI treatment to remove virus from water, but limited efficacy of plant-derived proteases to reduce infectivity of virus. ZVI removed approximately 2 to 4 $\log_{10}$ of TV and MNV, respectively, from water filtered through sand columns with ZVI incorporated. Buffers of various pH and osmolality did not enhance elution of virus as compared to elution by water from ZVI suggesting virus irreversibly associates with ZVI or is inactivated. Bromelain treatment (2500 ppm) of MNV for 10 minutes at 50°C resulted in a 2.5-$\log_{10}$ reduction in infectivity; whereas, infectivity of TV was not reduced by plant-derived protease treatments.

Recovery of TV suspended in enteric, skin, soil, and phyllospheric bacteria was not impeded as compared to TV alone suggesting a lack of direct, stable binding between virus and bacteria. Exposure of human norovirus to the metabolic growth products (cell-free supernatant, CFS) of *Bifidobacterium*

bifidum, a bacterium previously reported to constitutively produce extracellular glycosidases that degrade histoblood group antigens (HBGA), did not enhance viral recovery from stool or in coculture with *Enterobacter cloacae* as compared to phosphate buffered saline. TV propagation in cell culture was not impeded by the addition of CFS of *B. bifidum*. Exposure of MNV and TV to the CFS of *Bacillus subtilis* 168 and *Enterococcus faecalis* 19433 resulted in 2-$\log_{10}$ PFU/ml and less than one-$\log_{10}$ PFU/ml reductions in propagation, respectively, relative to viral propagation in media alone. Exposure of MNV and TV to the CFS of ten other bacterial species associated with the human intestinal tract, human skin, soil, or phyllosphere did not decrease viral propagation.

Suspension of TV in commercially-prepared bacterial peptidoglycan (PEP) of *Bacillus subtilis* increased viral thermal and chlorine inactivation resistance. TV was undetectable ($>$3.7-$\log_{10}$ PFU/ml reduction) after 60°C treatment when suspended in PBS and LPS, but was reduced by only 1.99 $\log_{10}$ PFU/ml in PEP. Chlorine treatment of 200 ppm rendered TV undetectable ($>$3-$\log_{10}$ PFU/ml reduction) in PBS and LPS; however, TV was still detected in PEP, reduced by 2.86 $\log_{10}$ PFU/ml. Suspension of TV in commercially-prepared lipopolysaccharide of *Escherichia coli* O111:B4 did not significantly affect viral resistance to the chlorine and thermal inactivation treatments tested.

Collectively, these data indicate ZVI has potential for remediation of viral-contaminated water, but plant-derived proteases were not antiviral at commercially-relevant conditions. Bacterial metabolic products had limited antiviral properties under the conditions tested, and the presence of bacteria may require more stringent treatment conditions to inactivate virus. These data are significant for understanding factors important for norovirus transmission and resistance and for identifying strategies to reduce norovirus contamination risk, thereby enhancing the safety of food and water.

INDEX WORDS: Norovirus, Zero-valent iron, Plant protease, Bacteria binding, Inactivation

Chapter 1

INTRODUCTION

1.1. HUMAN NOROVIRUS - PUBLIC HEALTH IMPACT AND OVERVIEW OF BIOLOGY

1.1.1. Public Health Impact

Human norovirus (HuNov) is the leading cause of acute gastroenteritis in the United States, estimated to cause 20 million illnesses annually, of which more than 56,000 require hospitalization and up to 800 result in death (Hall *et al.*, 2014). The number of domestically-acquired norovirus illnesses in the United States attributed to foodborne transmission is estimated at 5.5 million (Scallan *et al*., 2011). Health care costs associated with norovirus are estimated at $777 million (Hall *et al.*, 2014). Worldwide, norovirus accounts for 95% of viral gastroenteritis outbreaks and more than 50% of all foodborne disease outbreaks (Karst, 2010).

The prevalence of norovirus illness is attributed to a number of factors including a low infectious dose, prolonged viral shedding, environmental stability of the virions, and short-lived host immunity (Karst, 2010). The 50% human infectious dose is estimated to range from 18 to 1,320 HuNov genomic equivalents among susceptible individuals (Vinjé, 2015; Atmar *et al.*, 2014).

Symptoms of norovirus infection include nonbloody diarrhea, vomiting, and abdominal pain. Illness caused by norovirus is generally self-limiting within a few days of onset; however, immunocompromised individuals may have more severe and prolonged illness. Infected individuals can shed millions of virus particles in vomitus and stool (Montazeri *et al.*, 2015), and shedding can continue for three weeks (Vinjé, 2015) to up to two months (Sarvestani *et al.* 2016) beyond resolution of symptoms. Treatment for norovirus illness is generally limited to rehydration therapy (Sarvestani *et al.*, 2016).

Norovirus is transmitted via the fecal-oral route through direct contact with an infected individual, consumption of contaminated food or water, and contact with contaminated surfaces (Hall *et al.*, 2014). Outbreaks have commonly occurred in semi-closed communities including care settings, schools, and cruise ships (Karst, 2010). Food is also a leading transmission vector in norovirus illness outbreaks, with restaurants the most common setting and food handlers the most-often implicated source (Centers for Disease Control and Prevention, 2014; Hall *et al.*, 2012; Hall *et al.*, 2014; Karst, 2010). Foods that require considerable handling and which do not receive treatment to inactivate virus prior to consumption are particularly vulnerable to serve as norovirus transmission vehicles. Leafy greens, fresh fruit and undercooked mollusks have most commonly been implicated as transmission vehicles in outbreaks for which a specific food could be identified (Callejón *et al.*, 2015; Hall *et al.*, 2014; Centers for Disease Control and Prevention, 2014). Of nearly 3,000 outbreaks for which

norovirus was the confirmed or suspected cause during the period of 2001 to 2008, approximately 10% were attributed to a single commodity of which leafy vegetables accounted for 33%, fruits/nuts for 16%, and mollusks for 13% (Hall *et al.*, 2012). Contamination is presumed to occur most often during food production, processing and service by infected food handlers (Hall *et al.*, 2012), although environmental contamination of produce and mollusks can occur via contaminated water (Li *et al.*, 2015).

1.1.2. Classification and Structure

HuNov is a member of the *Caliciviridae* family and *Norovirus* genus which consists of noroviruses that infect other animal species including murine, bovine, porcine, feline, and canine species. The *Norovirus* genus is classified into genogroups GI to GVI and proposed GVII (Vinjé, 2015) based on similarity of major capsid proteins; genogroups are further divided into genotypes. Norovirus that infect humans are among genogroups I, II, and IV. Norwalk (GI) is considered the prototype and has been implicated in approximately 11% of outbreaks, while norovirus (GII.4) is associated with the majority (89%) of illness outbreaks (Siebenga *et al.*, 2009; Vinjé, 2015). The higher and cyclical incidence of norovirus GII.4 illness is attributed to rapid genomic evolution as well as ability to interact with a greater variety of host cell surface receptors (Sarvestani *et al.*, 2016; Vinjé, 2015).

HuNov is nonenveloped, icosahedral in shape, and approximately 27 to 30 nm in diameter (Karst, 2010). Its linear, single-stranded, positive-sense RNA genome

is comprised of approximately 7.5 kb, consisting of three open reading frames (ORF). The first ORF encodes at least six non-structural proteins (p48, NTPase, p22, VPg, Pro, Pol) involved in viral replication (Vinjé, 2015). The functions of the nonstructural proteins are not well-defined but have proposed roles in formation of a replication complex, RNA polymerization, and post-translational protein cleavage (Thorne and Goodfellow, 2014; Sarvestani *et al.*, 2016). The second ORF encodes the major structural capsid protein VP1, and the third ORF encodes the minor structural protein VP2. The 60,000-Da capsid protein, VP1, (Vinjé, 2015) has a shell (S) domain structurally conserved among noroviruses, and a more variable arch-shaped protruding (P) domain. The protruding domain consists of two regions, the P1 domain along the sides of the arches and the P2 domain located at the outermost region of the arch protrusions. Norovirus capsid P2 amino acids bind histo-blood group antigens (HBGAs) (Singh, *et al.*, 2015; Tan and Jiang, 2005), carbohydrates linked to lipids or proteins on cell surfaces, including the surfaces of host mucosal epithelial cells (Karst, 2010). HBGAs serve as receptors for HuNov, and binding patterns vary among HuNov outbreak strains, contributing to the variable susceptibility to infection within the human population. However, there are HuNov that do not bind HBGAs (Karst, 2010), and HuNov may have other critical host cell receptors for infection (Murakami *et al.*, 2013).

Norovirus replicates in the duodenum and upper jejunum of the small intestine (Vinjé, 2015). Upon cell entry, the viral genome is uncoated, and the proteins

encoded in ORF1 are translated as a polyprotein and cleaved into the nonstructural proteins. Formation of a replication complex, aided by two of the nonstructural proteins, has been proposed to occur in the host cell perinuclear space (Thorne and Goodfellow, 2014). Negative-strand RNA is generated and serves as template for replication of positive-sense, genomic RNA, which can then be translated or encapsidated. Capsid proteins are translated from subgenomic RNA replicated by viral RNA-dependent RNA polymerase (RdRp) and are presumed to self-assemble to form virions ready for cellular egress (Thorne and Goodfellow, 2014).

1.1.3. Laboratory Propagation and Detection

Research to advance the characterization of human norovirus has been limited by the inability to efficiently propagate the virus outside the human host. Considerable research has been undertaken to develop efficient models by which virus can be propagated and viral infectivity assessed to support study of viral life cycle, environmental transmission, inactivation treatments, as well as potential clinical interventions. Duizer and colleagues (2004) reported extensive but ultimately unsuccessful attempts to support infection and propagation of 15 norovirus strains in 27 human and other animal cell lines, including intestinal epithelial, kidney, and cells of other tissues, combined with various additives and incubation conditions. Three-dimensional organoid intestinal cell culture models for HuNov infection in INT-407 and Caco-2 cell lines have been reported (Straub *et al.*, 2007; Straub *et al.*, 2011), but not readily replicated (Papafragkou *et al.*,

2013). As a positive sense RNA virus, HuNov RNA has been transfected into human hepatoma cells, resulting in viral replication and egress, but not subsequent spread of infection to neighboring naïve cells (Guix *et al.*, 2007) in spite of cellular expression of the α-(1,2) fucosyltransferase gene (FUT2) responsible for the HBGA-secretor phenotype. HuNov has been demonstrated to infect B cells in the presence of stool and when co-cultured with *Enterobacter cloacae* or HBGA H-antigen (Jones *et al.*, 2014). Most recently, HuNov GII.4 was demonstrated to infect human intestinal enteroids (HIEs), which are derived from stem cells isolated from human intestinal crypts and consist of nontransformed, multiple, intestinal cell types (Ettayebi *et al.*, 2016). In this study, HuNov infection occurred in enterocytes from FUT2 secretor-positive individuals and was enhanced by treatment of HIEs with human bile (Ettayebi *et al.*, 2016). HuNov strain differences were noted in that, unlike HuNov GII.4, HuNov GII.3 infected some HIEs derived from FUT2 secretor-negative individuals, and HIE infection was reliant on treatment of HIEs with human bile (Ettayebi *et al.*, 2016). In contrast with observations with B cells (Jones *et al.*, 2014), infection of HIEs occurred independent of bacteria (Ettayebi *et al.*, 2016).

In vitro cultivation of HuNov is anticipated to advance research in the interactions between HuNov and host as well as HuNov inactivation susceptibility. However, in spite of the major advancements in identifying cells supportive of HuNov replication and necessary cofactors, routine HuNov propagation by these methods has not been adopted to-date. Further

development is needed to overcome likely cost and accessibility barriers to widespread utilization.

In vivo studies with human norovirus are limited and more applicable to understanding clinical features of human norovirus infection rather than applied food safety research. Human challenge studies have been conducted to better characterize human infection susceptibility, infectious dose, symptom characteristics and duration, and viral shedding in vomitus and stool (Atmar *et al.*, 2014; Frenck *et al.*, 2012; Kirby *et al.*, 2016; Okhuysen *et al.*, 1995; Teunis *et al.*, 2015). Nonhuman animal models of gnotobiotic pigs (Souza *et al.*, 2007), gnotobiotic calves (Souza *et al.*, 2008), and 'humanized' mice (Taube *et al.*, 2013) have been developed to characterize norovirus infection, pathogenesis, and host immune response.

Studies of human norovirus for transmission or interventions for food safety often involve obtaining virus from human stool and detecting viral RNA by reverse-transcription, quantitative polymerase chain reaction (RT-qPCR). RT-PCR is currently considered the gold standard for HuNov detection in stool, environmental, food and water samples associated with outbreaks (Vinjé, 2015). The fairly conserved RdRp gene is typically targeted for detection, and the presence or absence of RNA is considered a measure of viral integrity as it is assumed that HuNov RNA is naturally degraded or can be experimentally degraded when unprotected by an intact capsid. The assay does not assess infectivity.

Alternatively, norovirus capsids can be detected by immunoassays or by HuNov binding to porcine gastric mucin (PGM), which has HGBA-like moieties. The PGM assay has been reported to mirror RT-qPCR and/or human challenge study data for viral inactivation by high hydrostatic pressure, heat, and UV light (Dancho *et al.*, 2012) and has subsequently been applied to evaluate HuNov resistance to chemical sanitizers (Kingsley *et al.*, 2014) and pressure treatment of produce (Li *et al.*, 2013).

Another model developed for study of human norovirus utilizes virus-like particles (VLPs) that consist of the self-assembling major capsid protein (VP1) of human norovirus but lack HuNov RNA. VLPs of HuNov have been expressed in *Escherichia coli* (Choi *et al.*, 2008), insect cells (Jiang *et al.*, 1992), yeast (Xia *et al.*, 2007), plants (Zhang *et al.*, 2006), and vesicular stomatitis virus (Ma and Li, 2011). VLPs are structurally and antigenically similar to complete viral particles and are particularly applicable in studies for which noninfectious material is desired, such as research on virus transmission, potential vaccines, and the binding of norovirus to biological and environmental surfaces.

1.1.4. Surrogates

Given the lack of routine cultivability of HuNov, researchers are largely reliant on surrogates to gain potential insight on HuNov environmental persistence, transmission, and resistance to inactivation strategies. Although various surrogate viruses have been used for HuNov, including feline calicivirus (FCV) and coliphage (Kniel, 2014), the growing body of literature of HuNov surrogates

involves murine norovirus (MNV) and Tulane virus (TV). MNV is a member of the *Caliciviridae* family and *Norovirus* GV genogroup and thus bears similarity to HuNov in size and genomic structure (Kniel, 2014). MNV binds sialic acids expressed on epithelial cells (Taube *et al.*, 2009; Taube *et al.*, 2012) and was recently shown to infect the intestinal epithelial tuft cells of mice (Wilen *et al.*, 2018). MNV can be cultivated in macrophage (Vashist *et al.*, 2009), and cell surface proteins in the CD300 family were found to serve as receptors for infection (Haga *et al.*, 2016; Orchard *et al.*, 2016). TV has been isolated from rhesus macaques and is also a member of the Caliciviridae family but of the Recovirus genus (Kniel, 2014; Vashist *et al*, 2009). It also bears similarity to HuNov in size and structure (Kniel, 2014), as well as its binding to HBGAs (Farkas *et al.*, 2008; Farkas *et al.*, 2010; Zhang *et al.*, 2015). TV can be cultivated in cells derived from monkey kidney (Zhang *et al.*, 2015). As capsid integrity and receptor binding are major determinants of viral infectivity, the similarity of surrogate capsids to HuNov is an important consideration in projecting resistance traits to those of HuNov.

1.2. VIRAL PERSISTENCE AND INACTIVATION RESISTANCE

1.2.1. Environmental Adherence and Persistence

Human norovirus prevalence is likely due in part to its ability to adhere to, persist on, and be transferred among a variety of food and food contact surfaces (Deboosere *et al.*, 2012). Several studies have explored whether certain foods are particularly vulnerable to HuNov adherence due to the nature of food surface

molecules and similarity to known HuNov receptors. VLPs of HuNov were found to bind gastrointestinal homogenates of oysters, mussels, and clams (Tian *et al.*, 2007). Pretreatment of the VLPs with HBGA antibodies or HBGAs from human saliva inhibited VLP adhesion to the homogenates suggesting HuNov may bind to HBGA-like molecules in these bivalves (Tian *et al.*, 2007). VLPs of norovirus were also found to adhere to the veins of romaine lettuce as well as extracts of cilantro, iceberg lettuce, spinach, and celery (Gandhi *et al.*, 2010). HuNov VLP binding to romaine lettuce leaf cell wall material was inhibited by carbohydrate disruption and lectin interaction with carbohydrates, but not by HBGA antibodies, thereby suggesting multiple carbohydrate moieties may be involved in HuNov binding to romaine lettuce leaves (Esseili *et al.*, 2012). Oyster extract, cranberry juice, pomegranate juice, and 100% raspberry juice demonstrated HuNov VLP saliva-binding inhibition; less binding inhibition was observed in the presence of lettuce, tomato, spinach, strawberry, blackberry, or blueberry extracts (Li *et al.*, 2012). In consideration of other studies with mussels and juices, the researchers speculated that oyster tissue may preferentially bind HuNov, whereas the juices may damage the VLPs (Li *et al.*, 2012).

Human norovirus has considerable persistence on foods, food contact surfaces, and in the environment. HuNov persists through the shelf-life of inoculated lettuce, strawberry, raspberry, parsley, and basil (Kotwal and Cannon, 2014; Verhaelen *et al.*, 2012) and on a variety of non-food surfaces, including stainless steel, ceramic, Formica, and plastic, for 42 to 56 days (D'Souza *et al.*,

2006; Escudero *et al.*, 2012; Kotwal and Cannon, 2014). HuNov adherent to surfaces, including hands, has also been demonstrated to readily transfer to foods such as lettuce and deli meats that contact contaminated surfaces (D'Souza *et al.*, 2006; Escudero *et al.*, 2012; Kotwal and Cannon, 2014).

Environmental stability of human norovirus has been demonstrated in various water sources and in the presence of other biological and synthetic substances. Human norovirus was shown to retain infectivity in ground water for at least 61 days (Seitz *et al.*, 2011). As evaluated by genome-based detection, HuNov persisted in water up to five weeks (Kotwal and Cannon, 2014) and in ground water over three years (Seitz *et al.*, 2011). Human norovirus persists in stool suspension for 120 days at -80°C (Kotwal and Cannon, 2014) and in pesticides for at least two hours (Verhaelen *et al.*, 2013). HuNov adherence to bacteria and the potential impact on infection, transmission, and resistance are presented in subsequent sections of this review.

1.2.2. Inactivation of Human Norovirus and Surrogates

Potential intervention strategies to inactivate or remove HuNov contamination from food and food contact surfaces are largely based on studies conducted with surrogates; however, there are studies on the effects of various treatments on the binding of HuNov to known receptors, the integrity of HuNov capsids, as evaluated by RNA detection, or HuNov infectivity in human volunteer studies. For cleaning of surfaces, the use of liquid soap followed by 1000 ppm of chlorine reduced HuNov contamination by 2.5 to 4.5 log PCR units (Tuladhar *et al.*,

2012). When applied to fresh vegetables, lettuce, celery, and white cabbage, sodium hypochlorite treatment of 1000 ppm resulted in a 3-log reduction of HuNov GII.4 genomic copies; the same HuNov reduction was obtained with 100 ppm sodium hypochlorite with the inclusion of sodium metasilicate pentahydrate (0.4%) treatment (Ha *et al.*, 2018). Chlorine efficacy for HuNov reduction on berries and herbs varied by the type of berry treated, with 200 ppm for 30 sec at 18°C yielding 3-log RT-PCRU reductions for blueberries, but less than 2-log RT-PCRU reductions for raspberries, strawberries, basil, and parsley (Butot *et al*., 2008). Treatment of HuNov with pomegranate extract and tannic acid had inhibitory effects on P protein binding in saliva ELISA (Li *et al.*, 2013). HuNov was shown to be resistant to ethanol commonly used in hand sanitizers (Cromeans *et al.*, 2014). Treatment of HuNov seeded in oysters with 600 MPa for 5 minutes rendered the virus noninfectious as determined in volunteer feeding studies (Leon *et al.*, 2011).

Resistance studies with surrogate viruses showed stability of MNV and TV over the pH range of 3 to 8, susceptibility to 2000 ppm chlorine with five minutes of exposure, and susceptibility to two minutes of heat treatment at 70°C (Hirneisen and Kniel, 2013) or 20 minutes at 56°C (Cromeans *et al.*, 2014). Tung *et al*. (2013) demonstrated the impact of treatment matrix on MNV chlorine resistance. MNV-1 was resistant to up to 500 ppm chlorine in the presence of 20% stool suspension, but in cell culture media with 1% fetal bovine serum, MNV reduction was 3-logs as measured by RT-PCR (Tung *et al*., 2013). Ultraviolet

(UV) light treatment of MNV (30 MJ/cm^2) yielded a 5-log reduction in infectivity (Lee *et al.*, 2008b). MNV was found to be sensitive to chlorine and povidone iodine disinfectants used for surfaces (Belliot *et al.*, 2008). MNV was also found to be sensitive to ethanol (Belliot *et al.*, 2008; Cromeans *et al.*, 2014); MNV sensitivity to isopropanol is inconsistent between studies in the literature (Belliot *et al.*, 2008; Cromeans *et al.*, 2014). TV was resistant to both ethanol and isopropanol (Cromeans *et al.*, 2014).

Direct treatment of produce with water or chlorine (200 ppm) reduced MNV contamination by about one log, whereas the combination of surfactant and chlorine yielded three-log reductions in MNV on strawberries, lettuce, cabbage, and raspberries (Predmore and Li, 2011). Waterless treatment of blueberries with gaseous chlorine dioxide (generated with 1 mg sodium chlorite) inactivated approximately two logs of TV with 15 minutes of exposure without compromising the appearance of the berries (Kingsley *et al.*, 2018). A novel approach to inactivate norovirus on blueberries or mixed berries (strawberry and raspberry) utilized heat-denatured egg white lysozyme at 1% solution in water for 1 min to achieve greater than a 3-log reduction in infectious MNV (Takahashi *et al.*, 2018). Although the mechanism of action for heat-denatured lysozyme is not fully understood, viral particle diameter and capsid integrity appeared to be affected (Takahashi *et al.*, 2015).

Plant-derived compounds, such as lemongrass oil, citral, allspice oil, grape seed extract, green tea extract, pomegranate juice, and cranberry juice have

variable efficacy against MNV from approximately one to three-log reductions with exposure times of one to 24 hours (D'Souza, 2014; Gilling *et al.*, 2014; Kim *et al*., 2017; Li *et al.*, 2012; Li *et al.*, 2013; Randazzo *et al*., 2017). The juices or extracts of cranberry, pomegranate, and grapeseed were shown to reduce initial titers (10^5 PFU/ml) of MNV by 3-$\log_{10}$ PFU/ml and FCV by approximately 5-$\log_{10}$ PFU/ml (Su *et al.*, 2010a; Su *et al.*, 2010b; D'Souza *et al.*, 2014). The inhibitory affects were not attributable to low pH as neutralized juices yielded similar reductions (Su *et al.*, 2010a; Su *et al.*, 2010b). Cranberry and grapeseed appeared to affect capsid integrity whereas pomegranate may have affected host cell binding *in vitro* (D'Souza, 2014; Li *et al.*, 2012; Li *et al.*, 2013). Cranberry and pomegranate juices also inhibited saliva-binding by HuNov GII.4 P particles (Li *et al.*, 2012) in an ELISA. Although raspberries and strawberries have served as vehicles for norovirus outbreaks, undiluted juices of raspberry and strawberry have also shown inhibition of HuNov GII.4 P particle saliva-binding (Li *et al.*, 2012).

With environmental water a potential source of contamination of drinking water and produce, water treatment methods are needed to remove or inactivate virus. Studies have demonstrated incomplete removal of HuNov in even tertiary-treated effluent that has undergone settlement, activated-sludge treatment, sand and biological filters, and UV disinfection *(*Campos *et al.*, 2016*;* López-Gálvez *et al.*, 2016*;* Ottoson *et al.*, 2006*)*. HuNov has demonstrated resistance to chlorine in postharvest leafy green wash water (Dunkin *et al.*, 2017), stool filtrate (Kingsley

et al., 2014), and secondary-treated wastewater effluent (Kingsley *et al.*, 2017). HuNov GII reduction of 2-$\log_{10}$ units as determined by RT-qPCR was observed in whole leaf and chopped Romaine lettuce wash water with 15 minutes exposure to an initial 1.5 mg/L free chlorine (Dunkin *et al.*, 2017). HuNov GII chlorine resistance was greater than that of HuNov GI and surrogate viruses MNV and bacteriophage MS2 (Dunkin *et al.*, 2017). To achieve HuNov reductions of approximately 4-$\log_{10}$ units as measured by viral binding assays, 189 ppm chlorine was required for HuNov suspended in stool filtrate (Kingsley *et al.*, 2014), and 100 ppm chlorine was necessary to reduce HuNov in secondary-treated wastewater effluent (Kingsley *et al.*, 2017). HuNov inoculated in treated drinking water required 30 minutes of exposure to 0.5 mg/L free chlorine to achieve a 3.6-$\log_{10}$ reduction as measured by RT-PCR; the same treatment inactivated more than four logs of infectious MNV (Kitajima *et al.*, 2010).

1.2.3. Inactivation Strategies Investigated for Dissertation Research

The research presented in this dissertation was undertaken to address knowledge gaps and commercial needs important to advancing the safety of foods through control of foodborne virus, especially norovirus. The overarching goals are to identify potential strategies to prevent or mitigate the contamination of water and ready-to-eat foods for which effective control strategies are currently unavailable. The first part of the dissertation research addresses emerging or novel methods to remove and/or inactivate virus for application to safety of water

and fresh produce using zero-valent iron and plant-derived proteases, respectively.

1.2.3.1. Zero-valent Iron

Zero-valent iron (ZVI) has been incorporated in commercial filtration systems to remove chemical contaminants from groundwater (Interstate Technology and Regulatory Council PRB: Technology Update Team, 2011). There is a growing body of literature demonstrating the efficacy of ZVI to remediate water of biological contaminants including bacteria and viruses. Addition of ZVI to water filtration systems has been shown to enhance the removal of pathogens of significance to food safety. Bacterial reductions ranged from two to six logs from water filtration systems containing ZVI and sand as compared to sand alone for *E. coli* O157:H7 (Derevianko, 2008), *E. coli* O157:H12 (Ingram *et al.* 2012), *Pseudomonas fluorescens*, and *Bacillus subtilis* (Diao and Yao, 2009). ZVI also enhanced removal of virus from water systems over sand filtration by four to six logs for bacteriophage MS-2, ΦX174, and f2 as well as adenovirus, rotavirus, and Aichi virus (Bradley *et al.*, 2011; Cheng *et al.*, 2016; You *et al.*, 2005; Shi *et al.*, 2012). A summary of the research on ZVI effects on biological systems of relevance to food safety is presented in Table 1.1.

ZVI retains or reduces toxicity of contaminants through a variety of proposed mechanisms including oxidation, reduction, and adsorption reactions. Some of the important reactive species produced by the exposure of ZVI to water are outlined in the following chemical equations (Cheng *et al.*, 2016; Fu *et al.*, 2014):

$$Fe^0 + O_2 + 2H^+ \rightarrow Fe^{2+} + H_2O_2$$

$$Fe^0 + H_2O_2 + 2H^+ \rightarrow Fe^{2+} + 2H_2O$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2 \downarrow$$

$$4Fe(OH)_2 + 2H_2O + O_2 \rightarrow 4Fe(OH)_3 \downarrow$$

Bactericidal effects of ZVI have been attributed to disruption of bacterial cell membranes and respiratory activity (Kim *et al.*, 2010; Lee *et al.*, 2008a) particularly under deaerated conditions (Kim *et al.*, 2010). The mechanism of ZVI action against virus has been proposed to be due to ionic interactions (Shi *et al.*, 2012). A study published during the more recent course of this dissertation work investigated important chemical species that contribute to the reduced recovery of bacteriophage f2 exposed to nanoscale ZVI (nZVI) (Cheng *et al.*, 2016). Cheng and colleagues (2016) demonstrated that hydroxyl radical (•OH) and superoxide radical ($\bullet O_2^-$) were important for antiviral effects as determined by the reduced efficacy of nZVI in the presence of scavengers dimethyl sulfoxide (DMSO) and superoxide dismutase (SOD) (Cheng *et al.*, 2016). This same study demonstrated that ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron were also important for nZVI reduction of recoverable bacteriophage f2 (Cheng *et al.*, 2016), and that nZVI antiviral efficacy was enhanced in aerobic and acidic conditions, findings consistent with inactivation studies of MS2 coliphage by nZVI (Kim *et al.*, 2011). TEM images further illustrated damage to the outer structure of bacteriophage, which was attributed to direct interaction with nZVI (Cheng *et al.*, 2016).

No previously-published studies were found on the effectiveness of ZVI for removal of norovirus or its surrogates from water systems. The research presented herein aims to fill this knowledge gap as well as to better characterize the mechanism of virus-ZVI interaction and to predict the reversibility of this interaction. The research bears significance in the utility of ZVI for remediation of norovirus contamination of water to improve the microbiological safety of water used for irrigation and food processing as well as to advance efforts for water reuse.

1.2.3.2. Plant-derived Proteases

Plant-based cysteine proteases, papain and ficin, are found in the latex of papaya and figs, respectively; bromelain is found predominantly in the stem of pineapple as well as the fruit and leaves (Gosalia *et al.*, 2005; Biozym 2012). In the plants, cysteine proteases are involved in protein degradation in response to external stimuli (González-Rábade *et al.*, 2011), and are proposed to provide insect resistance (González-Rábade *et al.*, 2011; Gosalia *et al.*, 2005).

The extracted enzymes are safe for human consumption and currently have application in food processing for meat, brewing, and dairy industries (Homaei *et al.*, 2010; González-Rábade *et al.*, 2011). Bromelain, papain, and ficin are also generally recognized as safe (GRAS) food ingredients (Code of Federal Regulations, 1983, 2013a, 2013b). The proteases have good water solubility and optimal functionality over a pH range of approximately 4 to 7.5. The proteases have catalytic activity at 30 to 40°C albeit less than at the optimal catalytic

temperature range of 50 to 60°C (Choudhury *et al.*, 2010; Whitaker and Lee, 1972; Xue *et al.*, 2010). An activator is not essential for catalytic activity of the proteases; however, activity is enhanced in the presence of cysteine (Homaei *et al.*, 2010; MP Biomedicals, 2012).

Plant cysteine proteases also have atoxigenic properties (Helting and Zwisler, 1974; Helting and Zwisler, 1977; Helting and Nau, 1984) as well as direct antimicrobial properties including fungistatic (López-García *et al.*, 2012), antihelminthic (de Amorin *et al.*, 1999; Mansur *et al.*, 2014), and antibacterial (Assatarakul *et al.*, 2012) activities (as summarized in Table 1.2). Bromelain also cleaves the hemagglutinin protein from influenza virus with subsequent application in antisera production (Wang *et al.*, 2012). No previously-published studies were found on the effect of the plant proteases on foodborne human norovirus, HuNov surrogates, or hepatitis A virus.

Papain, bromelain, and ficin are broad-acting enzymes. Preferential cleavage occurs at the carboxyl end of lysine and arginine (Gosalia *et al.*, 2005), as well as histidine, glycine, glutamine, and tyrosine for papain (Biozym, 2012), glutamine, threonine (Choe *et al.*, 2006), alanine, tyrosine, and glycine (MP Biomedicals, 2012) for bromelain, and phenylalanine and tyrosine (MP Biomedicals, 2013) for ficin. An adjacent (P2 position) hydrophobic amino acid enhances papain activity, whereas a basic amino acid, such as arginine, at the P2 position enhances bromelain activity (Choe *et al.*, 2005; Gosalia *et al.*, 2005).

The VP1 region of the capsid of human norovirus (GII.4/5M/USA/2004; GenBank AFL70023.1) as deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/) is approximately 540 amino acids, of which more than 30 amino acids (6%) are lysine (K) or arginine (R). At least nine of these lysine and arginine also have an adjacent amino acid reported to enhance the catalytic action of the proteases. In consideration of the other amino acids (histidine, glycine, glutamine, tyrosine, threonine, alanine, and phenylalanine) cleaved by the proteases to a lesser degree than lysine and arginine targets, up to 24%, 37%, and 14% of the peptide bonds of the VP1 region of the norovirus capsid may be vulnerable to hydrolysis by papain, bromelain, and ficin, respectively. Additionally, several norovirus capsid amino acids important for interactions with receptor histo-blood group antigens (HBGA), including lysine, glycine, alanine, and histidine (Bu *et al.*, 2008; Choi *et al.*, 2008; Tan *et al.*, 2009) may theoretically be targets for cleavage by the plant proteases. Although the capsid amino acid sequences of MNV and TV bear little similarity to that of HuNov, the total compositions of amino acids theoretically vulnerable to proteolysis are similar. Based on the primary structures of the proteins, the viral capsids contain several targets potentially vulnerable to proteolysis by the plant proteases. However, the accessibility of the peptide bonds to hydrolysis as influenced by capsid protein secondary and tertiary structures as well as the conditions that may favor hydrolysis are unknown.

The research presented herein aims to evaluate the efficacy of the plant-derived proteases, bromelain, papain, and ficin, individually or combined, on HuNov, TV, MNV, and hepatitis A virus (HAV) in consideration of the ultimate need for improved washing strategies, preferably those that are generally recognized as safe (GRAS) and plant-sourced, for removal of viruses from ready-to-eat produce.

Table 1.1. Prior studies on ZVI effects on microorganisms

Target	Treatment	Efficacy	Reference
Rotavirus, Bacteriophage MS-2	▪ Sand column filter with 10% (v/v) ZVI ▪ Virus suspended in groundwater ▪ Rotavirus also suspended in treated wastewater effluent	▪ ZVI treated MS2 reduced approximately 5-log from initial 10^9 PFU/ml ▪ ZVI treated rotavirus reduced by 4 to 5-log focus forming units per ml (FFU/ml) from an initial $10^{4 \text{ to } 5}$ FFU/ml in treated wastewater effluent with 20 mg/L total organic carbon (TOC)	Bradley *et al.*, 2011
Bacteriophage f2	▪ Nanoscale ZVI (nZVI) (0.2 or 0.5 mM) in virus suspension ▪ 25°C ▪ 0 to 60 min ▪ pH 5, 7, 9 ▪ Aerobic or anaerobic	▪ 4-log reduction from initial bacteriophage 10^6 PFU/ml, 0.5 mM nZVI 25C, 60 min, aerobic conditions, pH 7 ▪ 6-log reduction at pH 5 ▪ Morphological damage at 20 min exposure detected by TEM ▪ Dissolved iron in aerobic systems predominantly ferric form ▪ Radical scavengers, especially SOD, inhibited nZVI phage removal	Cheng *et al.*, 2016
Bacteriophage ϕX174 and MS-2	▪ Column of iron and sand (1:1 v/v mix) ▪ Virus suspended in artificial ground water (AGW) ▪ Contact time 20 min	▪ 4 to 5-log reductions from initial bacteriophage 10^5 PFU/ml	You *et al.*, 2005
Bacteriophage ϕX174 and MS-2, Aichi virus (AiV), Adenovirus 41 (Ad41)	▪ Column of iron (15%) and sand ▪ Virus suspended in AGW, filtered water from treatment plants with and without pre-chlorination	▪ 4.5 to 6-log reductions from initial virus 10^6 PFU/ml in all water types	Shi *et al.*, 2012

Pseudomonas fluorescens, *Bacillus subtilis var. niger*, *Aspergillus versicolor*	▪ nZVI (0.1, 1, 10 mg/ml) ▪ 5 min ▪ Aerobic conditions	▪ Approximately 4 log reductions from initial bacteria 10^4 CFU/ml ▪ No inactivation of fungi	Diao and Yao, 2009
E. coli O157:H12	▪ Biosand filter with ZVI ▪ Bacteria suspended in groundwater	▪ Approximately 6 log reduction from initial bacteria 10^8 CFU/ml	Ingram *et al.* 2012
E. coli O157:H7	▪ ZVI:Sand (1:1) filter ▪ Bacteria suspended in AGW at pH 5, 7, 9	▪ Up to 2 log reduction from initial bacteria 10^6 CFU/ml for new columns ▪ Approximately 5-log reduction when passed through columns aged one month	Derevianko, 2008

Table 1.2. Prior research on antimicrobial effects of plant-derived proteases

Target	Plant Protease	System	Efficacy	Reference
Helminthes (rodent cestodes)*: Hymenolepis diminuta*, *Hymenolepis microstoma*	Bromelain (fruit and stem), Papain (papaya latex)	In vitro juvenile, artificially excysted scoleces, adult worms; treatment of 37°C/2h/ up to 3000 µM concentration, exogenous cysteine unnecessary	Fruit bromelain most effective at 260 µM as measured by decreased motility and tegument integrity	Mansur, *et al.*, 2014
Helminthes: *Syphacia obvelata, Aspiculuris tetraptera, Vampirolepis nana*	Latex of *Ficus insipid* Willd. and *Ficus carica* L.	Mice – administered 3 to 4 ml/kg/day for three consecutive days	Weakly antihelminthic against *S. obvelata*; toxicity with hemorrhagic enteritis observed	De Amorin *et al.,* 1999
Helminthe: *Heligmosomoides polygyrus*, adult stage	Papain, Ficin, Stem bromelain	Direct application to worm *in vitro*, 25 to 100 µM, 37°C	Reduced motility and cuticle damage occurred within 15 to 45 min exposure. Effectiveness dependent on cysteine activator	Stepek *et al.*, 2005
Fusarium verticillioides (rice isolate)*, F. oxysporum, F. proliferatum* (rice and maize isolates)	Bromelain (pineapple stems)	0.3 µM (proteolytic activity of 0.87 U/mg in haemoglobin assay)	90% growth inhibition (fungistatic) of *F. verticillioides* (10^4 spores/ml) due to proteolytic activity (as determined by	López-García *et al.*, 2012

			protease inhibitor)	
H1N1 Influenza A viruses	Bromelain	50 U/ml bromelain with beta-mercaptoethanol (1:1000, v/v) in TE buffer; 16h; 37°C; agitated	Bromelain-cleaved HA recovered from some virus (amino acid dependent), antibody inducing and protective against infection in ferrets	Wang *et al.*, 2012
Alicyclobacillus acidoterrestris, vegetative cells and spores		100 or 1000 ppm enzyme in deionized water, 20°C, 24h, apple and orange juices	For vegetative cells: papain at 1,000 ppm yielded 3 to 4 log reductions in all menstra; bromelain yielded 3-log and 1-log reductions in apple and orange juice, respectively. No inactivation of spores.	Assatarakul *et al.*, 2012
Tetanus toxin of *C. tetani*	Papain	Tetanus toxin (300,000 Lf units) in phosphate buffer, pH 6.5 and cysteine-HCL with 30 U/mg papain; 55°C; 4h; fractions collected from Sephadex column and analyzed	One purified fraction reacted with antiserum to toxin and protected guinea pigs from challenge to native toxin	Helting and Zwisler, 1974

1.3. INTERACTIONS OF VIRUS AND BACTERIA

Human norovirus encounters diverse and abundant bacterial populations in both the host intestinal tract and in the environment. The microbiome of the human gastrointestinal tract consists of an estimated 10^{11} to 10^{12} microbes/ml of luminal content (Palmer *et al.*, 2007), with individuals harbouring at least 160 different intestinal bacterial species (Qin *et al*., 2010). Culturable bacteria associated with the surfaces of edible leafy greens number 10^{7} cells/g (Lindow and Brandl, 2003) with nearly 500 genera present at leaf maturity as determined by 16S rRNA gene sequencing (Wiken Dees *et al.*, 2015). However, the role of bacteria in norovirus environmental persistence, transmission, infection, and detection in complex matrices remains largely underexplored. The limited data on norovirus and bacteria interactions suggest bacteria may both favor and limit HuNov persistence and transmission, depending on the bacterial species and system.

1.3.1. Human Norovirus and Surrogate Interactions with Bacteria

1.3.1.1. Binding between human norovirus and bacteria

Studies indicate that direct molecular interactions occur between human norovirus and some fecal bacteria. One of the earlier studies demonstrated that bacterial proteins isolated from activated sludge bind with ligands of conserved HuNov amino acid capsid sequences (Sano *et al.*, 2010). VLPs of HuNov were found to bind to lactic acid bacteria and *E. coli* Nissle within one hour of exposure at 37°C (Rubio-del-Campo *et al*., 2014). More recent research demonstrated the

association of HuNov GII.4 Sydney, GII.4 New Orleans, and GI.6 with bacteria isolated from human stool as well as laboratory bacterial strains that had been cultured repeatedly (Almand *et al.*, 2017). The association of virus with fecal bacteria was more pronounced when bacteria were propagated in minimal media than in nutrient-rich media (Almand *et al.*, 2017), although both nutrient conditions would be found in the human gastrointestinal tract. Although the specific molecular structures involved in binding between virus and bacteria were not elucidated in this study, the researchers surmised that specific receptor-ligand binding was involved based on the comparative inconsistent bacterial binding of TV and lack of bacterial binding by turnip crinkle virus, which are similar to HuNov in size, shape, and charge (Almand *et al.*, 2017). Transmission electron microscopy (TEM) imaging revealed association of HuNov VLPs with the membranes and pili of bacteria (Almand *et al.*, 2017).

Some bacteria express HBGA-like moieties on their surfaces, and Miura and colleagues (2013) provided evidence for a role of these HBGA-like moieties in binding HuNov. An exopolysaccharide with HBGA-like moieties produced by an enteric bacterium closely related to *Enterobacter cloacae* was found to bind HuNov VLPs, and enzymatic cleavage of the terminal N-acetyl-galactosamine residues of EPS resulted in reduced binding of the VLPs (Miura *et al.*, 2013).

1.3.1.2. Bacterial role in norovirus infection

Bacteria have been reported to support norovirus infection both *in vitro* and *in vivo*, but the mechanisms have not been fully elucidated. As previously noted,

stool containing bacteria, synthetic H-type HBGA, and *E. cloacae* expressing HBGA aided norovirus infection of B cells; whereas, filtration or UV-treatment of stool diminished HuNov B-cell infection (Jones *et al.*, 2014). In this same study, MNV were also found to infect B cells *in vivo*, and in support of the apparent influence of bacteria on norovirus infectivity, MNV infection was reduced when intestinal bacteria of mice were depleted by administration of antibiotics (Jones *et al.*, 2014). In other studies using the mouse model, the ability of MNV to establish persistent infection in mice was inhibited by administration of antibiotics to mice (Baldridge *et al.*, 2015). Persistent MNV infection could be restored in these antibiotic-treated mice by fecal transplantation from non-antibiotic treated mice but not by fecal transplantation from antibiotic-treated mice (Baldridge *et al.*, 2015).

Infection of intestinal tuft cells by MNV occurred in both wild type and germ-free mice indicating that bacteria are not essential to MNV infection of these cells (Wilen *et al.*, 2018); however, consistent with prior studies (Baldridge *et al.*, 2015; Jones *et al.*, 2014), administration of antibiotics inhibited MNV infection of mice (Wilen *et al.*, 2018). Antibiotic administration was also associated with reduced detection of tuft cell markers in the mouse colon suggesting that intestinal bacteria may contribute to the regulation of tuft cells and thereby their subsequent availability for MNV infection (Wilen *et al.*, 2018).

By contrast, in the HIE *in vitro* system, bacteria were not found to be essential for HuNov infection of HIEs; infection was supported by addition of bile (Ettayebi

et al., 2016). In the gnotobiotic pig model, HuNov was found to infect enterocytes rather than B cells, and *E. cloacae* administration actually reduced HuNov fecal shedding and detection of HuNov in intestinal tissues as compared to gnotobiotic pigs that were not administered *E. cloacae* (Lei *et al.*, 2016).

1.3.1.3. Bacterial role in resistance of norovirus and norovirus surrogates

Reports on the direct impact of bacteria on norovirus resistance to inactivation treatments were not found; however, reports of increased viral resistance in matrices that contain bacteria call attention to the need to investigate the potential role of bacteria on viral resistance. As previously noted, HuNov surrogates, MNV and FCV, were resistant to 500 ppm chlorine in the presence of 20% stool suspension, but in cell culture media with 1% fetal bovine serum, reduction was 3-log RT-qPCR (Tung *et al*., 2013). Both media contain organic material, albeit different kinds, and the components and interactions that may have provided either a protective effect for MNV and FCV and/or decreased chlorine efficacy were not determined (Tung *et al*., 2013).

Another study investigated the chlorine resistance of FCV spiked into various HuNov GII.4 fecal samples diluted into hard water supplemented with 0.3% bovine serum albumin (BSA) (Nowak *et al*., 2011). FCV was more resistant to chlorine inactivation in the fecal matrices than in cell culture media, although the magnitude of difference varied by the fecal suspension (Nowak *et al*., 2011). This difference was not attributed to overall protein load or properties of the different HuNov in the fecal material, but rather some other protective stool component

such as gastric mucin (Nowak et al., 2011). The bacterial content was not characterized nor was their potential role discussed (Nowak *et al*., 2011).

1.3.1.4. Antagonistic interactions of bacteria and human norovirus or surrogates

In contrast with studies that suggest bacteria may provide protective effects to HuNov or its surrogates, some studies suggest that some bacteria or their metabolic products may compromise virus persistence in foods or their attachment and infectivity in cell culture. Infective MNV and FCV were reduced during fermentation of oysters (Seo *et al*., 2014) and vegetables (Lee *et al*., 2012) over periods of 15 and 20 days, respectively. MNV reductions were modest at less than 2-log PFU/ml; whereas, FCV reductions ranged from 2 to 4 logs PFU/ml (Lee *et al*., 2012; Seo *et al*., 2014). The viral reductions were attributed to increases in lactic acid bacteria, organic acids, and enzymes, amylase, lipase, and proteinases (Lee *et al.*, 2012; Seo *et al*., 2014). However, controls were not incorporated for each variable or for the storage time, and thus it was not possible to discern which of the factors was responsible for the change in virus titers.

Various studies have demonstrated reduced cellular attachment or infectivity of FCV, MNV, and HuNov VLPs due to lactic acid bacteria or their products. Pretreatment of FCV with the bacterial growth medium filtrate of *Lactococcus lactis* subsp. lactis reduced viral infectivity in cell culture by approximately one-log $TCID_{50}$ per 0.1 ml, while treatment throughout the infection period resulted in

approximately a 7-log $TCID_{50}$ per 0.1 ml reduction (Aboubakr *et al*., 2014). Similarly, pretreatment of MNV with the bacterial growth medium filtrate of *Bifidobacterium longum* reduced infectivity by 0.7 log PFU/ml over the control medium (Loncke, 2016). The attachment of HuNov VLPs to human colon adenocarcinoma cells (HT-29) was decreased when co-inoculated with lactic acid bacteria, although VLP binding to cells increased when bacteria were added prior to or after the VLPs (Rubio-del-Campo *et al*., 2014).

1.3.2. Interactions of Bacteria and Other Viruses

Although investigations on norovirus and bacteria interactions are limited, studies with other virus and bacteria reveal similar apparent contradictions in the role of bacteria for viral resistance and infectivity.

The potential importance of bacteria on the inactivation resistance of viruses has been reported. Studies have demonstrated that viruses can accumulate in bacterial biofilms of drinking water systems (Lehtola *et al.*, 2007; Skraber *et al.*, 2005), with the quantity of virus recovered also affected by surface material (less recovered from copper pipes than polyethylene pipes) (Lehtola *et al.*, 2004) and level of chlorine (Quignon *et al.*, 1997). Virus may persist in biofilms for days to months as was demonstrated with poliovirus-1 and bacteriophages, B40-8 and MS2 (Skraber *et al.*, 2005), and are presumed to be afforded protection from environmental and chemical stresses as has been reported for bacteria embedded in biofilm matrix.

Bacterial lipopolysaccharide (LPS) and peptidoglycan, in the absence of live bacteria, have been demonstrated to confer enhanced resistance of poliovirus to chemical and thermal stress (Robinson *et al.*, 2014). Mahoney poliovirus incubated with LPS or peptidoglycan (1 mg/ml) for one hour at 37°C enhanced virus inactivation resistance to subsequent bleach treatment (0.1 mg/ml) with approximately 3 to 4-log greater recovery of virus incubated with the bacterial compounds than with phosphate buffered saline. Exposure to LPS or peptidoglycan also shifted virus thermal resistance by 3°C (Robinson *et al.*, 2014).

The importance of bacteria for viral infection may not be limited to norovirus. Consistent with findings of MNV infection in mice (Baldridge *et al.*, 2015; Jones *et al.*, 2014), antibiotic treatment of rotavirus-infected mice resulted in decreased viral shedding, rotavirus antigen production, and detectable rotavirus genomic material in intestinal tissues (Uchiyama *et al.*, 2014).

However, elucidating the role of bacteria in supporting or limiting viral infections is complicated by findings that some antibiotics can exert direct effects on host antiviral response, and thus the depletion of bacteria through antibiotic treatment may not necessarily be the underlying cause for decreased viral infection. The antibiotic, neomycin, was found to enhance resistance of mouse infection to various viruses including herpes simplex virus, influenza virus, and Zika virus (Gopinath *et al*., 2018). This protection was afforded to both conventional and germ-free mice, prophylactically and after viral infection

(Gopinath *et al*., 2018). Germ-free mice that were not treated with neomycin exhibited viral disease symptoms upon infection suggesting that bacteria were not needed to support virus infection (Gopinath *et al*., 2018). The antiviral effect of neomycin was attributed to antibiotic-induced upregulation of transcription of immune molecules and recruitment of immune cells (Gopinath *et al*., 2018).

Conversely, antagonistic interactions of bacteria or their metabolic products and other viruses have also been reported. Antiviral effects of metabolic by-products of environmental and intestinal bacteria, especially probiotic bacteria, have been investigated for several enveloped and nonenveloped viruses and are summarized in Table 1.3. These studies were undertaken for various purposes, including to determine virus persistence in contaminated environmental waters (Ward *et al*, 1986; Ward, 1982; Fujioka *et al*., 1980), to determine viral reductions in human and animal waste to assess disposal risk (Deng and Cliver, 1995b), and to identify potential antiviral treatments *in vivo* (Hotta *et al*., 1977; Pant *et al*., 2007) and *in vitro* (Cliver and Hermann, 1972; Kim *et al*., 2014; Choi *et al*., 2009; Aboubakr *et al*., 2014; Loncke *et al*., 2016).

The investigative approaches to study viral persistence in the presence of environmental water or waste products included monitoring viral decay in the test medium naturally containing bacteria as compared to viral decay in sterile water or the test medium that had been treated to remove or inactivate bacteria (Deng and Cliver, 1995b; Ward *et al*., 1986). For studies that investigated the interaction between specific virus and bacteria, bacteria were grown in microbiological

media, and bacterial cells were separated from the growth media. Virus were then exposed to the bacterial cells or the spent cell-free growth media directly or at various stages during infection in cell culture or *in vivo* and monitored for viral inactivation or reduced pathological effects, respectively.

Several studies demonstrated that virus decayed more rapidly in natural samples containing live bacteria. Coxsackievirus (CVB5) (Ward *et al*., 1986), Echovirus (Ward *et al*., 1986), and poliovirus (Fukioka *et al*., 1980; Ward *et al*., 1986) persisted longer in bacteria-free water as rendered through autoclaving, filtration, or UV irradiation than water containing viable bacteria. Similarly, HAV persisted longer in mixed human and animal wastes than in autoclaved waste (Deng and Cliver, 1995b). In studies with specific bacteria, virus were reported to bind to bacteria as was observed for rotavirus with *Lactobacillus rhamnosus* and *Bifidobacterium lactis* (Salminen *et al*., 2010). Some virus demonstrated decreased infectivity in cell culture when co-inoculated with bacteria as for Coxsackie virus co-inoculated with *Bacillus subtilis* (Cliver and Herrmann, 1972) and rotativirus with *Escherichia coli* Nissle (Kandasamy *et al*., 2016). Bacterial metabolic products, in the absence of live bacteria, were also reported to reduce virus infectivity in cell culture such as for poliovirus exposed to a peptide derived from *Enterococcus mundtii* (Todorov *et al*., 2005) and enterovirus exposed to cell-free supernatants of *Bifidobacterium bifidum* growth (Choi *et al*., 2009). Administration of some bacteria or their spent and filtered growth medium has also been shown to reduce viral pathological effects *in vivo* such as for

adenovirus in mice treated with spent media of *Streptococcus faecalis* (Hotta *et al*., 1977) and rotavirus in mice administered *Lactobacillus rhamnosus* (Pant *et al*., 2007).

Several mechanisms of inactivation of viruses by bacterial products have been proposed. Some of the reported antiviral effects were inhibited with the addition of proteases or protease inhibitors, thereby suggesting bacterial proteolysis of virus as was proposed for the cell-free filtrate of *Micrococcous luteus* against poliovirus-1 (Deng and Cliver, 1992) and *Bacillus subtilis* and *B. brevis* against hepatitis A virus (HAV) (Deng and Cliver, 1995a). Other antiviral effects were proposed to be related to low-molecular-mass products of less than 1,000 Da as was reported for cell-free filtrates of *Ps. aeruginosa* against coxsackie virus (Cliver and Hermann, 1972) and *Ps. alcaligenes* against HAV (Deng and Cliver, 1995a). Other mechanisms of reported antiviral activity were speculated to be a function of cell culture assay such as in the inhibition of infection by the modification or blockage of cellular receptors or inhibition of a later stage of viral replication (Botić *et al*., 2007; Hotta *et al*., 1977; Serkedjieva *et al*., 2000; Todorov *et al*., 2005; Todorov *et al*., 2010; Varyukhina *et al*., 2012; Wachsman *et al*., 2003; Wachsman *et al*., 1999). For example, spent culture supernatants of *Bacteroides thetaiotamicron* and *Lactobacillus casei* applied to mucus-secreting HT29-MTX cells prior to rotavirus infection resulted in decreased viral infection; this reduction was attributed to cell-surface glycan modification by soluble factors produced by the bacteria (Varyukhina *et al*., 2012). Although not necessarily

characterized in these *in vitro* studies, some virus and bacteria bind the same cell surface receptors. Rotavirus, human norovirus, and some *Lactobacillus* species bind human A-, B- or H-antigens (Liu *et al*., 2012; Uchida *et al*., 2006a; Uchida *et al*., 2006b). In addition to potential direct interactions between bacteria and virus as well as competition for host, probiotic bacteria may also indirectly affect virus pathology by enhancing host antiviral immune responses (Salminen *et al*., 2010) that regulate intestinal homeostatis and induction of mucosal protective factors (Kawahara *et al*., 2017).

While some bacteria or their exopolysaccharides bear similarity in surface moieties to HBGAs and may consequently bind HuNov, with potential implications for transmission and infection, some bacteria secrete glycosidases that can degrade HBGAs for use as an energy source (Hoskins *et al.*, 1985). Some Bifidobacteria are among these glycosidase secretors (Hoskins *et al.*, 1985), and individuals positive for mucosal ABH type HBGA expression have a greater abundance and diversity of intestinal Bifidobacteria than non-secretor individuals (Waklin *et al.*, 2011). *Bifidobacterium bifidum* strain VIII-210 and *Ruminococcus torques* strain IX-70 were found to have blood-group degrading (BGD) glycosidase activity with approximately 30 to 90% of the BGD activity being extracellular depending on the strain and antigen (Hoskins *et al*., 1985). Production of these glycosidases occurred in the absence of mucin (Hoskins *et al*., 1985). Blood group degrading enzyme activity was also observed by fecal extracts and the culture supernatant of these fecal microorganisms derived from

blood group B secretors (Cromwell and Hoskins, 1977). The enzyme activity of these fecal bacteria was shown to degrade B-like antigens on the surface of *E. coli* 086 (Cromwell and Hoskins, 1977). The effect of intestinal bacteria with glycosidase activity on bacteria that bind HuNov has not been reported.

1.3.3. Viral and Bacterial Interactions Investigated for Dissertation Research

Prior research revealed complex associations between bacteria and virus, including norovirus, which bear significance for food safety. Collectively, these data suggest bacteria could affect viral and host interactions, transmission between human waste and the environment, viral environmental persistence, viral resistance to removal or inactivation in food, and viral detection from complex matrices. Further investigations are warranted into the fecal bacteria that associate closely with norovirus as well as the bacteria that may compete for or disrupt norovirus receptor associations. Data are notably lacking on interactions between norovirus and phyllospheric bacteria. Data are also limited on the effect of bacteria on norovirus persistence in food and resistance to inactivation strategies used in food processing.

The second part of this dissertation addresses interactions between norovirus or norovirus surrogates and bacteria associated with the human intestinal tract, human skin, phyllosphere, and soil. Both cooperative and antagonistic interactions are considered, those which may favor or limit, respectively, the persistence and inactivation resistance of norovirus and its surrogates.

1.3.3.1. Viral propagation in presence of bacterial metabolic products

The objective of the first study was to evaluate the antiviral activity of bacterial metabolic products. Bacteria were grown in microbiological media and the spent media was filtered of bacterial cells. These cell-free supernatants (CFS) were evaluated for antiviral activity by the viral yield reduction assay from the propagation of HuNov surrogates, Tulane virus and murine norovirus.

1.3.3.2. Bacterial interactions and virus resistance

The objective of the second study was to evaluate direct assocations between virus and bacteria and the impact of bacteria on viral inactivation resistance. Binding between Tulane virus and intestinal, soil, and phyllospheric bacteria was investigated as well as binding of HuNov to fecal bacteria as influenced by CFS of glycosidase-producing bacteria. The effect of bacterial cell constituents on the resistance of TV to chlorine and thermal inactivation was also evaluated.

Table 1.3. Prior research on antiviral effects of bacteria

Virus	Bacteria or System	Findings	Reference
Non-enveloped viruses			
Adenovirus	*Streptococcus faecalis*	Bacterial growth supernatant administered orally to mice reduced virus-induced tumor development incidence to approximately half that of when virus was administered alone.	Hotta *et al.*, 1977
Coxsackie virus type A-9 (CA-9)	*Pseudomonas aeruginosa*, *Bacillus subtilis*	Co-inoculation of virus and *B. subtilis* or *Ps. aeruginosa* resulted in > one-log and two-log reductions, respectively, in viral infectivity. Cell-free filtrates of *Ps. aeruginosa* growth inactivated CA-9 in previously filter-sterilized lake water samples. Various molecular weight exclusion filters suggested some inactivation may be attributable to low molecular weight substances. Cell cytotoxicity, bacterial adsorption of virus appeared to be uninvolved in antiviral effect. Uptake of radiolabeled virus by *Ps. aeruginosa* suggested some effect to viral particle integrity and potential growth substrate.	Cliver and Herrmann, 1972
Coxsackievirus B3 (CVB3)	*Bifidobacterium adolescentis*, human isolate	Bacterial cells (10^9 CFU/ml, suspended in PBS and sonicated) reduced CVB3 plaque formation in human cervical cancer (HeLa) cells and detectable CVB3 RNA	Kim *et al.*, 2014

		levels by one CT-value in real-time PCR.	
Coxsackievirus B5 (CVB5)	Environmental waters (creek, river, well)	Infective virus (plaque assay in BGM cells) decreased >99.99% during 7 days of incubation at 27°C in environmental waters as compared to sterile distilled water.	Ward *et al.*, 1986
Echovirus, type 12 (Echo-12)	Environmental waters (creek, river, well) and bacterial isolates (unidentified)	Infective virus (plaque assay in RD cells) decreased > 99.999% during 7 days of incubation at 27°C in environmental waters as compared to sterile distilled water. Treatments to inactivate microorganisms (filtration, UV irradiation, heat, hypochlorous acid, sodium azide) in environmental water lead to decrease of antiviral properties. Cleavage of viral capsid proteins was detected, followed by cleavage of viral RNA.	Ward *et al.*, 1986
Enterovirus (CA16, CB3, CB4)	*Bifidobacterium bifidum*	Cell-free supernatants of bacterial growth in MRS broth or yogurt fermented by *B. bifidum* inhibited viral infection as measured by reduction of cytopathic effect to rhesus monkey kidney (Vero) cells or Madin-Darby canine kidney (MDCK) cells	Choi *et al.*, 2009
Feline calicivirus (FCV)	*Lactococcus lactis* subsp. *lactis* LM0230	Pre-treatment of FCV with bacterial growth medium filtrate (BGMF) (in MRS broth) for 24 h reduced FCV infectivity in Crandell-Reese feline kidney (CRFK) cells up to 1.3 $\log_{10}TCID_{50}$/0.1 ml. Pre-treatment of FCV with bacterial cell suspension (BCS)	Aboubakr *et al.*, 2014

		for 24 h to allow viral-bacterial adsorption reduced recoverable FCV by 1.8 $log_{10}TCID_{50}$/0.1 ml. Co-treatment of CRFK and FCV with undiluted pH-7 adjusted BGMF reduced FCV infectivity by 7.5 $log_{10}TCID_{50}$/0.1 ml. Co-treatment of CRFK with FCV and BCS reduced FCV infectivity by up to 6.9 $log_{10}TCID_{50}$/0.1 ml. Pretreatment of CRFK with BGMF or BCS did not reduce FCV infectivity.	
Hepatitis A virus (HAV)	Bacteria isolated from dairy cattle manure: *Bacillus brevis*, *B. subtilis*, *B. cereus*, *Ps. alcaligenes*	HAV inactivation in bacteria in fluid thioglycollate medium with D_{10} values at 30°C of 5.7, 6.9, and 6.9 days in presence of *B. brevis*, *B. subtilis*, and *Ps. alcaligenes*, respectively, compared to control at 35.1 days. *B. brevis* antiviral effects were attributed to serine and cysteine proteases. *B. subtilis* antiviral effects were attributed to proteases. *Ps. alcaligenes* antiviral effects attributed to molecular weight product < 1 kDa.	Deng and Cliver, 1995a
Hepatitis A virus (HAV)	Mixed waste: human dairy cattle, swine manure	HAV persisted longer when suspended in human waste alone than in human waste mixed with animal waste. HAV inactivation D-values in mixed human/animal wastes at 25°C and 37°C were less in raw waste and filtered waste than in	Deng and Cliver, 1995b

		autoclaved waste suggesting microbial metabolites were antiviral.	
Hepatitis C virus (HCV)	*Lactobacillus bulgaricus* 761N	Bacterial growth in skim milk medium crude extracellular extract pretreatment of human liver hepatocellular carcinoma (HepG2) cells reduced HCV infectivity by up to 80% compared to control as determined by RT-qPCR. Bacterial growth semi-purified fraction (14.4 kDa) pre-treatment of HepG2 cells prior to HCV infection as well as treatment after HCV infection reduced the viral load by 99.9% compared to control as determined by RT-qPCR.	El-Adawi *et al.*, 2015
Human norovirus, P-particle (VP1 capsid protein)	Lactic acid bacteria (LAB)	P-particles bound to LAB and *E. coli* Nissle 1917 within 1 h at 37°C as determined by centrifugation and washing of the bacterial pellets to remove unbound P-particles followed by detection of P-particles in bacterial pellets by Western blot assay. Exclusion and displacement assays of P-particles in human colon adenocarcinoma (HT-29) monolayers with LAB demonstrated decreased attachment of P-particles when added to cells simultaneously with bacteria, but increased P-particle binding to HT-29 when bacteria were added prior to or after the addition of P-particles.	Rubio-del-Campo *et al.*, 2014
Murine norovirus (MNV-1)	*Bifidobacterium longum*	MNV incubated 48h at 37C in *B. longum* cell-free filtrate prior to enumeration.	Loncke, 2016

		Reduction of 2.4 log PFU/ml MNV (approx. 0.7 log PFU/ml greater reduction than in TSB control)	
Poliovirus (PV3, strain Sabin)	*Enterococcus mundtii* ST4V isolate of soya beans	Bacterial peptide (3.95 kDa, 400 μg/ml) added after initial viral infection inhibited viral propagation by 50% in Vero cells. Peptide also antibacterial; effects reduced or negated after treatment with proteinase K, pronase, pepsin, and trypsin, but not α-amylase indicative of protein not glycosylated.	Todorov *et al.*, 2005
Poliovirus (PV1)	Mixed human and swine wastes *Micrococcus luteus*, *Staphylococcus epidermidis*, *Bacillus sp., Streptococcus sanguis* (isolated from swine manure)	PV inactivation D-values in mixed human/swine wastes at 25°C and 37°C were less in raw waste and filtered waste than in autoclaved waste suggesting microbial metabolites were antiviral. Bacterial isolates added to autoclaved manure demonstrated greater PV inactivation (greater than one log infectivity) than controls. A mix of protease (trypsin, serine, metal) inhibitors partially inhibited viral inactivation by cell-free filtrate of *M. luteus*	Deng and Cliver,1992
Poliovirus (PV1)	Activated sludge	Mixed liquor suspended solids – antiviral alone and when resuspended in broth and incubated prior to addition of virus. Supernatant only effective after 48 h. Centrifugation, autoclaving, and filtering leads to loss of antiviral effect. Antiviral activity determined by plaque assay in HeLa cells	Ward, 1982

		Living microorganism proposed to have antiviral property though not determined	
Poliovirus (PV1)	Environmental waters (creek, river, well)	Infective virus (plaque assay in HeLa cells) decreased >99.9% during 7 days of incubation at 27°C in environmental waters as compared to sterile distilled water.	Ward *et al.*, 1986
Poliovirus (PV1)	Seawater	Infective virus (plaque assay in African green monkey kidney cells (BGM)) suspended in untreated and filtered (1.0 μm pore) seawater decreased by 2 to 4 logs within 4 days as compared to persistence in seawater treated by boiling, autoclaving, filtering through 0.45 μm pore. Antiviral activity was attributed to microorganisms.	Fujioka *et al.*, 1980
Rotavirus (RV)	*Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG	LAB applied and aspirated from macrophage (TLT) and intestinal epithelial (GIE, H4, and PSI) cells afforded protection from subsequent RV-induced cytopathic effect. The protection did not correlate with attachment of LAB to host cells.	Maragkoudakis *et al.*, 2010
Rotavirus (human, Wa)	*Bifidobacterium longum* subsp. *infantis* CECT7210	Infant stool isolate combined with virus or host cell prior to virus infection resulted in nearly 50% reduction in viral foci in human colon adenocarcinoma (HT-29) cells	Muñoz *et al.*, 2011
Rotavirus (human, Wa)	*Lactobacillus ruminis*, *Bifidobacterium longum*	Bacterial cells (10^9 CFU/ml, suspended in PBS and sonicated) reduced RV plaque formation in Vero cells to 50 to 70% of control with Vero cell viability reduced to 80% and	Kang *et al.*, 2015

		88% of control for *L. ruminis* and *B. longum*, respectively. *L. ruminis* reduced RV plaque formation in human colon adenocarcinoma (Caco-2) cells by 60% of control.	
Rotavirus, human Wa	*Escherichia coli* Nissle (EcN), *Lactobacillus rhamnosus* GG (LGG)	Co-inoculation of EcN and RV reduced attachment of RV to Caco-2 cells (by 50%) as compared to non-probiotic treated cells and LGG-treated cells. EcN demonstrated greater association to Caco-2 cells and to HRV than LGG. EcN treated gnotobiotic pigs had reduced incidence of diarrhea (by 34%), duration of diarrhea (by >3 days), and reduced duration of fecal viral shedding (0.6 days) as compared to non-probiotic treated controls. EcN induced IL-6, IL-10, and IgA; LGG did not.	Kandasamy *et al.*, 2016.
Rotavirus, human Wa and porcine RF strains	*Bacteroides thetaiotamicron, Lactobacillus casei*	Spent culture supernatants (SCS) of *B. thetaiotamicron* and *L. casei*, and exogenous galactosyltransferase (but not galactosidase) applied to mucus-secreting HT29-MTX cells prior to RV infection showed reduced viral infection measured by immunofluorescence. Reduction in rotavirus infection attributed to cell-surface glycan modification by SCS resulting in decreased sialic acid, mannose, and fucose, and	Varyukhina *et al.*, 2012

		increased in galactose on cell surface.	
Rotavirus, Nebraska calf diarrhea virus (NCDV)	*Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb-12, other *Lactobacillus* sp.	NCDV binds *L. rhamnosus* GG and *B. lactis* as measured by radioactivity of virus-bound radiolabeled bacteria	Salminen *et al*., 2010
Rotavirus, rhesus (RRV)	*Lactobacillus rhamnosus* GG, *L. paracasei*, *L. reuteri*, *L. johnsonii*, , *Sreptococcus thermophilus*	Lactobacillus rhamnosus GG (LGG) administered to RRV-challenged BALB/c mice pups reduced diarrhea prevalence by 50%, duration by 64%, and severity by 71% as compared to untreated infected control mice. Heat-killed LGG did not provide the same degree of protection.	Pant *et al.*, 2007
Enveloped Viruses			
Arbor Viruses, Group A (Sindbis virus, Chikungunya virus)	*Corynebacterium sp.*	Bacterial growth medium filtered supernatant had limited effect on infectivity of Sindbis virus in human amnion cells. Bacterial concentrate (subjected to sonication, freeze/thaw, filtration) inhibited viral infectivity and cytopathogenicity. Time of exposure experiments suggested the inhibitory factor does not inactivate the virus directly or impede host cell penetration, or viral release. Proposed mechanism of inhibition of intracellular viral replication. The antiviral component presumed proteinaceous based	Carver and Naficy, 1962

		on inactivity after trypsin treatment. The bacterial concentrate was ineffective against unrelated viruses (including ECHO 9 and 11, vaccinia, Coxsackie B5, HSV, Poliovirus 1, adenovirus 2).	
Herpes simplex virus (HSV-1)	*Streptococcus faecalis*	Virus suspended in bacterial growth supernatant adsorbed 65% less to HeLa cells than virus suspended in PBS. Bacterial growth supernatant administered orally to mice reduced virus-induced mortality ratio by approximately 60% compared to virus administered alone. Active principle presumed to be a protein as pronase and trypsin treatment reduced efficacy whereas treatment with lipase, RNase, and heat (95°C for 5 min) did not.	Hotta *et al.*, 1977
Herpes simplex virus (HSV-1)	*Enterococcus faecium* ST5Ha, isolate of smoked salmon	Bacterial peptide (4.5 kDa, 50 μg/ml) added after initial viral infection inhibited viral propagation by 50% in Vero cells.	Todorov *et al.*, 2010
Herpes simplex virus (HSV-1)	*Lactobacillus rhamnosus*	Vero cell viability exposed to HSV-1 with *L. rhamnosus* was greater (by 20%, as measured by MTT mitochondrial function assay) than HSV-1 alone or with *E. coli*. PFU reduction and induction of NO, IFNγ or TNF-α did not differ between bacteria.	Khani *et al.*, 2012
Herpes simplex viruses (HSV-1, HSV-2)	*Enterococcus mundtii* ST4V,	Bacterial peptide (3.95 kDa, 400 μg/ml) added after initial viral infection inhibited viral	Todorov *et al.*, 2005

	isolate of soya beans	propagation by >99% in Vero cells.	
Herpes simplex virus (HSV-1, HSV-2)	*Enterococcus faecium CRL35*	Bacterial polypeptide (3.5 kDa, 100 μg/ml) applied for 18h during viral propagation after initial infection period (1 h) in Vero and Baby hamster kidney (BHK-21) cells reduced viral yield by 2 log PFU/ml	Wachsman *et al.*, 1999
Herpes simplex virus (HSV-1, HSV-2)	*Enterococcus faecium CRL35*	Bacterial polypeptide (100 μg/ml) applied 8h p.i. of Vero cells inhibits synthesis of viral late glycoprotein D, preventing infection spread	Wachsman *et al.*, 2003
Human immunodeficiency virus (HIV)	*Lactobacillus sp.* and *Pediococcus pentosaceus* (isolates of human breastmilk)	HIV-1 infectivity in TZM-bl cells was reduced by up to 55% with viral exposure to bacteria or up to 42% with exposure to bacterial cell-free supernatants.	Martin *et al.*, 2010
Influenza virus A (H7N1)	*Lactobacillus delbrueckii*	Crude extract (B1) of bacterial growth in media yielded protein (6 kDa) sensitive to some proteases. B1 (0.08 μg/ml) reduced viral infection in chicken embryo fibroblast (CEF) cells (>5 log $TCID_{50}$/ml reduction) when present for entire replicative cycle. B1 did not inactivate virus, decrease host cell sensitivity, or inhibit viral adsorption to cell. Viral internalization was slightly affected. Intracellular replication inhibition was proposed.	Serkedjieva *et al.*, 2000
Influenza virus A and B	*Lactobacillus plantarum*	Cell-free supernatants of bacterial growth in MRS broth or yogurt fermented by	Choi *et al.* 2009

		Lactobacillus sp. inhibited viral infection as measured by reduction of cytopathic effect to Vero or MDCK cells	
Influenza virus, Avian (AI-H9N2)	*Leuconostoc mesenteroides* (Kimchi isolate)	Antiviral activity of bacterial cell-free supernatant observed in MDCK cells. AI-H9N2 hemagglutination was inhibited in eggs treated with bacterial cell-free supernatant and heat-treated supernatant. SPF chickens administered live *L. mesenteroides* in feed had greater weight gains, less viral genomic material detectable in cloacae, and an increase in IFN-γ when challenged with AI-H9N2 as compared to positive control chickens.	Seo *et al.*, 2012
Measles virus (MV/BRAZIL/001/91)	*Enterococcus mundtii* ST4V isolate of soya beans	Bacterial peptide (3.95 kDa, 400 μg/ml) added after initial viral infection inhibited viral propagation by >95% in Vero cells.	Todorov *et al.*, 2005
Transmissible gastroenteritis coronavirus (TGEV)	*Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG	LAB applied and aspirated from macrophage (TLT) and intestinal epithelial (GIE, H4, and PSI) cells afforded protection from subsequent TGEV-induced cytopathic effect. The protection did not correlate with attachment of LAB to host cells.	Maragkoudakis *et al.*, 2010
Vesicular stomatitis virus (VSV)	*Bifidobacterium longum, Lactobacillus plantarum, Lactobacillus reuteri*	Bacterial metabolic products protected IPEC-J2 cells from VSV infection (65% cellsurvival compared to untreated control cells). VSV adsorbed to probiotic bacteria.	Botić *et al.*, 2007

Vesicular stomatitis virus (VSV)	*Lactobacillus paracasei, Bifidobacterium longum*	Prior treatment (90 min) of heat-killed (100°C, 10 min) bacteria applied to pig alveolar macrophage derived cells (3D4/21) afforded 10 to 30% greater cell survival after VSV challenge than control cells treated with VSV alone as determined by the cytopathic effect reduction (CPE) assay. Bacteria adherence to 3D4/21 cells ranged from 9 to 27% of original inoculum depending on the bacterial strain.	Ivec *et al.*, 2007

REFERENCES

1. Aboubakr, H. A., A. A. El-Banna, M. M. Youssef, S. A. A. Al-Sohaimy, and S. M. Goyal. 2014. Antiviral effects of *Lactococcus lactis* on feline calicivirus, a human norovirus surrogate. *Food Environ Virol*. 6: 282-289.

2. Almand, E. A., M. D. Moore, J. Outlaw, and L-A. Jaykus. 2017. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS ONE* 12(3): e0173124.doi:10.1371/journal.pone.0173124.

3. Assatarakul, K., J. J. Churey, and R.W. Worobo. 2012. Antimicrobial activity of papain and bromelain juice supplements against *Alicyclobacillus acidoterrestris* and associated physicochemical properties of apple and orange juice. *Institute of Food Technologists Annual Meeting Abstracts*, Abstract 043-05.

4. Atmar, R. L., A. R. Opekun, M. A. Gilger, M. K. Estes, S. E. Crawford, F. H. Neill, S. Ramani, H. Hill, J. Ferreira, and D. Y. Graham. 2014. Determination of the 50% human infectious dose for Norwalk virus. *J. Infect. Dis.* 209: 1016-1022.

5. Baldridge, M. T., T.J. Nice, B.T. McCune, C.C. Yokoyama, A. Kambal, M. Wheadon, M. S. Diamond, Y. Ivanova, M. Artyomov, and H. W. Virgin. 2015. Commensal microbes and interferon-λ determine persistence of enteric murine norovirus infection. *Science* 347: 266-269. Belliot, G., A. Lavaux, D. Souihel, D. Agnello, and P. Pothier. 2008. Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Appl. Environ. Microbiol.* 74(10): 3315-3318.

6. Botić, T., T.D. Klingberg, H. Weingartle, and A. Cencic. 2007. A novel eukaryotic cell culture model to study antiviral activity of potential probiotic bacteria. *Int. J. Food Microbiol.* 12: 227-234.

7. Biozym. Data Sheet – Papain (EC 3.4.22.2). Available at http://www.biozyme.de/datasheets/papain.php. Accessed 12 September 2012

8. Bradley, I., A. Straub, P. Maraccini, S. Markazi, and T. H. Nguyen. 2011. Iron oxide amended biosand filters for virus removal. *Water Res.* 45:4501-4510.

9. Bu, W., A. Mamedova, M. Tan, M. Xia, X. Jiang, and R. S. Hedge. 2008. Structural basis for the receptor binding specificity of Norwalk virus. *J. Virol.* 82(11): 5340-5347.

10. Butot, S., T. Putallaz, and G. Sánchez. 2008. Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. *Int. J. Food Microbiol.* 126: 30-35.

11. Callejón, R. M., M. I. Rodriguez-Naranjo, C. Ubeda, R. Hornedo-Ortega, M. C. Garcia-Parrilla, and A. M. Troncoso. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and Causes. *Foodborne Path. Dis*. 12: 32-38.

12. Campos, C. J. A., J. Avant, J. Lowther, D. Till, and D. N. Lees. 2016. Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes. *Water Res.* 103:224-232.

13. Carver, D.H. and K. Naficy.1962. Inhibition of Arbor viruses (Group A) by a protein-like constituent of a *Corynebacterium*. *Proc. Soc. Exp. Biol. Med.* 111(2): 356-360.

14. Centers for Disease Control and Prevention (CDC). 2014. Surveillance for foodborne disease outbreaks, United States, 2012, Annual Report. US Department of Health and Human Services, CDC. Atlanta, GA.

15. Cheng, R., G. Li, L. Shi, X. Xue, M. Kang, and X. Zheng. 2016. The mechanism for bacteriophage f2 removal by nanoscale zero-valent iron. *Water Res.* 105:429-435.

16. Choe, Y., F. Leonetti, D. C. Greenbaum, F. Lecaille, M. Bogyo, D. Brömme, J. A. Ellman, and C. S. Craik. 2006. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J. Biol. Chem*. 281(18): 12824-12832.

17. Choi, J.-M., A. M. Hutson, M. K. Estes, and B. V. Venekataram Prasad. 2008. Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus. *PNAS* 105(27): 9175-9180.

18. Choi, H-J., J-H. Song, Y-J. Ahn, S-H. Baek, and D-H. Kwon. 2009. Antiviral activities of cell-free supernatants of yogurts metabolites against some RNA viruses. *Eur. Food Res. Technol.* 228: 945-950.

19. Choudhury, D., S. Biswas, S. Roy, and J.K. Dattagupta. 2010. Improving thermostability of papain through structure-based protein engineering. *Protein Eng. Des. Sel.* 23(6): 457-467.

20. Cliver, D.O. and J.E. Herrmann. 1972. Proteolytic and microbial inactivation of enteroviruses. *Water Res.* 6: 797-805.

21. Code of Federal Regulations. 2013a. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1024, Bromelain. Available at: www.ecfr.gov. Accessed 13 June 2017.

22. Code of Federal Regulations. 2013b. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1316, Ficin. Available at: www.ecfr.gov. Accessed 13 June 2017.

23. Code of Federal Regulations. 1983. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1585, Papain. Available at: www.ecfr.gov. Accessed 13 June 2017.

24. Cromeans, T., G. W. Park, V. Costantini, D. Lee, Q. Wang, T. Farkas, A. Lee, and J. Vinjé. 2014. Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Appl. Environ. Microbiol.* 80(18): 5743-5751.

25. Cromwell, C. L. and L. C. Hoskins. 1977. Antigen degradation in human colon ecosystems. *Gastroenterol.* 73: 37-41.

26. Dancho, B. A., H. Chen, and D. H. Kingsley. 2012. Discrimination between infectious and non-infectious human norovirus using porcine gastric mucin. *Int. J. Food Microbiol.* 155: 222-226.

27. de Amorin, A., H. R. Borba, J. P. P. Carauta, D. Lopes, and M. A. C Kaplan. 1999. Anthelmintic activity of the latex of *Ficus* species. *J. Ethnopharmacol.* 64: 255-258.

28. Deboosere, N., A. Pinon, Y. Caudrelier, A. Delobel, G. Merle, S. Perelle, S. Temmam, J. Loutreul, T. Morin, M. Estienney, G. Belliot, P. Potheir, C. Gantzer, and M. Vialette. 2012. Adhesion of human pathogenic enteric viruses and surrogate viruses to inert and vegetal food surfaces. *Food Microbiol.* 32: 48-56.

29. Deng, M. Y. and D. O. Cliver, 1992. Inactivatioon of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manure. *Appl. Environ. Microbiol.* 58(6): 2016-2021.

30. Deng, M. Y. and D. O. Cliver. 1995a. Antiviral effects of bacteria isolated from manure. *Microbial. Ecol.* 30: 43-54.

31. Deng, M. Y. and D. O. Cliver. 1995b. Persistence of inoculated hepatitis A virus in mixed human and animal wastes. *Appl. Environ. Microbiol*. 61: 87-91.

32. Derevianko, S. 2008. Effect of Zero-Valent Iron on the Removal of *Escherichia coli* O157:H7 from Water. M.S. Thesis. University of Delaware, Newark, DE.

33. Diao, M. and M. Yao. 2009. Use of zero-valent nanoparticles in inactivating microbes. *Water Res.* 43:5243-5251.

34. D'Souza, D.H. 2014. Phytocompounds for the control of human enteric viruses. *Curr. Opin. Virol*. 4: 44-49.

35. D'Souza, D.H., A. Sair, K. Williams, E. Papafragkou, J. Jean, C. Moore, and L Jaykus. 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. *Int. J. Food Microbiol.* 108: 84-91.

36. Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. G. Koopmans, and M. K. Estes. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* 85: 79-87.

37. Dunkin, N., S. Weng., J. G. Jacangelo, and K. J. Schwab. 2017. Inactivation of human norovirus genogroups I and II and surrogates by free chlorine in postharvest leafy green wash water. *Appl. Environ. Microbiol.* 83:e01457-17.

38. El-Adawi, H., I. Nour, F. Fattouh, and N. El-Deeb. 2015. Investigation of the antiviral bioactivity of *Lactobacillus bulgaricus* 761N extracellular extract against hepatitis C virus (HCV). *Int. J. Pharmacol*. 11: 19-26.

39. Escudero, B.I., H. Rawsthorne, C. Gensel, and L. A. Jaykus. 2012. Persistence and transferability of noroviruses on and between common surfaces and foods. *J. Food Prot.* 75(5): 927-935.

40. Esseili, M. A., Q. Wang, and I. J. Saif. 2012. Binding of human GII.4 norovirus virus-like particles to carbohydrates of Romaine lettuce leaf cell wall materials. *Appl. Environ. Microbiol.* 78:786-794.

41. Ettayebi K., S. E. Crawford, K. Murakami, J. R. Broughman, U. Karandikar, V. R. Tenge, F. H. Neill, S. E. Blutt, X. L. Zeng, L. Qu, B. Kou, A. R Opekun, D. Burrin, D. Y. Graham, S. Ramani, R. L. Atmar, and M. K. Estes. 2016. Replication of human noroviruse in stem cell-derived human enteroids. *Science* 353: 1387-1393.

42. Farkas, T., R. W. Cross, E. Hargitt III, N. W. Lerche, A. L. Morrow, and K. Sestak. 2010. Genetic diversity and histo-blood group antigen interactions of Rhesus enteric caliciviruses. *J. Virol.* 84(17): 8617-8625.

43. Farkas, T., K. Sestak, C. Wei, and X. Jiang. 2008. Characterization of a Rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *J. Virol.* 82(11): 5408-5416.

44. Frenck, R., D. I. Bernstein, M. Xia, P. Huang, W. Zhong, S. Parker, M. Dickey, M. McNeal, and X. Jiang. 2012. Predicting susceptibility to norovirus GII.4 by use of a challenge model involving humans. *J. Infect. Dis.* 206: 1386-1393.

45. Fu, F., D. D. Dionysiou, and H. Liu. 2014. The use of zero-valent iron for groundwater remediation and wastewater treatment: A review. *J. Haz. Mat.* 267:194-205.

46. Fujioka, R. S., P. C. Loh, and L. S. Lau. 1980. Survival of human enteroviruses in the Hawaiian ocean environment: evidence for virus-inactivating microorganisms. *Appl. Environ. Microbiol*. 39: 1106-1110.

47. Gandhi, K. M., R. E. Mandrell, and P. Tian. 2010. Binding of virus-like particles of Norwalk virus to Romaine lettuce veins. *Appl. Environ. Microbiol.* 76:7997-8003.

48. Gilling, D.H., M. Kitajima, J. R. Torrey, and K. R. Bright. 2014. Mechanisms of antiviral action of plant antimicrobials against murine norovirus. *Appl. Environ. Microbiol.* 80(16): 4898-4910.

49. González-Rábade, N., J. A. Badillo-Corona, J. S. Aranda-Barradas, and M. d. C. Oliver-Salvador. 2011. Production of plant proteases *in vivo* and *in vitro* – A review. *Biotechnol. Adv*. 29: 983-996.

50. Gopinath, S., M. V. Kim, T. Rakib, P. W. Wong, M. van Zandt, N. A. Barry, T. Kaisho, A. L. Goodman, and A. Iwasaki. 2018. Topical application of aminoglycoside antibiotics enhances host resistance to viral infections in a microbiota-independent manner. *Nature Microbiol.* 3: 611-621.

51. Gosalia, D.N., C. M. Salisbury, J. A. Ellman, and S. L. Diamond. 2005. High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays. *Mol. Cell. Proteomics* 4:626-636.

52. Guix, S., M. Asanaka, K. Katayama, S. E. Crawford, F. H. Neill, R. L. Atmar, and M. K. Estes. 2007. Norwalk virus RNA is infectious in mammalian cells. *J. Virol.* 81(22): 12238-12248.

53. Ha, J-H., S. H. Kim, H-M. Lee, S-J. Kim, and H-W. Lee. 2018. Efficacy of combination treatment with sodium metasilicate and sodium hypochlorite for inactivation of norovirus on fresh vegetables. *Foodborne Path. Dis.* 15: 73-80.

54. Haga, K., A. Fujimoto, R. Takai-Todaka, M. Miki, Y. H. Doan, K. Murakami, M. Yokoyama, K. Murata, A. Nakanishi, and K. Katayama. 2016. Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells. *Proc. Natl. Acad. Sci*. E6248-E6255. www.pnas.org/cgi/doi/10.1073/pnas.1605575113

55. Hall, A. J., V. G. Eisenbart, A. Lehman Etingüe, L. H. Gould, B. A. Lopman, and U. D. Parashar. 2012. Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg. Infect. Dis.* 18: 1566-1573.

56. Hall, A. J., M. E. Wikswo, K. Pringle, L. H. Gould, and U. D. Parashar. 2014. Vital signs: Foodborne norovirus outbreaks – United States, 2009-2012. *MMWR* 63: 1-6.

57. Helting, T.B. and H.-H. Nau. 1984. Analysis of the immune response to papain digestion products of tetanus toxin. *Acta Path. Microbiol. Immunol. Scand.* Sect. C 92: 59-63.

58. Helting, T. and O. Zwisler. 1974. Enzymatic breakdown of tetanus toxin. *Biochem. Biophys. Res. Comm.* 57(4): 1263-1270.

59. Helting, T.B. and O. Zwisler. 1977. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J. Biol. Chem*. 252(1): 187-193.

60. Hirneisen, K.A. and K. E. Kniel. 2013. Comparing human norovirus surrogates: murine norovirus and Tulane virus. *J. Food Prot.* 76(1): 139-143.

61. Homaei, A.A., R. H. Sajedi, R. Sariri, S. Seyfzadeh, and R. Stevanato. 2010. Cysteine enhances activity and stability of immobilized papain. *Amino Acids* 38: 937-942.

62. Hoskins, L.C., M. Agustines, W. B. McKee, E. T. Boulding, M. Kriaris, and G. Niedermeyer. 1985. Mucin degradation in human colon ecosystems, Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J. Clin. Invest.* 75: 944-953.

63. Hotta, S., M. Kojima, M. Fujisaki, S. Uchida, H. Kuroda, and C. Hamada, 1977. An antiviral substance extracted from *Streptococcus faecalis*. *Nature* 268: 733-734.

64. Ingram, D.T., M. T. Callahan, S. Ferguson, D. G. Hoover, P. C. Chiu, D. R. Shelton, P. D. Millner, M. J. Camp, J. R. Patel, K. E. Kniel, and M. Sharma. 2012. Use of zero-valent iron biosand filters to reduce *Escherichia coli* O157:H12 in irrigation water applied to spinach plants in a field setting. *J. Appl. Microbiol.* 112:551-560.

65. Interstate Technology and Regulatory Council PRB: Technology Update Team. 2011. Permeable reactive barrier: Technological Update. Interstate Technology and Regulatory Council, Washington, DC, USA.

66. Ivec, M., T. Botić, S. Koren, M. Jakobsen, H. Weingartl, and A. Cencić. 2007. Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. *Antiviral Res.* 75: 266-274.

67. Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* 66: 6527-6532.

68. Jones, M.K., M. Watanabe, S. Zhu, C. L. Graves, L. R. Keyes, K. R. Grau, M. B. Gonzalez-Hernandes, M. N. Iovine, C. E. Wobus, J. Vinjé, S. A. Tibbetts, S. M. Wallet, and S. M. Karst. 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science*: 346: 755-759.

69. Kandasamy, S. A. N. Vlasova, D. Fischer, A. Kumar, K. S. Chattha, A. Rauf, L. Shao, S. N. Langel, G. Rajashekara, and L. J. Saif. 2016.

Differential effects of *Escherichia coli* Nissle and *Lactobacillus rhamnosus* Strain GG on human rotavirus binding, infection, and B cell immunity. *J. Immunol*. 196: 1780-1789.

70. Kang, J. Y., D. K. Lee, N. J. Ha, and H. S. Shin. 2015. Antiviral effects of *Lactobacillus ruminis* SPM0211 and *Bifidobacterium longum* SPM1205 and SPM1206 on rotavirus-infected Caco-2 cells and a neonatal mouse model. *J. Microbiol.* 53: 796-803.

71. Karst, S.M. 2010. Pathogenesis of noroviruses, emerging RNA viruses. *Viruses* 2:748-781.

72. Kawahara, T., Y. Makizaki, Y. Oikawa, Y. Tanaka, A. Maeda, M. Shimakawa, S. Komoto, K. Moriguchi, H. Ohno, and K. Taniguchi. 2017. Oral administration of *Bifidobacterium bifidum* G9-1 alleviates rotavirus gastroenteritis through regulation of intestinal homeostasis by inducing mucosal protective factors. *PLoS ONE* 12(3): e0173979. https://doi.org/10.1371/journal.pone.0173979

73. Khani, S. M. Motamedifar, H. Golmoghaddam, H. M. Hosseini, and Z. Hashemizadeh. 2012. *In vitro* study of the effect of a probiotic bacterium *Lactobacillus rhamnosus* against herpes simplex virus type 1. *Braz. J. Inf. Dis.* 16: 129-135.

74. Kim, M. J., D. K. Lee, J. E. Park, I. H. Park, J. G. Seo, and N. J. Ha. 2014. Antiviral activity of *Bifidobacterium adolescentis* SPM1605 against Coxsackievirus B3. *Biotechnol. Biotechnol. Equip.* 28: 681-688.

75. Kim, J. Y., H. Park, C. Lee, K. L. Nelson, D. L. Sedlak, and J. Yoon. 2010. Inactivation of *Escherichia coli* by nanoparticulate zerovalent iron and ferrous ion. *Appl. Environ. Microbiol.* 76:7668-7670.

76. Kim, J. Y., C. Lee, D. C. Love, D. L. Sedlak, J. Yoon, and K. L. Nelson. 2011. Inactivation of MS2 coliphage by ferrous ion and zero-valent iron nanoparticles. *Environ. Sci. Technol.* 45: 6978-6984.

77. Kim, Y. W., H. J. You, S. Lee, B. Kim, D. K. Kim, J.-B. Choi, J.-A. Kim, H. J. Lee, I. S. Joo, J. S. Lee, D. H. Kang, G. Lee, G. P. Ko, and S.-J. Lee. 2017. Inactivation of norovirus by lemongrass essential oil using a norovirus surrogate system. *J. Food Prot*. 80: 1293-1302.

78. Kingsley, D. H., J. P. Fay, K. Calci, R. Pouillot, J. Woods, H. Chen, B. A. Niemira, and J. M. Van Doren. 2017. Evaluation of chlorine treatment levels for inactivation of human norovirus and MS2 bacteriophage during

sewage treatment. *Appl. Environ. Microbiol*. 83:e01270-17, https://doi.org/10.1128/AEM01270-17

79. Kingsley, D. H., R. E. Pérez-Pérez, B. A. Niemira, and X. Fan. 2018. Evaluation of gaseous chlorine dioxide for the inactivation of Tulane virus on blueberries. *Int. J. Food Microbiol*. 273: 28-32.

80. Kingsley, D. H., E. M. Vincent, G. K. Meade, C. L. Watson, and X. Fan. 2014. Inactivation of human norovirus using chemical sanitizers. *Int. J. Food Microbiol.* 171:94-99.

81. Kirby, A. E., A. Streby, and C. L. Moe. 2016. Vomiting as a symptom and transmission risk in norovirus illness: Evidence from human challenge studies. *PloS ONE* 11(4): e0143759.doi:10.1371/journal.pone.0143759.

82. Kitajima, M., Y. Tohya, K. Matsubara, E. Haramoto, E. Utagawa, and H. Katayama. 2010. Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. *Lett. Appl. Microbiol.* 51:119-121.

83. Kniel, K. E. 2014. The makings of a good human norovirus surrogate. *Curr. Opin. Virol.* 4: 85-90.

84. Kotwal, G. and J. L. Cannon. 2014. Environmental persistence and transfer of enteric viruses. *Curr. Opin. Virol.* 4: 37-43.

85. Lee, C., J. Y. Kim, W. I. Lee, K. L. Nelson, J. Yoon, and D. L. Sedlak. 2008a. Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. *Environ. Sci. Technol.* 42:4927-4933.

86. Lee, J., K. Zoh, and G. Ko. 2008b. Inactivation and UV disinfection of murine norovirus with TiO2 under various environmental conditions. *Appl. Environ. Microbiol.* 74(7): 2111-2117.

87. Lee, M. H., S.-H. Yoo, S.-D. Ha, and C. Choi. 2012. Inactivation of feline calicivirus and murine norovirus during *Dongchimi* fermentation. *Food Microbiol.* 31: 210-214.

88. Lehtola, M. J., I. T. Miettinen, M. M. Keinänen, T. K. Kekki, O. Laine, A. Hirvonen, T. Vartiainen, and P. J. Martikainen. 2004. Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. *Water Res.* 38: 3769-3779.

89. Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkänen, L. Maunula, C-H. von Bonsdorff, P. J. Martikainen, S. A Wilks, C. W. Keevil, and I. T. Miettinen. 2007. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and Caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl. Environ. Microbiol.* 73(9): 2854-2859.

90. Lei, S., H. Samuel, E.Twitchell, T. Bui, A. Ramesh, K. Wen, M. Weiss, G. Li, S. Yang, and L. Yuan. 2016. *Enterobacter cloacae* inhibits human norovirus infectivity in gnotobiotic pigs. *Sci. Rep* 6: 25017; doi.10.1038/srep25017.

91. Leon, J.S., D. H. Kingsley, J. S. Montes, G. P. Richards, G. M. Lyon, G. M. Abdulhafid, S. R. Seitz, M. L. Fernandez, P. F. Teunis, G. J. Flick, and C. L. Moe. 2011. Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing. *Appl. Environ. Microbiol.* 77(15): 5476-5482.

92. Li, D., L. Baert, and M. Uyttendaele. 2013. Inactivation of food-borne viruses using natural biochemical substances. *Food Microbiol.* 35: 1-9.

93. Li, D., L. Baert, M. Xia, W. Zhong, X. Jiang, and M. Uyttendaele. 2012. Effects of a variety of food extracts and juices on the specific binding ability of norovirus GII.4 P particles. *J. Food Prot.* 75(7): 1350-1354.

94. Li, D., A. De Keuckelaere, and M. Uyttendaele. 2015. Fate of foodborne viruses in the “farm to fork” chain of fresh produce. *Compr. Rev. Food Sci. Food Saf.* 14:755-770.

95. Lindow, S.E. and M. T. Brandl. 2003. Microbiology of the Phyllosphere. *Appl. Env. Microbiol.* 69(4): 1875-1883.

96. Liu, Y., P. Huang, M. Tan, Y. Liu, J. Biesiada, J. Meller, A. A. Castello, B. Jiang, and X. Jiang. 2012. Rotavirus VP8*: Phylogeny, host range, and interaction with histo-blood group antigens. *J. Virol.* 86: 9899-9910.

97. Loncke, D. 2016. Viral-bacterial interactions: The effect of histo-blood group antigen (HBGA)-expressing bacteria and probiotics on Noroviruses. Master’s Thesis, Ghent University, Belgium.

98. López-Gálvez, F., P. Truchado, G. Sánchez, R. Aznar, M. I. Gil, and A. Allende. 2016. Occurrence of enteric viruses in reclaimed and surface irrigation water: relationship with microbiological and physiochemical indicators. *J. Appl. Microbiol.* 121: 1180-1188.

99. López-García, B., M. Hernández, and B. S. Segundo. 2012. Bromelain, a cysteine protease from pineapple (*Ananas comosus*) stem, is an inhibitor of fungal plant pathogens. *Lett. Appl. Microbiol.* 55: 62-67.

100. Ma, Y. and J. Li. 2011. Vesicular stomatitis virus as a vector to deliver virus-like particles of human norovirus: a new vaccine candidate against an important noncultivable virus. *J. Virol.* 85: 2942-2952.

101. Mansur, F., W. Luoga, D. J. Buttle, I. R. Duce, A. Lowe, and J. M. Behnke. 2014. The anthelmintic efficacy of natural plant cysteine proteinases against two rodent cestodes *Hymenolepis diminuta* and *Hymenolepis microstoma in vitro. Vet. Parasitol.* 201: 48-58.

102. Maragkoudakis, P. A., W. Chingwaru, L. Gradisnik, E. Tsakalidou, and A. Cencic. 2010. Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection. *Int. J. Food Microbiol.* 141: S91-S97.

103. Martin, V., A. Maldonado, L. Fernández, J. M. Rodríguez, and R. I. Connor. 2010. Inhibition of human immunodeficiency virus type 1 by lactic acid bacteria from human breastmilk. Breastfeeding Medicine 5: 153-158.

104. Miura, T., D. Sano, A. Suenaga, T. Yoshimura, M. Fuzawa, T. Nakagomi, O. Nakagomi, and S. Okabe. 2013. Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses. *J. Virol.* 87(17): 9441-9451.

105. Montazeri, N., D. Goettert, E. C. Achberger, C. N. Johnson, W. Prinyawiwatkul, and M. E. Janes. 2015. Pathogenic enteric viruses and microbial indicators during secondary treatment of municipal wastewater. *Appl. Environ. Microbiol.* 81:6436-6445.

106. MP Biomedicals. Bromelain (catalogue number 101142). Available at: http://www4.mpbio.com/ecom/docs/proddata.nsf/440121766f8ee75e8525645d0068b043/38fbf8044ecbac558525668e005162bf?OpenDocument&Highlight=0,101142. Accessed 12 September 2012.

107. MP Biomedicals. Ficin (catalogue number 198761). Available at: http://www.mpbio.com/product_info.php?family_key=02198761&lot_number=&submit_lot=Find+By+Lot+Number#cofatab. Accessed 3 June 2013.

108. Muñoz, J.A.M, E. Chenoll, B. Casinos, E. Bataller, D. Ramón, S. Genovés, R. Montava, J.M. Ribes, J. Buesa, J. Fábrega, and M. Rivero.

2011. Novel probiotic *Bifidobacterium longum* subsp. Infantis CECT 7210 strain active against rotavirus infections. *Appl. Environ. Microbiol.* 77(24): 8775-8783.

109. Murakami, K., C. Kurihara, T. Oka, T. Shimoike, Y. Fujii, R. Takai-Todaka, Y. Park, T. Wakita, T. Matsuda, R. Hokari, S. Miura, and K. Katayama. 2013. Norovirus binding to intestinal epithelial cells is independent of histo-blood group antigens. *PLoS One* 8(6): e66534.

110. Nowak, P., J. R. Topping, K. Bellamy, V. Fotheringham, J. J. Gray, J. P. Golding, G. Wiseman, and A. I. Knight. 2011. Virolysis of feline calicivirus and human GII.4 norovirus following chlorine exposure under standardized light soil disinfection conditions. *J. Food Prot.* 74: 2113-2118.

111. Okhuysen, P. C., X. Jiang, L. Ye, L., P. C. Johnson, and M. K. Estes. 1995. Viral shedding and fecal IgA response after Norwalk virus infection. *J. Infect. Dis.* 171: 566-569.

112. Orchard, R. C., C. B. Wilen, J. G. Doench, M. T. Baldridge, B. T. McCune, Y.-C. J. Lee, S. Lee, S.M. Pruett-Miller, C. A. Nelson, D. H. Fremont, and H. W. Virgin. 2016. Discovery of a proteinaceous cellular receptor for a norovirus. *Science*. 353: 933-936.

113. Ottoson, J., A. Hansen, B. Björlenius, H. Norder, and T. A. Stenström. 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. *Water Res.* 40:1449-1457.

114. Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown, P.O. 2007. Development of the human infant intestinal microbiota. *PLoS Biol.* 5, 1556-1572.

115. Pant, N., H. Marcotte, H. Brüssow, L. Svensson, and L. Hammarström. 2007. Effective prophylaxis against rotavirus diarrhea using a combination of *Lactobacillus rhamnosus* GG and antibodies. *BMC Microbiol*. 7: 86. doi:10.1186/1471-2180-7-86.

116. Papafragkou, E., J. Hewitt, G. W. Park, G. Greening, and J. Vinjé. 2013. Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models. *PLOS ONE* 8(6): e63485. doi:10.1371/journal.pone.0063485.

117. Predmore, A. and J. Li. 2011. Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers. *Appl. Environ. Microbiol.* 77(14): 4829-4838.

118. Qin, J., R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.-M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Doré, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, MetaHIT Consortium, P. Bork, S. D. Ehrlich, and J. Wang. 2010. A human gut microbial gene catalogue established by metagenomics sequencing. *Nature* 464: 59-67.

119. Quignon, F., M. Sardin, L. Kiene, and L. Schwartzbrod. 1997. Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Appl. Environ. Microbiol.* 63(3): 978-982.

120. Randazzo, W., I. Falcó, R. Aznar, and G. Sánchez. 2017. Effect of green tea extract on enteric viruses and its application as natural sanitizer. *Food Microbiol*. 66: 150-156.

121. Robinson, C. M., P. R. Jesudhasan, and J. K. Pfeiffer. 2014. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe* 15: 36-46.

122. Rubio-del-Campo, A., J. M. Coll-Marqués, M. J. Yebra, J. Buesa, G. Pérez-Martinez, V. Monedero, J. Rodríguez-Díaz. 2014. Noroviral P-particles as an in vitro model to assess the interactions of noroviruses with probiotics. *PLoS ONE* 9(2): e89586. Doi::10.1371/journal.pone.0089586

123. Salminen, S., S. Nybom, J. Meriluoto, M. C. Collado, S. Vesterlund, and H. El-Nezami. 2010. Interactions of probiotics and pathogens – benefits to human health? *Curr. Opin. Biotechnol.* 21: 157-167.

124. Sano, D., K. Wada, T. Imai, Y. Masago, and T. Omura. 2010. Norovirus-binding proteins recovered from activated sludge micro-organisms with an affinity to a noroviral capsid peptide. *J. Appl. Microbiol.* 109: 1923-1928.

125. Sarvestani, S. T., B. Cotton, S. Fritzlar, T. B. O'Donnell, and J. M. Mackenzie. 2016. Norovirus infection: replication, manipulation of host, and interaction with the host immune response. *J. Interferon Cytokine Res*. 36: 215-225.

126. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A.Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States – major pathogens. *Emerg. Infect. Dis*. 17: 7-15.

127. Seitz, S.R., J. S. Leon, K. J. Schwab, G. M. Lyon, M. Dowd, M. McDaniels, G. Abdulhafid, M. L. Fernandez, L. C. Lindesmith, R. S. Baris, and C. L. Moe, C.L. 2011. Norovirus infectivity in humans and persistence in water. *Appl. Environ. Microbiol.* 77(19): 6884-6888.

128. Seo, B.J., I. A. Rather, V. J. R. Kumar, U. H. Choi, M. R. Moon, J. H. Lim, and Y. H. Park. 2012. Evaluation of *Leuconostoc mesenteroides* YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. *J. Appl. Microbiol.* 113: 163-171.

129. Seo, D. J., M. H. Lee, J. Seo, S.-D. Ha, and C. Choi. 2014. Inactivation of murine norovirus and feline calicivirus during oyster fermentation. *Food Microbiol.* 44: 81-86.

130. Serkedjieva, J., S. Danova, and I. Ivanova. 2000. Antiinfluenza virus activity of a bacteriocin produced by *Lactobacillus delbrueckii*. *Appl. Biochem. Biotechnol.* 88: 285-298.21.

131. Shi, C., J. Wei, Y. Jan, K. E. Kniel, and P. C. Chiu. 2012. Removal of viruses and bacteriophages from drinking water using zero-valent iron. *Sep. Purif. Technol.* 84:72-78.

132. Siebenga, J. J., H. Vennema, D. P. Zheng, J. Vinjé, B. E. Lee, X. L. Pang, E. C. Ho, W. Lim, A. Choudekar, S. Broor, T. Halperin, N. B. Rasool, J. Hewitt, G. E. Greening, M. Jin, Z. J. Duan, Y. Lucero, M. O'Ryan, M. Hoehne, E. Schreier, R. M. Ratcliff, P. A. White, N. Iritani, G. Reuter,G., and M. Koopmans. 2009. Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007. *J. Infect. Dis.* 200: 802-812.

133. Singh, B.K., M. M. Leuthold, and G. S. Hansman. 2015. Human noroviruses' fondness for histo-blood group antigens. *J. Virol.* 89(4): 2024-2040.

134. Skraber, S., J. Schijven, C. Gantzer, and A. M. de Roda Husman. 2005. Pathogenic viruses in drinking-water biofilms: a public health risk? *Biofilms* 2: 105-117.

135. Souza, M., M. S. P. Azevedo, K. Jung, S. Cheetham, and L. J. Saif. 2008. Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus. *J. Virol.* 82(4): 1777-1786.

136. Souza, M., S. M. Cheetham, M. S. P. Azevedo, V. Costantini, and L. J. Saif. 2007. Cytokine and antibody responses in gnotobiotic pigs after infection with human norovirus genogroup II.4 (HS66 strain). *J. Virol.* 81(17): 9183-9192.

137. Stepek, G., D. J. Buttle, I. R. Duce, A. Lowe, and J. M. Behnke. 2005. Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus, in vitro*. *Parasitol*. 130: 203-211.

138. Straub, T. M., R. A. Bartholomew, C. O. Valdez, N. B. Valentine, A. Dohnalkova, R. M. Ozanich, C. J. Bruckner-Lea, and D. R. Call. 2011. Human norovirus infection of Caco-2 cells grown as a 3-dimensional tissue structure. *J. Water Health* 9: 225-240.

139. Straub, T. M., K. Höner zu Bentrup, P. Orosz-Coghlan, A. Dohnalkova, B. K. Mayer, R. A. Bartholomew, C. O. Valdez, C. J. Bruckner-Lea, C. P. Gerba, M. Abbaszadegan, and C. A. Nickerson. 2007. In vitro cell culture infectivity assay for human noroviruses. *Emerg. Infect. Dis.* 13: 396-403.

140. Su, X., A. B. Howell, and D. H. D'Souza. 2010a. The effect of cranberry juice and cranberry proanthocyanidins on the infectivity of human enteric viral surrogates. *Food Microbiol.* 27: 535-540.

141. Su, X., M. Y. Sangster, and D. H. D'Souza. 2010b. *In vitro* effects of pomegranate juice and pomegranate polyphenols on foodborne viral surrogates. *Foodborne Path. Dis.* 7(12): 1473-1479.

142. Takahashi, H., M. Nakazawa, C. Ohshima, M. Sato, T. Tsuchiya, A. Takeuchi, M. Kunou, T. Kuda, and B. Kimura. 2015. Heat-denatured lysozyme inactivates murine norovirus as a surrogate human norovirus. *Sci. Rep*. 5: 11819.

143. Takahashi, M., Y. Okakura, H. Takahashi, M. Imamura, A. Takeuchi, H. Shidara, T. Kuda, and B. Kimura. 2018. Heat-denatured lysozyme could be a novel disinfectant for reducing hepatitis A virus and murine norovirus on berry fruit. *Int. J. Food Microbiol.* 266: 104-108.

144. Tan, M., and X. Jiang, X. 2005. The P domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors. *J. Virol.* 79(22): 14017-14030.

145. Tan, M., M. Xia, Y. Chen, W. Bu, R. S. Hedge, J. Meller, X. Li, and X. Jiang. 2009. Conservation of carbohydrate binding interfaces – evidence of human HBGA selection in norovirus evolution. *PLoS ONE* 4(4): e5058.

146. Taube, S., A. O. Kolawole, M. Höhne, J. E. Wilkinson, S. A. Handley, J. W. Perry, L. B. Thackray, R. Akkina, and C. E. Wobus. 2013. A mouse model for human norovirus. *mBio* 4(4):e00450-13.doi:10.1128/mBio.00450-13.

147. Taube, S., J. W. Perry, E. McGreevy, K. Yetming, C. Perkins, K. Henderson, and C. E. Wobus. 2012. Murine noroviruses bind glycolipid and glycoprotein attachment receptors in a strain-dependent manner. *J. Virol.* 86: 5584-5593.

148. Taube, S., J. W. Perry, K. Yetming, S. P. Patel, H. Auble, L. Shu, H. F. Nawar, C. H. Lee, T. D. Connell, J. A. Shayman, and C. E. Wobus. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. *J. Virol.* 83: 4092-4101.

149. Teunis, P. F. M., F. H. A. Sukhrie, H. Vennema, J. Bogerman, M. F. C. Beersma, and M. P. G. Koopmans. 2015. Shedding of norovirus in symptomatic and asymptomatic infections. *Epidemiol. Infect.* 143: 1710-1717.

150. Thorne, L. G. and I. G. Goodfellow. 2014. Norovirus gene expression and replication. *J. Gen. Virol.* 95: 278-291.

151. Tian, P., A. L. Engelbrektson, X. Jiang, W. Zhong, and R. E. Mandrell. 2007. Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. *J. Food Prot.* 70(9): 2140-2147.

152. Todorov, S.D., M.B. Wachsman, H. Knoetze, M. Meincken, and L.M.T. Dicks. 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. *Int. J. Antimicrob. Agents* 25: 508-513.

153. Todorov, S.D., M. Wachsman, E. Tomé, X. Dousset, M.T. Destro, L.M.T. Dicks, B.D. Gombossy de Melo Franco, M. Vaz-Velho, and D. Drider.

2010. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. *Food Microbiol.* 27: 869-879.

154. Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R Beumer, and E. Duizer. 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl. Environ. Microbiol.* 78(21): 7769-7775.

155. Tung, G., D. Macinga, J. Arbogast, and L.-A. Jaykus. 2013. Efficacy of commonly used disinfectants for inactivation of human noroviruses and their surrogates. *J. Food Prot*. 76: 1210-1217.

156. Uchida, H., Y. Kawai, H. Kinoshita, H. Kitazawa, K. Miura, K. Shiba, A. Horii, K. Kimura, N. Taketomo, M. Oda, T. Yajima, and T. Saito. 2006a. Lactic acid bacteria (LAB) bind to human B- or H-antigens expressed on intestinal mucosa. *Biosci. Biotechnol. Biochem*. 70: 3073-3076.

157. Uchida, H., H. Kinoshita, Y. Kawai, H. Kitazawa, K. Miura, K. Shiiba, A. Horii, K. Kimura, N. Taketomo, M. Oda, T. Yajima, and T. Saito. 2006b. Lactobacilli binding human A-antigen expressed in intestinal mucosa. *Res. Microbiol.* 157: 659-665.

158. Uchiyama, R., B. Chassaing, B. Zhang, and A. T. Gewirtz. 2014. Antibiotic treatment suppresses rotavirus infection and enhances specific humoral immunity. *J. Inf. Dis.* 210: 171-182.

159. Varyukhina, S., M. Freitas, S. Bardin, E. Robillard, E. Tavan, C. Sapin, J.-P. Grill, and G. Trugnan. 2012. Glycan-modifying bacteria-derived soluble factors from *Bacteroides thetaiotaomicron* and *Lactobacillus casei* inhibit rotavirus infection in human intestinal cells. *Microbes Infect*. 14: 273-278.

160. Vashist, S., D. Bailey, A. Putics, and I. Goodfellow. 2009. Model systems for the study of human norovirus biology. *Future Virol*. 4: 353-367.

161. Verhaelen, K., M. Bouwknegt, F. Lodder-Verschoor, S. A. Rutjes, and A. M. de Roda Husman. 2012. Persistence of human norovirus GII.4 and GI.4, murine norovirus, and human adenovirus on soft berries as compared with PBS at commonly applied storage conditions. *International J. Food Microbiol.* 160: 137-144.

162. Verhaelen, K., M. Bouwknegt, S. A. Rutjes, and A. M. de Roda Husman. 2013. Persistence of human norovirus in reconstituted pesticides – Pesticide application as a possible source of viruses in fresh produce chains. *International J. Food Microbiol.* 160: 323-328.

163. Vinjé, J. 2015. Advances in laboratory methods for detection and typing of norovirus. *J. Clin. Microbiol.* 53: 373-381.

164. Wachsman, M.B., M.E. Farías, E. Takeda, F. Sesma, A.P. de Ruiz Holgado, R.A. de Torres, and C.E. Coto. 1999. Antiviral activity of enterocin CRL35 against herpesviruses. *Int. J. Antimicrob. Agents* 12: 293-299.

165. Wachsman, M.B., V. Castilla, A.P. de Ruiz Holgado, R.A. de Torres, F. Sesma, and C.E. Coto. 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. *Antiviral. Res.* 58: 17-24.

166. Waklin, P., H. Mäkivuokko, N. Alakulppi, J. Nikkilä, H. Tenkanen, J. Räbinä, J. Partanen, K. Aranko, and J. Mättö. 2011. Secretor genotype (*FUT2* gene) is strongly associated with the composition of Bifidobacteria in the human intestine. *PLoS One* 6(5): e20113. doi:10.1371/journal.pone.0020113

167. Wang, W., A. L. Suguitan, Jr., J. Zengel, Z. Chen, and H. Jin. 2012. Generation of recombinant pandemic H1N1 influenza virus with the HA cleavable by bromelain and identification of the residues influencing HA bromelain cleavage. *Vaccine* 30: 872-878.

168. Ward, R. L. 1982. Evidence that microorganisms cause inactivation of viruses in activated sludge. *Appl. Environ. Microbiol*. 43: 1221-1224.

169. Ward, R. L., D. R. Knowlton, and P. E. Winston. 1986. Mechanism of inactivation of enteric viruses in fresh water. *Appl. Environ. Microbiol*. 52: 450-459.

170. Whitaker, J.R. and L.-S. Lee. 1972. Ficin- and papain-catalyzed reactions. Effect of temperature on reactivity of the essential sulfhydryl group of ficin in the presence and absence of competitive inhibitors. *Arch. Biochem. Biophys.* 148: 208-216.

171. Wiken Dees, M., E. Lysøe, B. Nordskog, and M. Bente Bruerber. 2015. Bacterial communities associated with surfaces of leafy greens: Shift in composition and decrease in richness over time. *Appl. Env. Microbiol.* 81(4): 1530-1539.

172. Wilen, C. B., S. Lee, L. L. Hsieh, R. C. Orchard, C. Desai, B. L. Hykes, Jr., M. R. McAllaster, D. R. Balce, T. Feehley, J. R. Brestoff, C. A. Hickey, C. C. Yokoyama, Y.-T. Wang, D. A. MacDuff, D. Kreamalmayer, M. R.

Howitt, J. A. Neil, K. Cadwell, P. M. Allen, S. A. Handley, M. van Lookeren Campagne, M. T. Baldridge, and H. W. Virgin. 2018. Tropism for tuft cells determines immune promotion of norovirus pathogenesis. *Science*. 360: 204-208.

173. Xia, M., T. Farkas, and X. Jiang. 2007. Norovirus capsid protein expressed in yeast forms virus-like particles and stimulates systemic and mucosal immunity in mice following an oral administration of raw yeast extracts. *J. Med. Virol.* 79: 74-83.

174. Xue, Y., C.-Y. Wu, C. J. Branford-White, X. Ning, H.-L Nie, and L.-M. Zhu. 2010. Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity. *J. Mol. Catal. B: Enzym.* 63: 188–193.

175. You, Y., J. Han, P. C. Chiu, and Y. Jin. 2005. Removal and inactivation of waterborne viruses using zerovalent iron. *Environ. Sci. Technol.* 39:9263-9269.

176. Zhang, X., N. A. Buehner, A. M. Hutson, M. K. Estes, and H. S. Mason. 2006. Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. *Plant Biotechnol. J.* 4: 419-432.

177. Zhang, D., P. Huang, L. Zou, T. L. Lowary, M. Tan, and X. Jiang. 2015. Tulane virus recognizes the A type 3 and B histo-blood group antigens. *J. Virol.* 89: 1419-1427.

Chapter 2

ENHANCED REMOVAL OF NOROVIRUS SURROGATES, MURINE NOROVIRUS AND TULANE VIRUS, FROM AQUEOUS SYSTEMS BY ZERO-VALENT IRON

2.1 Abstract

Viral contamination can compromise the safety of water utilized for direct consumption, produce irrigation, and post-harvest washing of produce. Zero-valent iron (ZVI) is used commercially for chemical remediation of water and has been demonstrated to remove some biological contaminants from water in laboratory and field studies. This study investigated the efficacy of ZVI to remove human norovirus surrogates, Tulane virus (TV) and murine norovirus (MNV), from water and to characterize the reversibility and nature of viral association with ZVI. Genomic material of TV and MNV recovered from the effluent of inoculated water treatment columns containing a 1:1 mixture of ZVI and sand was, respectively, 2 and 3 logs less than that recovered from the effluent of treatment columns containing only sand. Elution buffers (citrate buffers pH 4 and 7 and virus elution buffer (VEB) pH 9.5 with and without added 1 M NaCl) did not increase recovery of infectious TV and MNV from ZVI as compared to elution with water alone. TV-inoculated lettuce washed with water in the presence of ZVI

yielded 1.5 to 2 log fewer infectious TV from wash water as compared to lettuce washed with water alone or in the presence of sand. These data demonstrate the enhanced removal of human norovirus surrogates, TV and MNV, from water by ZVI and provide indications that unrecovered viruses are not readily disassociated from ZVI by buffers of various pH and ionic strength. These findings warrant further investigation into larger-scale simulations of ZVI-mediated water remediation of viral contaminants for potential application in the treatment of water used for drinking, irrigation and food processing.

2.2. Introduction

Human norovirus (HuNov) is the leading global cause of acute gastroenteritis *(17, 21)* and is transmitted via the fecal-oral route through direct contact with an infected individual, contact with contaminated surfaces, and consumption of contaminated food or water *(17)*. Norovirus transmission through drinking water and contamination of food crops by environmental water have been implicated in norovirus illness outbreaks *(2, 8, 15, 28)*, and contaminated water has the potential to cause widespread illness given its commonly broad distribution and application.

Human enteric viruses can contaminate ground and surface waters by the unintentional release of sewage from faulty containment systems, land application of municipal biosolids, release of inadequately treated water *(15, 34)*, and run-off from heavy precipitation events *(9)*. Several surveys of groundwater utility wells have revealed the presence of enteric virus genomic material *(38)*,

including that of HuNov. Studies indicate that norovirus is very stable in water, remaining infectious in ground water for at least 61 days and its genomic material detectable approximately three years in wastewater, groundwater, and drinking water *(22, 39)*.

Measures to adequately remove HuNov from water must account for viral load, stability, and low infectious dose. Individuals infected with norovirus can shed millions of virions in their feces *(31)*, potentially challenging wastewater treatment systems to remove elevated viral loads to prevent subsequent environmental contamination. Several studies have demonstrated high prevelance of HuNov in wastewater and incomplete removal of HuNov in even tertiary-treated effluent that has undergone settlement, activated-sludge treatment, sand and biological filters, and UV disinfection *(5, 29, 35)*. Illness outbreaks have been associated with contaminated groundwater wells due to inadequate removal of HuNov by sand filter treatment at onsite wastewater treatment systems *(22)*. Secondary-treated wastewater effluent *(24)* and stool filtrate *(25)* treated by chlorine at traditional levels and treatment times may not be adequate to inactivate HuNov as assessed by viral binding and genomic detection assays. Municipal treatment of environmental water for preparation of drinking water may include further sedimentation and filtration followed by disinfection to reduce pathogenic microorganisms not excluded by filtration *(3, 34)*. HuNov appears to be sensitive to sodium hypochlorite (0.5mg/L, 30 min exposure) in treated drinking water as determined by reduction of detectable viral

RNA *(26)*; however, byproducts of chlorinated disinfection may present human health hazards *(42)*. Investigation of additional technologies to remove or inactivate norovirus in wastewater, environmental water, and drinking water are warranted to minimize risk of illness outbreaks associated with contaminated water used for direct consumption or food contact.

Zero-valent iron (ZVI) is used to remediate groundwater of chemical contaminants *(42)* and may also have application for the removal of biological contaminants *(7, 13, 33)*. Granular ZVI is typically derived from scrap iron filings *(7)* and has been characterized as non-toxic, abundant, inexpensive, and easy to maintain *(13)*. ZVI retention or inactivation of *Escherichia coli* O157:H7, *E. coli* O157:H12 *(7, 19)*, *Pseudomonas fluorescens*, and vegetative cells of *Bacillus subtilis (10)* have been reported. Bactericidal effects of ZVI have been attributed to disruption of bacterial cell membranes and respiratory activity *(23, 27)*. Enhanced removal of bacteriophages, ϕX174 and MS-2 *(4, 41, 46)*, and human viruses, rotavirus *(4)*, Aichi virus (AiV) and adenovirus 41 (Ad41) *(41)* has been demonstrated for water filtered through sand with ZVI incorporated. The antiviral mechanism of ZVI has not been fully elucidated; however, ionic interactions between ZVI and virus have been postulated *(41)*.

The applicability of ZVI for treatment of water for direct consumption or food production necessitates the retention or inactivation of human norovirus. The objectives of this study were to evaluate the effect of ZVI incorporation in sand filtration columns on the removal of human norovirus surrogates, murine

norovirus (MNV) and Tulane virus (TV), from an aqueous system and to determine the reversibility and nature of viral retention in ZVI.

2.3 Materials and Methods

2.3.1 *Virus preparation.* MNV-1 (provided by Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was inoculated into cultures of RAW 264.7 (ATCC TIB-71) cells grown 24 h to confluency. Cells were incubated at 37ºC and 5% CO_2 for approximately 48 h in Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech, Inc., Manassas, VA) containing 4.5 g l^{-1} of glucose, L-glutamine and sodium pyruvate amended with fetal bovine serum (FBS, HyClone, Logan, UT) at 10% v/v, 4 mM glutamax (Invitrogen), sodium bicarbonate (Mediatech), and antibiotic/antimycotic (100 U penicillin, 100 μg ml^{-1} streptomycin, 0.25 μg ml-1 amphotericin B, HyClone) to propagate virus. MNV was recovered from cells by three freeze/thaw cycles (-80ºC/25 to 37ºC) and centrifugation (233 x *g*). The supernatant that contained the virus was aliquoted and stored at -80ºC until further use.

LLC-MK2 cells (ATCC CCL-7) were grown 24 h to confluency, inoculated with TV (provided by Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH), and incubated approximately 55 h at 37ºC with 5% CO_2 in Medium 199/EBSS (M199 with Earles Balanced Salts and L-glutamine, HyClone) amended with 10% FBS and antibiotic/antimycotic mix as previously described. TV was collected from LLC-MK2 cells as described for MNV and stored at -80ºC until use.

2.3.2. *Enumeration of viral RNA.* Viral RNA was extracted with the QIAamp® Viral RNA Extraction Kit (Qiagen, Hilden, Germany) for column studies according to manufacturer protocols. Reverse transcription was performed at 37°C for 60 min in an Eppendorf thermocycler (Hamburg, Germany) with the Sensiscript® RT Kit (Qiagen) according to manufacturer protocols. MNV *(18)* and TV *(42)* primers targeted the viral polymerase genes (MNV-F, 5'-CTTCGCAAGACACGCCAATTTCAG and MNV-R, 5'-GCATCACAATGTCAGGGTCAACTC, Sigma-Aldrich® Corp., St. Louis, MO, USA; TV-F, 5'-TCGCGCAGCGCACTTA and TV-R, 5'-CAAGAATCCAGAACAACCAATATCA). Amplification of cDNA was carried out with the QuantiTect SYBR Green PCR kit (Qiagen) using the same primers as for reverse transcription and two µl of template cDNA in a reaction volume of 20 µl per manufacturer protocol. Real time PCR was performed in a Rotor-Gene Q (Qiagen) with hot start at 95°C for 15 min, 40 cycles of 94°C/15 sec denaturation, 60°C/30 sec annealing, and 72°C/30 sec extension. Amplicons were confirmed by melt curves and/or target band size, 100 base pairs (bp) for TV and 318 base pairs for MNV, by electrophoresis in a 1% PCR grade, intermediate-melting agarose gel (Fisher Scientific, Fair Lawn, NJ, USA) in Tris-acetate-EDTA (TAE) buffer (Fisher Scientific) with 4% ethidium bromide. TV and MNV serially diluted in HBSS were used to generate standard curves with the lowest dilution of detection designated 1 RT-qPCR unit (RT-qPCRU) *(1, 43)*.

2.3.3. *Enumeration of infectious virus.* Infectious TV and MNV was quantified by plaque assay *(40, 45)*. LLC-MK2 or RAW cells were seeded in 6-well cell culture plates and grown to approximately 80% or greater confluency at 37°C and 5% CO_2 with media as previously described. Prior to infection, spent media was aspirated and cells were overlayed with 500 μl fresh media containing 10% FBS. Samples to be tested for virus were serially diluted in Hanks Balanced Salt Solution, 1x with calcium and magnesium (HBSS, Mediatech), and 500 μl was added to each well with replicate wells for each dilution. Suspensions were mixed well and infected into host cells for 3 h minimum at 37°C and 5% CO_2 in LLC-MK2 cells for TV and RAW cells for MNV. After infection, inoculants were aspirated and wells were rinsed once with HBSS. Cells were overlayed with 2 ml of a 1:1 mixture of 3% peqGOLD Universal agarose (PEQ Lab, Erlangen, Germany) and cell culture medium. For MNV plaque overlay, the cell culture medium combined with agarose was 2X MEM/EBSS (with L-glutamine, HyClone) containing 2% FBS and glutamax, sodium bicarbonate, and antibiotic/antimycotic as previously described herein for DMEM. For TV plaque overlay, the cell culture medium combined with agarose was M199/EBSS cell culture medium containing 2% FBS and antibiotics/antimycotics. After solidification, agarose was overlaid with medium containing 2% FBS, and incubated for approximately 48 h to allow plaque formation. The fluid medium was aspirated, and 10% formaldehyde in phosphate buffered saline (PBS, pH 7.4) was added to each well for a minimum of 2 h to fix cells. The fixative was aspirated and agarose removed from the

wells. Cells were overlayed with crystal violet (0.05% in 10% ethanol, Fisher Scientific, Kalamazoo, MI, USA) for a minimum of 30 min. Stain was aspirated, cells rinsed, and plaques counted visually.

2.3.4. *Column construction.* Control and test columns were constructed in pairs as previously described *(41, 46)*. A steel mesh (60 x 60.0075) disc was placed at the bottom of each acrylic column (3.8 cm i.d. by 10 cm) and overlaid with 1 cm layer of Accusand sand (Unimin, Le Sueur, MN, USA). The columns were wet-packed to the top with sterile deionized water and either sand alone, referred to hereafter as sand, for the control column or a 1:1 (w/w) mixture of sand and ZVI (particle size 0.005 to 0.125 in; Peerless Metal Powders and Abrasive Company (Detroit, MI, USA) for the test column, referred to hereafter as ZVI. Columns were sealed with tubing affixed at the center of the top and bottom.

2.3.5. *Column treatments.* TV and MNV were suspended individually in HBSS at 10^2 to 10^4 RT-qPCR units (10^4 to 10^5 PFU/ml), respectively. Columns were flushed with sterile, deionized water immediately prior to inoculation. Viral suspensions (100 ml) were loaded onto each of two columns, one sand column and one ZVI column. Viral suspensions were introduced to the top of the columns at a rate of 1 ml per min using a TRIS™ peristaltic pump (Teledyne ISCO, Inc. Lincoln, NE, USA). Columns were then flushed with sterile deionized water (300 ml per column). Eighty 5-ml fractions of eluate were collected in sterile glass tubes with a Foxy Jr. fraction collector (Teledyne ISCO, Inc.) and stored at 4°C less than 24 hours prior to testing every fifth fraction for the presence of viral

RNA (Viral RNA Extraction Kit, Qiagen). Two independent trials were conducted for each virus.

2.3.6. *Elution studies.* Inoculated sand and ZVI were treated with elution buffers to determine potential reversibility of association of infectious virus with ZVI. TV and MNV were treated in batch systems in 15-ml centrifuge tubes with 1 cm^3 sand or ZVI in sterile deionized water. The sand and ZVI were inoculated with 0.5 ml of TV (approximately 4 log PFU/ml) or MNV (approximately 5 log PFU/ml) in sterile deionized water. Tubes were gently agitated horizontally on a Nutator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 30 minutes at 21°C. Treated water was aspirated and reserved for plaque assay. To recover virus, either sterile deionized water or an elution buffer (0.5 ml) was added to the sand and ZVI. Tubes were agitated for 30 min at 21°C. The contents were then vortexed vigorously for 10 sec, and the fluid was aspirated and reserved for plaque assay. Buffers used to elute infectious virus included: 0.1 M citrate buffer, pH 4.0 and 7.0 (citric acid and sodium phosphate, dibasic, Fisher Scientific) and virus elution buffer (VEB, pH 9.5, consisting of 100 mM Tris (Fisher Scientific), 50 mM glycine (Fisher Scientific), 50 mM $MgCl_2$ (ICN Biomedicals Aurora, OH, USA), 3% beef extract (Fisher Scientific), all with and without added 1 M NaCl (Fisher Scientific). After vortexing, minute particles of sand and ZVI remained in the aspirate; therefore, control rinsates were prepared by vortexing sand and ZVI in sterile deionized water. Plaque assay detection of inoculated positive controls in the presence of each of the buffers and the rinsates of vortexed sand and ZVI

were verified as compared to detection of virus in HBSS and water. Negative controls included uninoculated buffers and rinsates of sand and ZVI to verify lack of cytotoxicity to host cells under the conditions for plaque assay. Two independent trials were conducted.

2.3.7. *Lettuce wash treatment.* Uncut Romaine lettuce was purchased from a local grocery store, and outer leaves were removed. Leaves were cut into 1.3 cm-diameter discs with a sterile cork borer, spot inoculated with 25 μl of TV (initial inoculum 4 $\log_{10}$ PFU/ml), and dried in a biosafety cabinet for approximately 3 h. Inoculated lettuce discs (three per treatment per trial) were transferred to 15-ml centrifuge tubes containing 1.5 ml sterile deionized water, 1.5 ml water and 1 cm^3 sand, or 1.5 ml water and 1 cm^3 ZVI. Control tubes of water alone or water with sand or ZVI were inoculated with 25 μl TV (initial inoculum 4 $\log_{10}$ PFU/ml) without lettuce. Uninoculated lettuce discs (one per trial per condition) were tested as negative controls. Tubes were placed horizontally on a rotator platform (Nutator Clay Adams Brand, TC Scientific) and mixed for 30 min at room temperature. Lettuce discs were then removed from tubes with sterile forceps and transferred to 1.5 ml of VEB and vortexed for 1 min. The VEB from the lettuce wash, the lettuce treatment water, water inoculated in the absence of lettuce, and water wash from uninoculated lettuce were evaluated for infectious TV by plaque assay. Two independent trials were conducted.

2.3.8. *Data analysis.* Statistical significance ($p<0.05$) of treatment effects for each virus was determined by analysis of variance and Student's *t*-test with statistical software JMP Pro, version 10.0.0 (SAS Institute Inc., Cary, NC).

2.4. Results and Discussion

The incorporation of ZVI in permeable reactive barriers (PRB) to remediate groundwater of chemical contaminants is an established practice in the United States *(20)*. Evidence for the utility of ZVI to remediate biological contaminants remains limited to laboratory-scale or small field investigations for a few bacterial species and virus types. To the authors' knowledge, this study provides the first evidence for ZVI removal of human norovirus surrogates from aqueous systems.

Previous studies have demonstrated ZVI removal of virus from treated wastewater effluent with approximately 5-log reductions of rotavirus realized in the presence of a total organic carbon load of 20 mg/ml *(4)*. Similar reductions of 4.5 to 6 logs of Aichi virus (AiV), Adenovirus 41 (Ad41), and bacteriophages MS2 and ϕX174 from water treatment plant samples were achieved within 20 minutes of passage through sand filters containing ZVI *(41)*.

The present study used the same column design, ZVI concentration, and slightly faster flow rate as reported by Shi and colleagues *(41)*. The recovery of MNV and TV in the eluate of buffered water filtered through simulated water-treatment columns are presented in break-through curves in Figures 1A and 1B, respectively. The initial fraction of collected eluate demonstrated that the columns had no detectable MNV or TV genomic material prior to inoculation of

the column. The first 20 fractions were collected after virus input; subsequent fractions were collected after flush with sterile water at three times the volume of input virus. Viral recovery in eluate of sand columns was at viral input levels, while the virus in eluate of ZVI columns was at or below detection. These represent approximately 3-log and greater than 2-log reductions of MNV and TV, respectively, by ZVI in column effluent relative to column influent. In conjunction with previous studies *(4, 41)*, these data provide additional evidence for the utility of ZVI to retain environmentally important enteric viruses of various size and structure, suggesting a potentially universal or broad method of interaction between ZVI and viruses.

The mechanism of ZVI inactivation of or association with virus has not been fully characterized. Water treatment columns in the present study and as reported by Shi *et al*. *(41)* have pores in the solid matrix greater in size than the viruses, indicating that size exclusion is not likely the primary mechanism of viral retention. This conclusion is further supported in the present study by the reduced recovery of MNV and TV in the presence of ZVI as compared to sand whether virus were exposed to ZVI in a packed column (Figure 1) or as loose particles in a batch test tube system (Figure 2). Rather, virus adsorption and inactivation has been attributed to multiple chemical species generated by ZVI in the presence of water, including iron oxides, intermediary oxidized iron and reactive oxygen species *(6, 41, 46)*, particularly under aerobic conditions *(6)*.

Recently, bacteriophage f2 was reported to adsorb to ZVI nanoparticles resulting in viral coat damage *(6)*.

In the present study, a batch treatment system in test tubes was utilized to study the potential reversibility of virus association with ZVI as well as to gain insight into the nature of virus and ZVI association. The batch system allowed for simultaneous comparison of different buffers to elute virus and to attempt to recover treated virus by both physical and chemical disruption by vortexing of the sand and ZVI in buffers. The elution buffers utilized in this study were selected based on the possibility that electrostatic interactions between the viral capsid and oxidized iron contribute to viral association with ZVI particles. Citrate buffer at pH values above (pH 7) and below (pH 4) the isoelectric points (http://web.expasy.org) of the major capsid proteins of TV (NCBI Accession Number ACB38132 (http://www.ncbi.nlm.nih/gov; computed pI 4.87) and MNV (NCBI Accession Number YP_720002.1; computed pI 4.78), was compared to water alone for the recovery of virus from sand and ZVI. The computed pI of 5.50 for the major VP1 capsid protein of human norovirus GII.4 (NCBI Accession Number AFL70023) and measured pI values (5.5 to 6.9) of human norovirus GI and GII virus-like particles *(16)* are in line with these surrogate viruses. Virus elution buffer (VEB) was selected for evaluation as it is a primary buffer used to separate virus from complex matrices *(32, 36)*. Elution buffers were also amended with NaCl as a further measure to disrupt potential electrostatic interactions between virus and iron as salt solutions have been reported to

desorb virus from anion-exchange filtration systems *(32)* and lettuce surfaces *(44)*. The batch studies of ZVI treatment of MNV- and TV-inoculated water yielded one-log reductions of infectious virus over those observed with sand alone. None of the subsequent washes of ZVI with eluents recovered virus to input levels (Figure 2). Slightly greater recovery of virus from ZVI was observed with VEB buffer (pH 9.5); however, the difference was not significantly greater ($p>0.05$) than that recovered by water elution. The addition of NaCl did not enhance recovery of virus. The comparable recovery of TV and MNV from the ZVI with buffer eluents as compared to water elution suggests that either the virus particles not recovered from the water treated by ZVI were inactivated, or viral association with ZVI is not readily reversed by buffers of various pH and ionic strength. These data are important as utility of ZVI for remediation depends on it not serving as a reservoir to concentrate viable virus with risk for later release into the environment.

In addition to the aforementioned elution buffers, the ZVI particles were also directly treated with a lysis buffer (Power Viral Environmental RNA/DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA; presently AllPrep PowerViral DNA/RNA kit (Qiagen, Hilden, Germany) containing reagents to protect released RNA in an effort to further assess the possibility that intact virus could be associated with the ZVI matrix. The results were inconclusive (data not shown) as an RNA control exposed to ZVI or sand could not be fully recovered suggesting that the presence of minute sand and ZVI particles in the water of batch treatment

systems adsorbed or degraded free RNA or interfered with downstream detection technologies .

Finally, the interaction of ZVI with virus inoculated onto lettuce was explored as leafy greens have been implicated as transmission vehicles in norovirus illness outbreaks, and direct association of virus-like particles of norovirus with leafy greens has been demonstrated *(14, 12)*. Similar to column and batch studies, recovery of TV from water containing ZVI was reduced by approximately 2 logs as compared to water alone or water with sand (Figure 3, black bars). When TV was inoculated onto lettuce discs and treated in water, the wash water with ZVI contained approximately 1.5 log fewer infectious TV than wash water alone or with sand (Figure 3, gray bars). Subsequent wash of inoculated lettuce with VEB (Figure 3, patterned bars) demonstrated approximately one-log lower recovery of TV from lettuce previously washed in water containing ZVI than that of lettuce previously treated with either water alone or in water with sand. While the aim of this study was not to simulate a commercial produce wash operation, the findings suggest that ZVI may have the potential to improve removal of virus from harvested produce possibly by decontaminating the wash water and thereby preventing reassociation of virus released from produce surfaces or by greater affinity of virus for ZVI than the produce surface. These data provide merit for investigating the use of ZVI to enhance the otherwise limited efficacy of water for removal of viral contaminants from produce *(11, 37)*, for remediating processing water that could contribute to cross-contamination *(11)*, and for reuse of

processing water in support of growing efforts to reclaim water discharged from food processing operations *(30)*.

In conclusion, this study demonstrated a greater removal of TV and MNV from aqueous systems by a mixture of ZVI and sand as compared to sand alone. These reductions were observed in both column and batch studies as detected by genomic and infectivity assays, respectively. Although we cannot conclude that the unrecovered virus were inactivated, this possibility is supported by the inability of elution buffers of various pH and ionic strengths to further release viruses from the matrices as would be expected if intact virus particles were bound to the matrices by electrostatic interactions. These data support findings from previous studies *(4, 41, 46)* that demonstrated higher levels of removal of virus from water by ZVI than by sand. The data also suggest the utility of further investigation into larger-scale simulations of water remediation of viral contaminants for potential application in the treatment of water used for drinking, irrigation and food processing. Further investigations are warranted on the efficacy of ZVI for remediation of water contaminated with human norovirus as compared to that containing its surrogates, even though they are similar in size, structure, pI, and binding properties. There also remains a critical need to evaluate removal of norovirus from water containing organic loads typical of reclaimed water, environmental waters, and food processing waters.

2.5. Acknowledgements

The authors gratefully acknowledge Rafael Castañeda Saldaña and Pauline Elizabeth Pantuliano for technical assistance with preparation of columns. This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 1111-2011-0494.

REFERENCES

1. Almand, E. A., M. D. Moore, J. Outlaw, and L-A. Jaykus. 2017. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS ONE* 12(3): e0173124.doi:10.1371/journal.pone.0173124.

2. Beer, K. D., J. W. Gargano, V. A. Roberts, V. R. Hill, L. E. Garrison, P. K. Kutty, E. D. Hilborn, T. J. Wade, K. E. Fullerton, and J. S. Yoder. 2015. Surveillance for waterborne disease outbreaks associated with drinking water – United States, 2011-2012. MMWR 64:842-851. Betts, R. 2014. Microbial update water. *Int. Food Hygiene* 25:12 -13.

3. Betts R. 2014. Microbial update water. *Int. Food Hygiene* 25:12 -13.

4. Bradley, I., A. Straub, P. Maraccini, S. Markazi, T. H. Nguyen. 2011. Iron oxide amended biosand filters for virus removal. *Water Res.* 45:4501-4510.

5. Campos, C. J. A., J. Avant, J. Lowther, D. Till, and D. N. Lees. 2016. Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes. *Water Res.* 103:224-232.

6. Cheng, R., G. Li, L. Shi, X. Xue, M. Kang, and X. Zheng. 2016. The mechanism for bacteriophage f2 removal by nanoscale zero-valent iron. *Water Res.* 105:429-435.

7. Chiu P. 2013. Applications of zero-valent iron (ZVI) and nanoscale ZVI to municipal and decentralized drinking water systems – a review, p. 237 – 249. *In* S. Ahuja and K. Hristovski (ed.) Novel solutions to water pollution, ACS Symposium Series, American Chemical Society, Washington, DC.

8. Craun, G. F., J. M. Brunkard, J. S. Yoder, V. A. Roberts, J. Carpenter, T. Wade, R. L. Calderon, J. M. Roberts, M. J. Beach, and S. L. Roy. 2010. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. *Clin. Microbiol. Rev.* 23:507-528.

9. Curriero, F. C., J. A. Patz, J. B. Rose, and S. Lele. 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948-1994. *Am. J. Pub. Health* 91: 1194-1199.

10. Diao, M. and M. Yao. 2009. Use of zero-valent nanoparticles in inactivating microbes. *Water Res.* 43:5243-5251.

11. Dunkin, N., S. Weng, J. G. Jacangelo, K. J. Schwab. 2017. Inactivation of human norovirus genogroups I and II and surrogates by free chlorine in postharvest leafy green wash water. *Appl. Environ. Microbiol.* 83:e01457-17.

12. Esseili, M. A., Q. Wang, and I. J. Saif. 2012. Binding of human GII.4 norovirus virus-like particles to carbohydrates of Romaine lettuce leaf cell wall materials. *Appl. Environ. Microbiol.* 78:786-794.

13. Fu, F., D. D. Dionysiou, and H. Liu. 2014. The use of zero-valent iron for groundwater remediation and wastewater treatment: A review. *J. Haz. Mat.* 267:194-205.

14. Gandhi, K. M., R. E. Mandrell, and P. Tian. 2010. Binding of virus-like particles of Norwalk virus to Romaine lettuce veins. *Appl. Environ. Microbiol.* 76:7997-8003.

15. Gibson, K. E. 2014. Viral pathogens in water: occurrence, public health impact, and available control strategies. *Curr. Opin. Virol.* 4:50-57.

16. Goodridge, L., C. Goodridge, J. Wu, M. Griffiths, and J. Pawliszyn. 2004. Isoelectric point determination of norovirus virus-like particles by capillary isoelectric focusing with whole column imaging detection. *Anal. Chem.* 76:48-52.

17. Hall, A. J., M. E. Wikswo, K. Pringle, L. H. Gould, and U. D. Parashar. 2014. Vital signs: Foodborne norovirus outbreaks – United States, 2009-2012. *MMWR* 63:1-6.

18. Hsu, C. C., C. E. Wobus, E. K. Steffen, L. K. Riley, and R. S. Livingston. 2005. Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. *Clin. Diagn. Lab. Immunol.* 12:1145-1151.

19. Ingram, D.T., M. T. Callahan, S. Ferguson, D. G. Hoover, P. C. Chiu, D. R. Shelton, P. D. Millner, M. J. Camp, J. R. Patel, K. E. Kniel, and M. Sharma. 2012. Use of zero-valent iron biosand filters to reduce *Escherichia coli* O157:H12 in irrigation water applied to spinach plants in a field setting. *J. Appl. Microbiol.* 112:551-560.

20. Interstate Technology and Regulatory Council PRB: Technology Update Team. 2011. Permeable reactive barrier: Technological Update. Interstate Technology and Regulatory Council, Washington, DC, USA.

21. Karst, S.M. 2010. Pathogenesis of noroviruses, emerging RNA viruses. *Viruses* 2:748-781.

22. Kauppinen, A., T. Pitkänen, and I. T. Miettinen. Persistent norovirus contamination of groundwater supplies in two waterborne outbreaks. *Food Environ. Virol.* DOI 10.1007/s12560-017-9320-6.

23. Kim, J. Y., H. J. Park, C. Lee, K. L. Nelson, D. L. Sedlak, and J. Yoon. 2010. Inactivation of *Escherichia coli* by nanoparticulate zerovalent iron and ferrous ion. *Appl. Environ. Microbiol.* 76:7668-7670.

24. Kingsley, D. H., J. P. Fay, K. Calci, R. Pouillot, J. Woods, H. Chen, B. A. Niemira, and J. M. Van Doren. 2017. Evaluation of chlorine treatment levels for inactivation of human norovirus and MS2 bacteriophage during sewage treatment. Appl. Environ. Microbiol. 83:e01270-17, https://doi.org/10.1128/AEM01270-17

25. Kingsley, D. H., E. M. Vincent, G. K. Meade, C. L. Watson, and X. Fan. 2014. Inactivation of human norovirus using chemical sanitizers. *Int. J. Food Microbiol.* 171:94-99.

26. Kitajima, M., Y. Tohya, K. Matsubara, E. Haramoto, E. Utagawa, and H. Katayama. 2010. Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. *Lett. Appl. Microbiol.* 51:119-121.

27. Lee, C., J. Y. Kim, W. I. Lee, K. L. Nelson, J. Yoon, and D. L. Sedlak. 2008. Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. *Environ. Sci. Technol.* 42:4927-4933.

28. Li, D., A. De Keuckelaere, and M. Uyttendaele. 2015. Fate of foodborne viruses in the “farm to fork” chain of fresh produce. *Compr. Rev. Food Sci. Food Saf.* 14:755-770.

29. López-Gálvez, F., P. Truchado, G. Sánchez, R. Aznar, M. I. Gil, and A. Allende. 2016. Occurrence of enteric viruses in reclaimed and surface irrigation water: relationship with microbiological and physiochemical indicators. *J. Appl. Microbiol.* 121: 1180-1188.

30. Maguire, N. and G. Claffey. 2015. The future is now for water reuse. *Food Technol.* 69:46-51.

31. Montazeri, N., D. Goettert, E. C. Achberger, C. N. Johnson, W. Prinyawiwatkul, and M. E. Janes. 2015. Pathogenic enteric viruses and microbial indicators during secondary treatment of municipal wastewater. *Appl. Environ. Microbiol.* 81:6436-6445.

32. Morales-Rayas R., P. F. G. Wolffs, and M. W. Griffiths. 2009. Anion-exchange filtration and real-time PCR for the detection of a norovirus surrogate in food. *J. Food Prot.* 72:2178-2183.

33. Naseri, E., A. I. Ndé-Tchoupé, H. T. Mwakabona, C. P. Nanseu-Njiki, C. Noubactep, K. N. Njau, and K. D. Wydra. 2017. Making Fe^0-based filters a universal solution for safe drinking water provision. *Sustainability* 9:1224-1255.

34. Okoh, A. I., T. Sibanda, and S. S. Gusha. 2010. Inadequately treated wastewater as a source of human enteric viruses in the environment. *Int. J. Environ. Res. Public Health* 7:2620-2637.

35. Ottoson, J., A. Hansen, B. Björlenius, H. Norder, and T. A. Stenström. 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. *Water Res.* 40:1449-1457.

36. Park, H., M. Kim, and G. Ko. 2010. Evaluation of various methods for recovering human norovirus and murine norovirus from vegetables and ham. *J. Food Prot.* 73:1651-1657.

37. Predmore, A. and J. Li. 2011. Enhanced removal of human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers. *Appl. Environ. Microbiol.* 77:4829-4838.

38. Reynolds, K. A., K. D. Mena, and C. P. Gerba. 2008. Risk of waterborne illness via drinking water in the United States. *Rev. Environ. Contam. Toxicol.* 192:117-158.

39. Seitz, S. R., J. S. Leon, K. J. Schwab, G. Marshall Lyon, M. Dowd, M. McDaniels, G. Abdulhafid, M. L. Fernandez, L. C. Lindesmith, R. S. Baric, and C. L. Moe. 2011. Norovirus infectivity in humans and persistence in water. *Appl. Environ. Microbiol.* 77:6884-6888.

40. Shearer, A. E. H., D. G. Hoover, and K. E. Kniel. 2014. Effect of bacterial cell-free supernatants on infectivity of norovirus surrogates. *J. Food Prot.* 77:145-149.

41. Shi, C., J. Wei, Y. Jan, K. E. Kniel, and P. C. Chiu. 2012. Removal of viruses and bacteriophages from drinking water using zero-valent iron. *Sep. Purif. Technol.* 84:72-78.

42. Tan M., C. Wei, P. Huang, Q. Fan, C. Quigley, M. Xia, H. Fang, X. Zhang, W. Zhong, J. S. Klassen, and X. Jiang. 2015. Tulane virus recognizes sialic acids as cellular receptors. *Sci. Rep.* 5, 11784; doi: 10.1038/srep11784

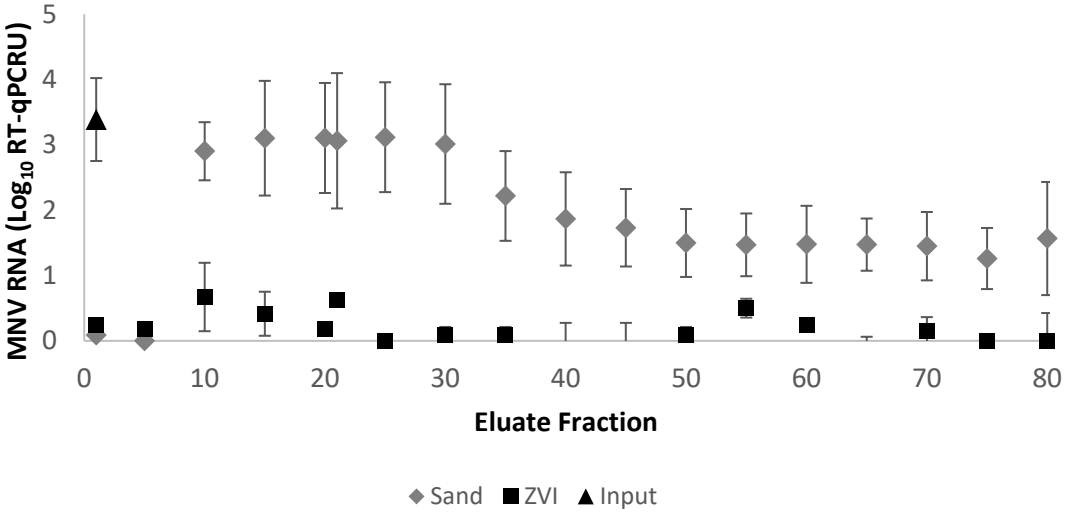
43. Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R. Beumer, and E. Duizer. 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl. Environ. Microbiol*. 78:7769-7775.

44. Vega, E., J. Garland, and S. D. Pillai. 2008. Electrostatic forces control nonspecific virus attachment to lettuce. *J. Food Prot.* 71:522-529.

45. Wang, Q. and K. E. Kniel. 2014. Effectiveness of calcium hypochlorite on viral and bacterial contamination of alfalfa seeds. *Foodborne Pathog. Dis*. 11:759-768.

46. You, Y., J. Han, P. C. Chiu, and Y. Jin. 2005. Removal and inactivation of waterborne viruses using zerovalent iron. *Environ. Sci. Technol.* 39:9263-9269.

A



B

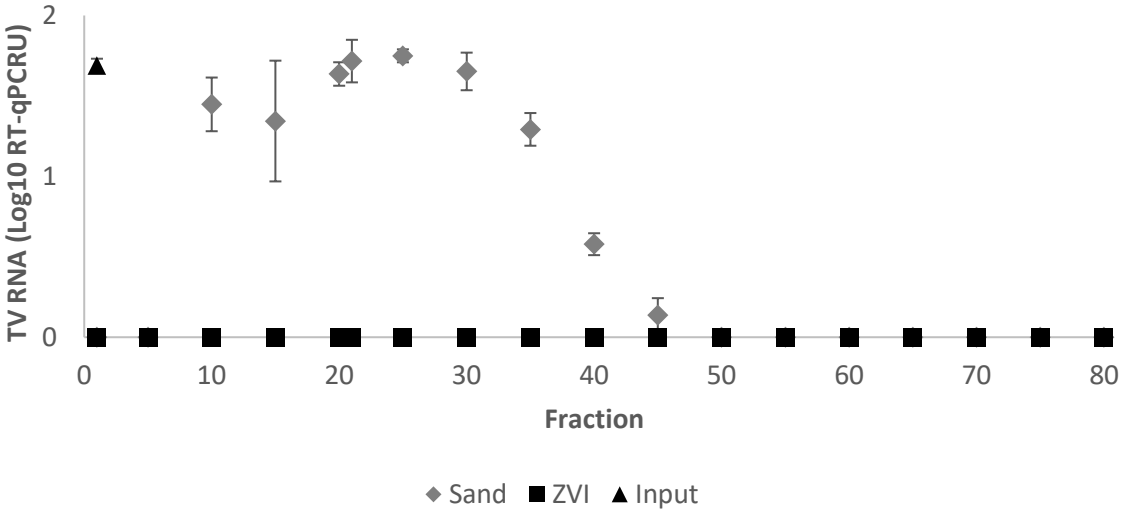
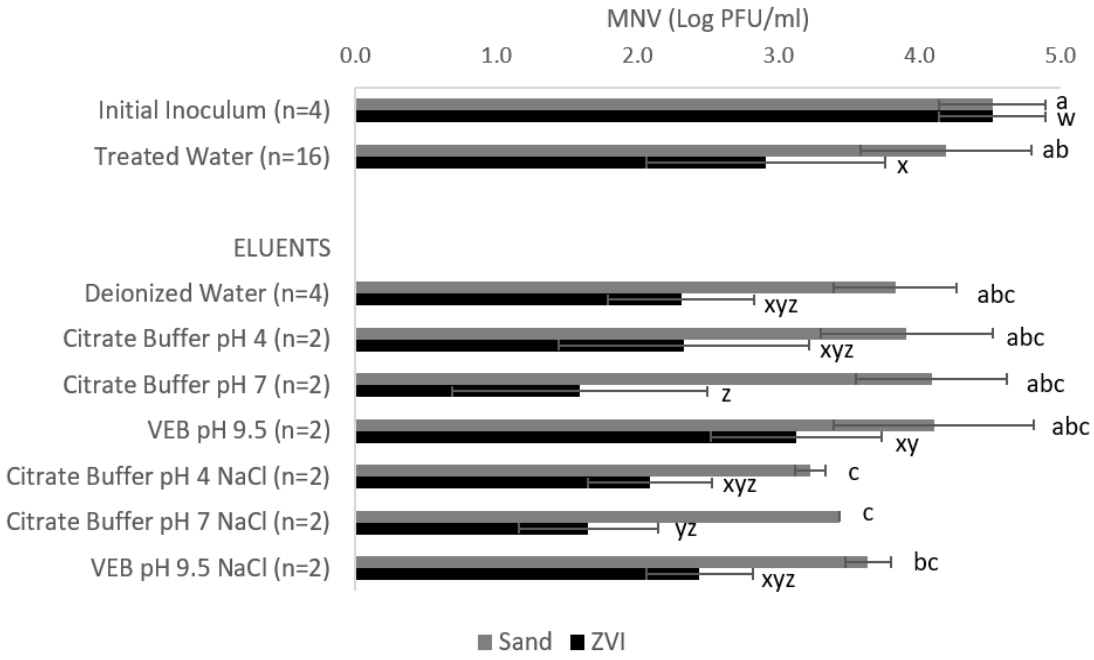


Figure 2.1. Recovery of A, MNV and B, TV genomic material in eluate of inoculated sand and ZVI columns. Data are averages from two samples; error bars represent standard deviation. TV RNA was not detected from eluate of the ZVI column and was below detection for the sand column at fraction 45.

A


MNV (Log PFU/ml)
0.0
1.0
2.0
3.0
4.0
5.0
Initial Inoculum (n=4)
Treated Water (n=16)
ELUENTS
Deionized Water (n=4)
Citrate Buffer pH 4 (n=2)
Citrate Buffer pH 7 (n=2)
VEB pH 9.5 (n=2)
Citrate Buffer pH 4 NaCl (n=2)
Citrate Buffer pH 7 NaCl (n=2)
VEB pH 9.5 NaCl (n=2)
a
w
ab
x
abc
xyz
abc
xyz
abc
z
abc
xy
c
xyz
c
yz
bc
xyz
Sand
ZVI

B

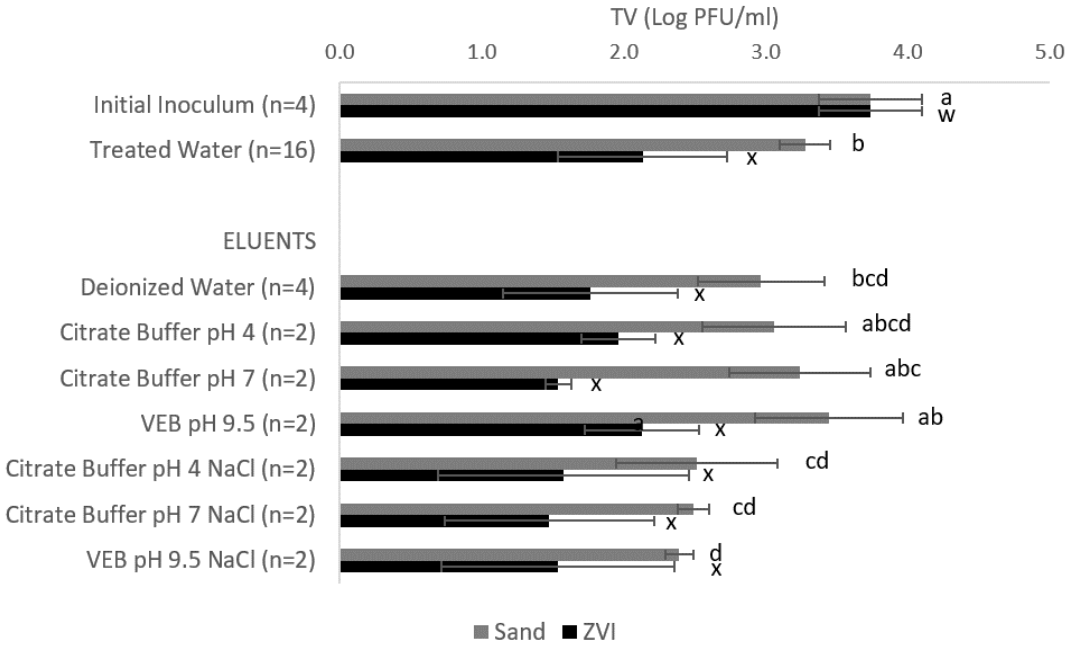

TV (Log PFU/ml)
0.0
1.0
2.0
3.0
4.0
5.0
Initial Inoculum (n=4)
Treated Water (n=16)
ELUENTS
Deionized Water (n=4)
Citrate Buffer pH 4 (n=2)
Citrate Buffer pH 7 (n=2)
VEB pH 9.5 (n=2)
Citrate Buffer pH 4 NaCl (n=2)
Citrate Buffer pH 7 NaCl (n=2)
VEB pH 9.5 NaCl (n=2)
a
w
b
x
bcd
x
abcd
x
abc
x
ab
x
cd
x
cd
x
d
x
Sand
ZVI

Figure 2.2. Recovery of infectious A, MNV and B, TV from inoculated batch studies of sand and ZVI water treatment and subsequent elution. Initial inoculum and recovery from sand and ZVI are noted in the top two bars followed by results of six elution buffers as compared to elution by water. Error bars represent standard deviation. Bars marked by different letters within a treatment (either sand (a, b, c, d) or ZVI (w, x, y, z)), for each virus considered separately, are significantly different ($p<0.05$).

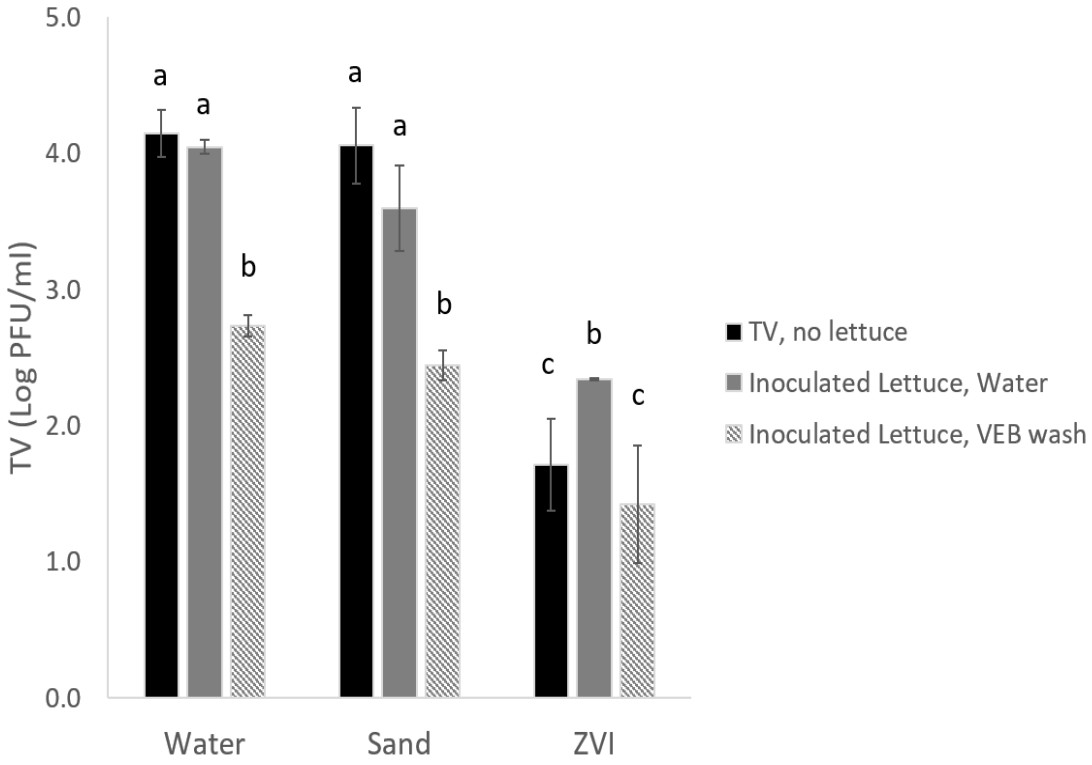


Figure 2.3. TV recovered from inoculated water without lettuce (black bars), either untreated or treated by sand or ZVI; wash water of inoculated lettuce (gray bars) treated by water alone or water with sand or ZVI; and VEB wash of lettuce after water wash (patterned bars) alone or with sand or ZVI. Data are averages from two samples; error bars represent standard deviation. Bars marked by different letters are significantly different ($p<0.05$).

Chapter 3

EFFECT PLANT-DERIVED PROTEASES FOR INACTIVATION OF HUMAN NOROVIRUS, TULANE VIRUS, MURINE NOROVIRUS, AND HEPATITIS A VIRUS

3.1. Abstract

Viruses are a leading cause of foodborne illness, and effective washing strategies are needed to remove or inactivate virus on fresh produce. Plant-derived proteases, bromelain, papain, and ficin are broad-acting enzymes with generally recognized as safe (GRAS) status for foods and current application in several food industries. This study investigated the efficacy of commercially-prepared bromelain, papain, and ficin, individually and combined (2500 ppm crude extract), for inactivation of hepatitis A virus (HAV), human norovirus (HuNov), and HuNov surrogates, Tulane virus (TV), and murine norovirus (MNV). Various treatment temperatures (45, 50, 55°C), times (10 or 60 min), and pH values (2.2, 5.5, 7.0) in the presence of cysteine (2 or 20 mM) were evaluated. Inactivation was assessed by infectivity in plaque assay for TV and MNV and by $TCID_{50}$ for HAV. Inactivation of HuNov was assessed by RT-qPCR. No reduction in infectious TV or HAV was attributed to the plant-derived proteases at any of

the conditions tested. Infectious MNV was reduced by one to 3 log_{10} PFU/ml; the most effective treatment was bromelain at pH 7 and 50°C for 10 minutes. A time course study with MNV in bromelain at 50°C indicated that a 2-log_{10} PFU/ml reduction could be achieved within 6 minutes, but extended treatment of 15 minutes was still insufficient to eliminate infectious MNV. HuNov was resistant to bromelain at 50°C for 10 min. The lack of or limited efficacy of bromelain, papain, and ficin on HAV, HuNov, TV, and MNV even at elevated temperatures and exposure times suggests the plant-derived proteases are not commercially applicable for inactivation of virus on raw produce. The variable susceptibilities observed among HuNov, TV and MNV illustrate limitations in utilization of surrogates for predicting pathogen behavior for a structure-specific treatment.

3.2. Introduction

The majority (58%) of foodborne illnesses in the United States are attributed to enteric viruses with human norovirus (HuNov) accounting for an estimated annual 5.5 million domestically-acquired illnesses (Scallan *et al*., 2011). Globally, norovirus is also the leading cause of viral gastroenteritis (Karst, 2010). Although hepatitis A virus (HAV) is estimated to cause considerably fewer foodborne illnesses of less than 2,000 annually in the U.S., the severity of illness is notable with the hospitalization rate greater than 30% (Scallan *et al*., 2011). Although any food commodity could serve as a transmission vehicle if contaminated in final handling, foods consumed raw and for which incomplete removal or inactivation methods exist are most commonly associated with illness.

These include fresh produce, particularly raw berries and leafy greens, and raw molluscan foods (Callejón *et al*., 2015; Chatziprodromidou *et al*., 2018; Hall *et al*., 2012).

Both HuNov and HAV are persistent in the environment, on food contact surfaces, and throughout the shelf life of fresh produce (reviewed by Kotwal and Cannon, 2014). Previously-investigated methods to remove or inactivate virus on fresh produce include UV-C treatment (Butot *et al*., 2018) and washes with water, chlorine, sodium hypochlorite, peroxyacetic acid (reviewed by Baert *et al*., 2009) and natural biochemical substances, including polyphenols, proanthocyanins, saponin, polysaccharides, peptides, and essential oils (reviewed by Li *et al*., 2013). These treatments have generally yielded one to three log reductions in HAV or HuNov or its surrogates (Baert *et al*., 2009; Butot *et al*., 2018; Li *et al*., 2013). Therefore, new treatments, preferably those derived from natural sources, are sought to inactivate virus or to inhibit viral adherence to raw produce.

Plant-derived cysteine proteases, papain and ficin, are found in the latex of papaya and figs; bromelain is found predominantly in the stem of pineapple as well as the fruit and leaves (Gosalia *et al*., 2005; Biozym 2012). The extracted enzymes are safe for human consumption, with Generally Recognized as Safe (GRAS) status for food (Code of Federal Regulations, 1983, 2013a, 2013b), and currently have application in food processing for meat, brewing, and dairy industries (Homaei *et al*., 2010; González-Rábade *et al*., 2011). Plant-derived proteases also have atoxigenic properties (Helting and Zwisler, 1974; Helting and

Zwisler, 1977; Helting and Nau, 1984) and antimicrobial activity including fungistatic (López-García *et al*., 2012), , antihelminthic (de Amorin *et al*., 1999; Mansur *et al*., 2014; Stepek *et al*., 2005), and antibacterial (Assatarakul *et al*., 2012; dos Anjos *et al.*, 2016) properties. Bromelain also cleaves the hemagglutinin protein from influenza virus with subsequent application in antisera production (Wang *et al.*, 2012). These proteases have several characteristics favorable for application to foods including good water solubility, optimal functionality over a pH range of approximately 4 to 7.5, and catalytic activity at 30 to 40°C, albeit less than at the optimal catalytic temperature range of 50 to 60°C (Choudhury *et al*., 2010; Whitaker and Lee, 1972; Xue *et al*., 2010). Moreover, while activity is enhanced in the presence of cysteine, an activator is not essential for catalytic activity (Homaei *et al*., 2010; MP Biomedicals, 2012).

Papain, bromelain, and ficin are broad-acting enzymes, all of which preferentially cleave the peptide bonds at the carboxyl end of lysine and arginine. Histidine, glycine, glutamine, tyrosine, threonine, alanine, and phenylalanine are additional proteolytic targets for one or more of the proteases (Choe *et al*., 2006; Gosalia *et al*., 2005; Biozyme, 2012; MP Biomedicals, 2012, MP Biomedicals, 2013). Based on the primary amino acid structure of the VP1 region of the capsid of human norovirus (GII.4/5M/USA/2004; GenBank AFL70023.1) as deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/), up to 24%, 37%, and 14% of the peptide bonds may be vulnerable to hydrolysis by papain, bromelain, and ficin, respectively. Additionally, several norovirus capsid amino acids important

for interactions with receptor histo-blood group antigens (HBGA) (Bu *et al*., 2008; Choi *et al*., 2008; Tan *et al*., 2009) are theoretical targets for cleavage by the plant proteases. Although the capsid amino acid sequences of surrogate viruses, murine norovirus (MNV) and Tulane virus (TV), bear limited similarity to that of HuNov, the total compositions of amino acids theoretically vulnerable to proteolysis are similar. Likewise, the HAV capsid polyprotein (Hepatovirus A; GenBank AAA45477.1) consists of 26%, 25%, 18% proteolytic targets of papain, bromelain, and ficin, respectively, according to primary amino acid sequence (http://www.ncbi.nlm.nih.gov/). However, the accessibility of the viral capsid peptide bonds to hydrolysis as influenced by capsid protein secondary and tertiary structures as well as the conditions that may favor hydrolysis are unknown.

The objective of this study was to investigate the efficacy of plant-derived proteases for inactivation of HAV, HuNov, and HuNov surrogates, MNV and TV.

3.3. Materials and Methods

3.3.1. *Viruses and host cells*. Murine norovirus (MNV-1, obtained from Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was propagated by inoculation in confluent 24-h RAW 264.7 cells (ATCC TIB-71) overlaid with Hanks Balanced Salt Solution with calcium and magnesium (HBSS; Mediatech, Inc., Manassas, VA) and were incubated at 37ºC and 5% CO_2 for 48 h. MNV was recovered by subjecting RAW cells to three freeze/thaw cycles (-

80ºC/25 to 37ºC), pelleting the cells by centrifugation (233 x *g*), and retaining the virus-containing supernatant at -80ºC until further use.

Tulane virus (TV, obtained from Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated by inoculation of 24-h confluent LLC-MK2 cells (ATCC CCL-7) in HBSS and incubated for 55 h at 37ºC with 5% CO_2. TV was recovered from LLC-MK2 cells as previously described for MNV and stored at -80ºC.

Hepatitis A virus (HAV, ATCC VR-1402) was propagated by inoculation in 24-h confluent FRhK-4 (ATCC CRL-1688) in Dulbecco's Modification of Eagle's Medium (DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate, Mediatech Inc. amended with 1x MEM Nonessential amino acids, Mediatech, Inc., and 1% antibiotic/antimycotic (100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml-1 amphotericin B, HyClone) and incubated for 21 d at 37C with 5% CO_2. HAV was recovered from FRhK-4 cells as previously described for MNV and stored at -80°C.

Human norovirus GII (HuNov) was extracted from human stool samples (provided by the Delaware Department of Public Health) by suspending stool (10%) in phosphate buffered saline, pH 7.4. The HuNov suspension was vortexed vigorously for 30 seconds, and the solid debris was pelleted by centrifugation at 1455 *x g* for 15 minutes. For subsequent protease treatment, the virus-containing supernatant was filtered (0.22 um) and stored at -20°C.

3.3.2. *Protease treatments.* TV and MNV (initial inoculum of 5 log PFU/ml) and HAV (6 log $TCID_{50}$) were treated by 2500 ppm of the proteases bromelain (MP Biomedicals, LLC, Solon, OH), papain (MP Biomedicals, LLC), ficin (MP Biomedicals, LLC), and an equal mix of all three in HBSS or citrate buffer, pH 5.0 with activator, tissue culture-grade L-cysteine-hydrochloride (2 mM) (Fisher Scientific, Fair Lawn, NJ). The treatment volume was 1250 μl in a 1500-μl sterile microcentrifuge tube submerged in a water bath. Treatment conditions varied by temperature (45, 50, or 55°C) and exposure time (2 to 60 min) (Table 1). Time to reach maximum treatment temperature was 2.75, 4.00, and 3.50 minutes for 45, 50, and 55°C, respectively, as monitored with a digital thermometer every 15 seconds. HuNov was treated with bromelain (2500 ppm) at 50°C for 10 min in a total treatment volume of 625 μl. Controls included untreated virus with no exposure to the proteases or heat to determine the initial inocula, and a heated control for which virus was exposed to heat but not the proteases. The pH was determined at 21°C with an Accumet AB200 pH meter (Fisher Scientific, Fair Lawn, NJ, USA). Enzyme activities of the 2500 ppm preparations were determined with the Protease Fluorescent Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) with fluorescein isothiocyanate (FITC)-labeled casein substrate and trypsin standard.

3.3.3*. Enumeration of infectious virus.* After treatment, tubes were placed on ice to stop enzymatic activity. Samples were diluted in HBSS and plaque assay was performed as previously described (Shearer *et al*., 2014; Wang and Kniel, 2014)

to enumerate infectious virus. Briefly, 500 µl per dilution of treated MNV and TV were added to 6-well cell culture plates in duplicate wells to confluent monolayers of RAW and LLC-MK2 cells, respectively, containing 500 µl per well cell culture media with 10% fetal bovine serum (FBS, HyClone, Logan, UT). Media for RAW cells was Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech, Inc.) containing 4.5 g l^{-1} of glucose, L-glutamine and sodium pyruvate amended with fetal bovine serum (FBS, HyClone, Logan, UT) at 10% v/v, 4 mM glutamax (Invitrogen), sodium bicarbonate (Mediatech), and antibiotic/antimycotic (100 U penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml-1 amphotericin B, HyClone). Media for LLC-MK2 cells was Medium 199/EBSS (M199 with Earles Balanced Salts and L-glutamine, HyClone) amended with 10% FBS and antibiotic/antimycotic mix as described for DMEM. After a minimum 2-h infection period at 37°C in 5% CO_2 static environment, inocula were aspirated and monolayers rinsed with HBSS. Cells were then overlaid with 2 ml of 1:1 (v/v) universal agarose peqGOLD Universal agarose (PEQ Lab, Erlangen, Germany) in cell culture media. For RAW cells, 2X MEM/EBSS (with L-glutamine, HyClone) containing 2% FBS and glutamax, sodium bicarbonate, and antibiotic/antimycotic as previously described for DMEM was used. For LLC-MK2 cells, M199/EBSS cell culture medium containing 2% FBS and antibiotics/antimycotics was used. Solidified agarose was overlaid with DMEM or M199 amended as previously described except containing 2% FBS for RAW or LLC-MK2 cells, respectively. Infected cells were incubated 48 h to allow plaque formation, after which, the

media were aspirated and cells were stained for 6 to 8 h with 1% neutral red in medium containing 2% FBS, or plaques were fixed with 10% formaldehyde in phosphate buffered saline for a minimum of 2 h and stained with crystal violet prior to plaque enumeration.

Infectious HAV was enumerated by tissue culture infectious dose ($TCID_{50,}$ Reed and Muench, 1938). FRhK-4 cells were seeded in 96-well cell culture plates and grown to confluency in DMEM as amended for HAV propagation and 10% FBS at 37C/ 5% CO2 for approximately 24 h. Prior to infection, spent media was aspirated and cells were washed with HBSS and overlaid with fresh HBSS. Eight wells were inoculated for each control and treated HAV and serially diluted and incubated for 2h at 37°C with 5% CO_2. The wells were overlaid with DMEM containing 2% FBS and incubated 21d at 37°C with 5% CO_2 prior to observation for cytopathic effects.

3.3.4. *Enumeration of viral RNA.* Viral RNA was extracted with the AllPrep® PowerViral® DNA/RNA Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. Reverse transcription (RT) was performed at 37°C for 60 min in an Eppendorf thermocycler (Hamburg, Germany) with the Sensiscript® RT Kit (Qiagen) according to manufacturer protocols. MNV primers (Hsu *et al*., 2005) targeted the viral polymerase gene (MNV-F, 5'-CTTCGCAAGACACGCCAATTTCAG and MNV-R, 5'-GCATCACAATGTCAGGGTCAACTC, Sigma-Aldrich® Corp., St. Louis, MO, USA). HuNov primers (Kojima *et al*., 2002) targeted the shell domain of capsid

protein VP1 (G2SK-F, 5'-CNTGGGAGGGCGATCGCAA and G2SK-R,5'-CCRCCNGCATRHCCRTTRTACAT, Sigma-Aldrich Corp.). Amplification of cDNA was carried out with the QuantiTect SYBR Green PCR kit (Qiagen) using the same primers as for reverse transcription and two μl of template cDNA in a reaction volume of 20 μl per manufacturer protocol. Real time PCR (qPCR) was performed in a Rotor-Gene Q (Qiagen) with hot start at 95°C for 15 min, 40 cycles of 94°C/15 sec denaturation, 60°C/30 sec annealing, and 72°C/30 sec extension. Amplicons were confirmed by melt curves and/or target band size, 318 base pairs for MNV and 344 bp for HuNov, by electrophoresis in a 1% PCR-grade, intermediate-melting agarose gel (Fisher Scientific, Fair Lawn, NJ, USA) in tris-acetate-EDTA (TAE) buffer (Fisher Scientific) with 4% ethidium bromide. MNV and unfiltered HuNov serially diluted in HBSS or sterile, nuclease-free water, respectively, were used to generate standard curves with the lowest dilution of detection designated 1 RT-qPCR unit (RT-qPCRU) (Almand *et al*., 2017, Tuladhar *et al*., 2012).

3.3.5. *Assay for cytotoxicity.* The effect of proteases and heat treatments in the absence of proteases on uninfected cells was evaluated under the same conditions as for treated virus-inoculated samples, and cytotoxic effect was characterized by sloughing of the monolayer. Wells with uninfected and virus-infected cells without any treatment served as controls.

3.3.6*. Data analysis.* All experiments were conducted in duplicate. Statistical significance ($p<0.05$) in viral recovery among treated samples and controls were

determined by analysis of variance and student's t-test with statistical software, JMP Pro Version 10.0.0 (SAS Institute Inc., Cary, NC).

3.4. Results and Discussion

The considerable impact of human norovirus and hepatitis A virus on public health and the limited efficacy of viral removal or inactivation strategies for fresh produce necessitate investigation of new methods to mitigate viral contamination of foods that are consumed raw. As plant-derived and broad-acting proteases with GRAS status for foods, bromelain, papain, and ficin were investigated for effectiveness against these foodborne viruses.

Bromelain, papain, and ficin were previously reported to have antimicrobial properties, yielding modest reductions in foodborne *Escherichia coli* O157:H7 (Eshamah *et al*., 2014), growth inhibition of plant fungi *Fusarium* spp. (López-García *et al*., 2012), and structural damage and reduced motility in rodent helminths *in vitro* (Mansur *et al*., 2014; Stepek *et al*., 2005). While structurally dissimilar to enteric viruses, these bacteria and helminths also must resist proteolytic degradation in the host gastrointestinal tract; yet they demonstrated susceptibility to the plant proteases. These effects were observed over treatment times of minutes to hours at temperatures below the optimal catalytic temperature range for the enzymes. For viral inactivation studied herein, treatment conditions (Table 1) favorable to enzyme activity were utilized including addition of the activator cysteine and elevated temperatures of 45 to 55°C. These conditions were selected based on prior thermal resistance studies (Hirneisen

and Kniel, 2013) and were sublethal to the viruses applied in the absence of proteases (Fig. 1). The commercially-prepared bromelain, papain, and ficin were used as received as crude extracts with concentrations normalized for treatments and at levels for which cytotoxicity did not preclude detection of infectivity reduction. However, the extracts demonstrated variable enzymatic activity as measured against a casein substrate (Table 1). Activity measured by this assay was not a good predictor for viral infectivity reduction (Table 1 and Fig. 1).

Regardless of treatment temperature, time, and pH, the proteases did not reduce infectivity of TV or HAV greater than the untreated control or samples treated with heat alone (Fig. 1a and 1c). By contrast, reductions in infectious MNV ranged from one $\log_{10}$ PFU/ml in the presence of papain at 45°C for 60 min to 3 $\log_{10}$ PFU/ml reductions after treatment with bromelain at all temperatures and the combined enzymes at 45°C (Fig. 1b). Detectable infectious MNV was also reduced by 2-$\log_{10}$ PFU/ml by heat treatment alone at 55°C for 10 min (Fig. 1b). No reductions in infectious MNV were observed at pH 5.5 (Fig 1b). The addition of 20 mM cysteine-HCl reduced the pH to 2.2, and with heat treatment at 50°C for 10 min, either with or without added proteases, the reduction in infectious virus was at least 3 $\log_{10}$ PFU/ml for MNV and TV; whereas HAV was reduced by less than 2 log $TCID_{50}$ (data not shown).

In spite of the broad-acting proteolytic properties of these enzymes, several potential limitations were anticipated for their application for inactivation of these enteric viruses. First, the protein secondary and tertiary structures may impede

enzyme association for sufficient peptide hydrolysis to degrade the capsid or disrupt surface molecules involved in host receptor interactions. Second, enteric viruses must resist a broad range of host digestive proteases. The reported susceptibility of other enteric organisms to plant proteases as compared to host digestive enzymes may have been as much a function of structural specificity for enzyme interaction as production of specific protease inhibitors to digestive enzymes (Stepek *et al*., 2005). Third, antimicrobial properties demonstrated in previous studies may have been due to degradation of bonds other than peptide bonds such as was reported for *Alicyclobacillus* spp. (dos Anjos *et al*., 2016). Finally, the viruses encode their own proteases involved in proteolytic processing of nonstructural proteins (Dinakarpandian *et al*., 1997; Farkas *et al*., 2008; Nakamura *et al*., 2005; Thorne and Goodfellow, 2014; Vashist *et al*., 2009; Viswanathan *et al*., 2013); the exposure of capsid proteins to these viral proteases during replication is not known.

The notable reduction in infectious MNV, however, warranted further investigation into conditions that may yield complete inactivation and the nature of the reduction, whether due to disruption of capsid integrity or host cell interaction. The different resistances of MNV and TV also prompted study with HuNov to discern which surrogate more closely represented the susceptibility of the human pathogen to these structure-dependent treatments.

A time-course study was undertaken to determine if MNV infectivity reduction could be achieved in less than 10 minutes or if complete infectivity reduction

could be achieved with longer exposure. The time necessary to realize a 2-log reduction in infectivity of MNV by bromelain at 50°C was at least 6 minutes (Fig. 2). Full inhibition of infectivity was not achieved with this treatment even when treatment time was extended to 15 minutes (Fig. 2). These conditions are not realistic for commercial application to fresh produce without anticipated compromise of product quality.

The basis for the reduction of MNV infectivity as a result of bromelain and heat treatment was further investigated in an effort to determine if MNV capsid proteins involved in adherence to host cellular receptors were compromised or if capsid integrity was sufficiently damaged to render the particles completely inactive. The first approach involved application of RNase to degrade free RNA (Hirneisen and Kniel, 2012; Kingsley, 2002) to predict capsid integrity; however, this assay yielded erroneous results as controls could not be detected (data not shown). Attempts to quench interfering bromelain activity by fetal bovine serum (FBS) were unsuccessful even with addition of FBS at 50% of the treatment tube volume (data not shown). Therefore, MNV RNA was directly added to the protease treatment tubes to detect RNA stability in the presence of bromelain. MNV RNA added directly to bromelain could not be detected when reverse transcription was performed immediately after exposure (data not shown). However, MNV RNA directly added to bromelain treatment tubes could be detected by RT-qPCR at time point zero when samples were first treated with the AllPrep® PowerViral® DNA/RNA Extraction Kit to reduce inhibitors (Table 2). At

longer time points of 5 and 10 minutes post inoculation of RNA in bromelain at 50°C, however, no RNA could be detected indicating that free RNA is degraded by bromelain and heat treatment (Table 2). With this established, intact MNV viral particles were subsequently treated by bromelain, and MNV was detected in parallel by plaque assay and RT-qPCR. This was done in order to determine if reduction in infectivity as measured by plaque assay also yielded reduction in MNV RNA, findings that would point to capsid degradation as indicated by release of RNA. Conversely, if reduction in infectivity did not correlate to reduction in RNA, the data would suggest MNV infectivity was reduced by means other than complete capsid degradation and release of genomic material, such as by alteration of host cell attachment capabilities. Although MNV infectivity was reduced by more than one log PFU/ml by combined bromelain and heat treatment (Fig. 3), unlike in previous experiments (Figs. 1 and 2), the reduction was not statistically significant in these experiments (Fig. 3). Likewise, the difference in MNV RNA detected from the bromelain and heat treatment compared to controls was not significantly different (Fig. 3). Due to the variable efficacy of MNV reduction between experiments, it is not possible to conclude if the modest reduction in MNV infectivity was a result of MNV capsid degradation and RNA release.

The most effective conditions observed for reduced infectivity of MNV were applied to HuNov GII to determine susceptibility to bromelain and to compare the pathogen resistance to that of its surrogates, TV and MNV, which exhibited

different susceptibilities to bromelain. HuNov was diluted ten-fold from stool into PBS and filtered of stool bacteria before further dilution into the treatment tubes. Due to the dilution and filtration preparatory steps, the initial inoculum of HuNov in the bromelain treatment study was very low and at the limit of detection by RT-qPCR for the first trial and below detection for the second trial. However, for the first trial, there was no decrease in detectable HuNov RNA from the untreated control to HuNov treated by heat alone, bromelain alone, or the combination of heat and bromelain (Table 3). Given that earlier studies indicated that MNV RNA added directly to the heated bromelain was undetectable after 5 and 10 minutes of treatment, release of HuNov RNA presumably did not occur suggesting that the treated HuNov capsid was intact. No further studies were undertaken to determine effect of enzyme on HuNov ability to adhere to known receptors or plant tissue.

In conclusion, the lack of or limited efficacy of bromelain, papain, and ficin on HAV, HuNov, TV, and MNV even at elevated temperatures and exposure times suggests the plant-derived proteases do not appear to be commercially applicable for inactivation of virus on raw produce. The variable susceptibilities observed among HuNov, TV and MNV further illustrate differences in related viruses and limitations of utilization of surrogates for predicting pathogen behavior.

3.5. Acknowledgement

This project was funded in part by the USDA NoroCORE grant # 1111-2011-0494.

REFERENCES

1. Almand, E. A., M. D. Moore, J. Outlaw, and L-A. Jaykus. 2017. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS ONE* 12(3): e0173124.doi:10.1371/journal.pone.0173124.

2. Assatarakul, K., Churey, J.J., and R.W. Worobo. 2012. Antimicrobial activity of papain and bromelain juice supplements against *Alicyclobacillus acidoterrestris* and associated physicochemical properties of apple and orange juice. *Institute of Food Technologists Annual Meeting Abstracts*, Abstract 043-05.

3. Baert, L., J. Debevere, and M. Uyttendaele. 2009. The efficacy of preservation methods to inactivate foodborne viruses. *Int. J. Food Microbiol*. 131: 83-94.

4. Biozym. Data Sheet – Papain (EC 3.4.22.2). Available at http://www.biozyme.de/datasheets/papain.php. Accessed 12 September 2012.

5. Bu, W., Mamedova, A., Tan, M., Xia, M, Jiang, X. and R. S. Hedge. 2008. Structural basis for the receptor binding specificity of Norwalk virus. *J. Virol.* 82(11): 5340-5347.

6. Butot, S., F. Cantergiani, M. Moser, J. Jean, A. Lima, L. Michot, T. Putallaz, T. Stroheker, and S. Zuber. 2018. UV-C inactivation of foodborne bacterial and viral pathogens and surrogates on fresh and frozen berries. *Int. J. Food Microbiol*. 275: 8-16.

7. Callejón, R. M., M. I. Rodríguez-Naranjo, C. Ubeda, R. Hornedo-Ortega, M. C. Garcia-Parrilla, and A. M. Troncoso. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. *Foodborne Path. Dis.* 12: 32-38.

8. Chatziprodromidou, I. P., M. Bellou, G. Vantarakis, and A. Vantarakis. 2018. Viral outbreaks linked to fresh produce consumption: a systematic review. *J. Appl. Microbiol.* 124: 932-942.

9. Choe, Y., F. Leonetti, D. C. Greenbaum, F. Lecaille, M. Bogyo, D. Brömme, J. A. Ellman, and C. S. Craik. 2006. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J. Biol. Chem*. 281(18): 12824-12832.

10. Choi, J.-M., A. M. Hutson, M. K. Estes, and B. V. Venekataram Prasad. 2008. Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus. *PNAS* 105(27): 9175-9180.

11. Choudhury, D., S. Biswas, S. Roy, and J.K. Dattagupta. 2010. Improving thermostability of papain through structure-based protein engineering. *Prot. Engin., Des. Sel.* 23(6): 457-467.

12. Code of Federal Regulations. 2013a. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1024, Bromelain. Available at: www.ecfr.gov. Accessed 13 June 2017.

13. Code of Federal Regulations. 2013b. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1316, Ficin. Available at: www.ecfr.gov. Accessed 13 June 2017.

14. Code of Federal Regulations. 1983. Direct food substances affirmed as generally recognized as safe, title 21, sec. 184.1585, Papain. Available at: www.ecfr.gov. Accessed 13 June 2017.

15. de Amorin, A., H. R. Borba, J. P. P. Carauta, D. Lopes, D., and M. A. C. Kaplan. 1999. Anthelmintic activity of the latex of *Ficus* species. *Journal of Ethnopharmacology* 64: 255-258.

16. Dinakarpandian, D., B. Shenoy, M. Pusztai-Cary, B. A. Malcolm, and P. R. Carey. 1997. Active site properties of the 3C proteinase from hepatitis A virus (a hybrid cysteine/serine protease) probed by Raman spectroscopy. *Biochem*. 36: 4943-4948.

17. dos Anjos, M. M., A. A. da Silva, I. C. de Pascoli, J. M. G. Mikcha, M. Machiriski, R. M. Peralta, and B. A. de Abreu. 2016. Antibacterial activity of papain and bromelain on *Alicyclobacillus* spp. *Int. J. Food Microbiol.* 216: 121-126.

18. Eshamah, H., I. Han, H. Naas, J. Acton, and P. Dawson. 2014. Antibacterial effects of natural tenderizing enzymes on different strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on beef. *Meat Sci*. 96: 1494-1500.

19. Farkas, T., K. Sestak, C. Wei, and X. Jiang. 2008. Characterization of a Rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *J. Virol.* 82: 5408-5416.

20. González-Rábade, N., J. A. Badillo-Corona, J. S. Aranda-Barradas, and M. d. C. Oliver-Salvador. 2011. Production of plant proteases *in vivo* and *in vitro* – A review. *Biotechnol. Adv*. 29: 983-996.

21. Gosalia, D.N., C. M. Salisbury, J. A. Ellman, and S. L. Diamond. 2005. High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays. *Mol. Cell. Proteomics* 4:626-636.

22. Hall, A. J., V. G. Eisenbart., A. L. Etingüe, L. H. Gould, B. A. Lopman, and U. D. Parashar. 2012. Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg. Infect. Dis.* 18: 1566-1573.

23. Helting, T.B. and H.-H. Nau. 1984. Analysis of the immune response to papain digestion products of tetanus toxin. *Acta Path. Microbiol. Immunol. Scand.* Sect. C 92: 59-63.

24. Helting, T. and O. Zwisler. 1974. Enzymatic breakdown of tetanus toxin. *Biochem. Biophys. Res. Commun.* 57: 1263-1270.

25. Helting, T.B. and O. Zwisler. 1977. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J. Biol. Chem*. 252: 187-193.

26. Hirneisen, K.A. and K. E. Kniel. 2013. Comparing human norovirus surrogates: murine norovirus and Tulane virus. *J. Food Prot.* 76: 139-143.

27. Hirneisen, K. A. and K. E. Kniel. 2012. Comparison of ELISA attachment and infectivity assays for murine norovirus. *J. Virol. Methods* 186: 14-20.

28. Homaei, A. A., R. H. Sajedi, R. Sariri, S. Seyfzadeh, and R. Stevanato, R. 2010. Cysteine enhances activity and stability of immobilized papain. *Amino Acids* 38: 937-942.

29. Hsu, C. C., C. E. Wobus, E. K. Steffen, L. K. Riley, and R. S. Livingston. 2005. Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. *Clin. Diagn. Lab. Immunol.* 12:1145-1151.

30. Karst, S.M. 2010. Pathogenesis of noroviruses, emerging RNA viruses. *Viruses* 2:748-781.

31. Kingsley, D. H., D. G. Hoover, E. Papafragkou, and G. P. Richards. 2002. Inactivation of hepatitis A virus and a calicivirus by high hydrostatic pressure. *J. Food Prot.* 10: 1605-1609.

32. Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* 100: 107-114.

33. Kotwal, G. and J. L. Cannon. 2014. Environmental persistence and transfer of enteric viruses. *Curr. Opin. Virol.* 4: 37-43.

34. Li, D., L. Baert, and M. Uyttendaele. 2013. Inactivation of food-borne viruses using natural biochemical substances. *Food Microbiol.* 35: 1-9.

35. López-García, B., M. Hernández, and B. S. Segundo. 2012. Bromelain, a cysteine protease from pineapple (*Ananas comosus*) stem, is an inhibitor of fungal plant pathogens. *Lett. Appl. Microbiol.* 55: 62-67.

36. Mansur, F., W. Luoga, D. J. Buttle, I. R. Duce, A. Lowe, and J. M. Behnke. 2014. The anthelmintic efficacy of natural plant cysteine proteinases against two rodent cestodes *Hymenolepis diminuta* and *Hymenolepis microstoma in vitro. Vet. Parasitol.* 201: 48-58.

37. MP Biomedicals. Bromelain (catalogue number 101142). Available at: http://www4.mpbio.com/ecom/docs/proddata.nsf/440121766f8ee75e8525645d0068b043/38fbf8044ecbac558525668e005162bf?OpenDocument&Highlight=0,101142. Accessed 12 September 2012.

38. MP Biomedicals. Ficin (catalogue number 198761). Available at: http://www.mpbio.com/product_info.php?family_key=02198761&lot_number=&submit_lot=Find+By+Lot+Number#cofatab. Accessed 3 June 2013.

39. Nakamura, K., Y. Someya, T. Kumasaka, G. Ueno, M. Yamamoto, T. Sato, N. Takeda, T. Miyamura, and N. Tanaka. 2005. A norovirus protease structure provides insights into active and substrate binding site integrity. *J. Virol.* 79: 13685-13693.

40. Reed, L. J. and H. Muench. 1938. A simple method for estimating fifty percent endpoints. *Amer. J. Hyg.* 27: 438-497.

41. SAS Institute Inc. 2012. JMP® Pro 10.0.0 64-bit Edition.

42. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A.Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States – major pathogens. *Emerg. Infect. Dis*. 17: 7-15.

43. Shearer, A.E.H., D. G. Hoover, and K. E. Kniel. 2014. Effect of bacterial cell-free supernatants on infectivity of norovirus surrogates. *J. Food Prot.* 77: 145-149.

44. Stepek, G., D. J. Buttle, I. R. Duce, A. Lowe, and J. M. Behnke. 2005. Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus, in vitro*. *Parasitol*. 130: 203-211.

45. Tan, M., M. Xia, Y. Chen, W. Bu, R. S. Hedge, J. Meller, X. Li, and X. Jiang. 2009. Conservation of carbohydrate binding interfaces – evidence of human HBGA selection in norovirus evolution. *PLoS ONE* 4(4): e5058.

46. Thorne, L. G. and I. G. Goodfellow. 2014. Norovirus gene expression and replication. *J. Gen. Virol.* 95: 278-291.

47. Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R. Beumer, and E. Duizer. 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl. Environ. Microbiol*. 78:7769-7775.

48. Vashist, S., D. Bailey, A. Putics, and I. Goodfellow. 2009. Model systems for the study of human norovirus biology. *Future Virol.* 4: 353-367.

49. Viswanathan, P., J. May, S. Uhm, C. Yon, and B. Korba. 2013. RNA binding by human norovirus 3C-like proteases inhibits protease activity. *Virol*. 438: 20-27.

50. Wang, Q. and K. E. Kniel. 2014. Effectiveness of calcium hypochlorite on viral and bacterial contamination of alfalfa seeds. *Foodborne Path. Dis.* 11(10): 759-768.

51. Wang, W., A. L. Suguitan, Jr., J. Zengel, Z. Chen, and H. Jin. 2012. Generation of recombinant pandemic H1N1 influenza virus with the HA cleavable by bromelain and identification of the residues influencing HA bromelain cleavage. *Vaccine* 30: 872-878.

52. Whitaker, J.R. and L.-S. Lee. 1972. Ficin- and papain-catalyzed reactions. Effect of temperature on reactivity of the essential sulfhydryl group of ficin

in the presence and absence of competitive inhibitors. *Arch. Biochem. Biophys.* 148: 208-216.

53. Xue, Y., C.-Y. Wu, C. J. Branford-White, X. Ning, H.-L. Nie, and L.-M. Zhu. 2010. Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity. *J. Mol. Catal. B: Enzym.* 63: 188–193.

Table 3.1. Treatment Conditions

Enzyme	Buffer	Protease (ppm)	Cysteine-hydrochloride (mM)	Treatment Temperature and Time	pH at 20°C	Active Protease (μg/ml)
Untreated Control	HBSS	0	2	21°C, 10 min 21°C, 60 min	7.10	None detected
	Citrate	0	2	21°C,10 min	5.55	None detected
Heated Control	HBSS	0	2	45°C, 60 min 50°C, 10 min 55°C, 10 min	7.10	None detected
	Citrate	0	2	50°C, 10 min	5.55	None detected
Bromelain	HBSS	2500	2	45°C, 60 min 50°C, 2 to 10 min 55°C, 10 min	7.01	14.1
	Citrate	2500	2	50°C, 10 min	5.49	174.4
Papain	HBSS	2500	2	45°C, 60 min 50°C, 10 min 55°C, 10 min	7.57	4.0
	Citrate	2500	2	50°C, 10 min	5.51	33.4
Ficin	HBSS	2500	2	45°C, 60 min 50°C, 10 min 55°C, 10 min	7.17	20.4
	Citrate	2500	2	50°C, 10 min	5.52	97.7
Bromelain Papain Ficin	HBSS	2500	2	45°C, 60 min 50°C, 10 min 55°C, 10 min	7.10	15.0
	Citrate	2500	2	50°C, 10 min	5.50	95.6

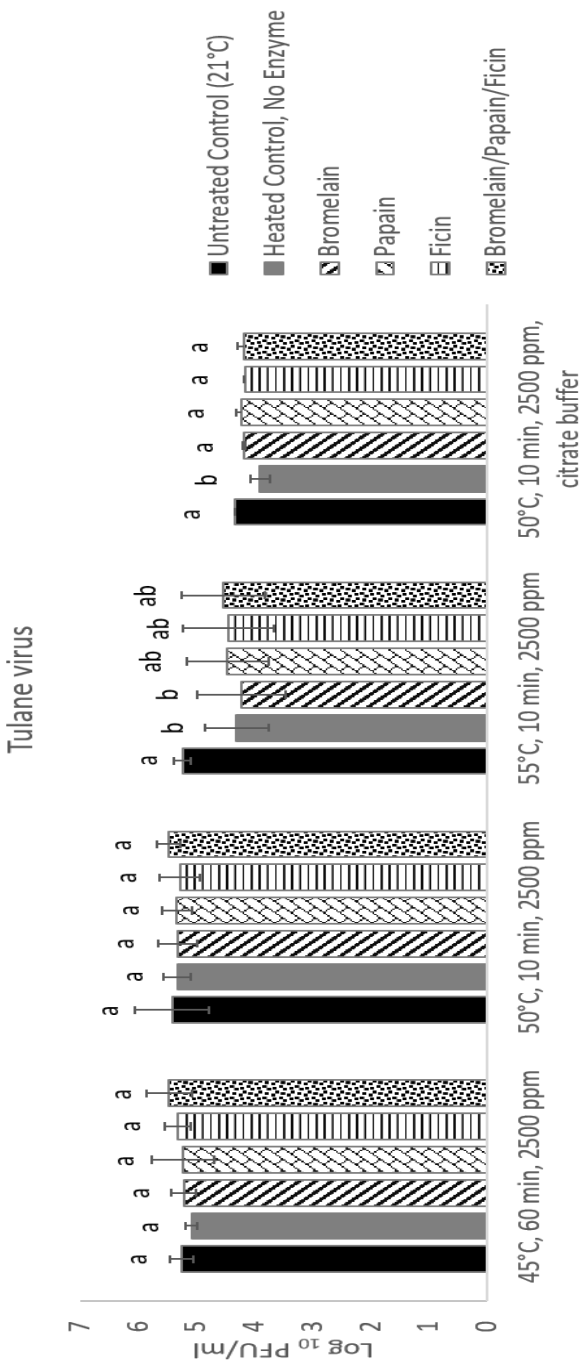
Table 3.2. Detection of murine norovirus RNA added directly to bromelain

Bromelain (ppm)	Temperature (°C)	Time (min)	RT-qPCRU (st dev)
0	Ice bath	10	8.8 (9.5)
2500	50	0	3.5 (4.2)
2500	50	5	None detected
2500	50	10	None detected

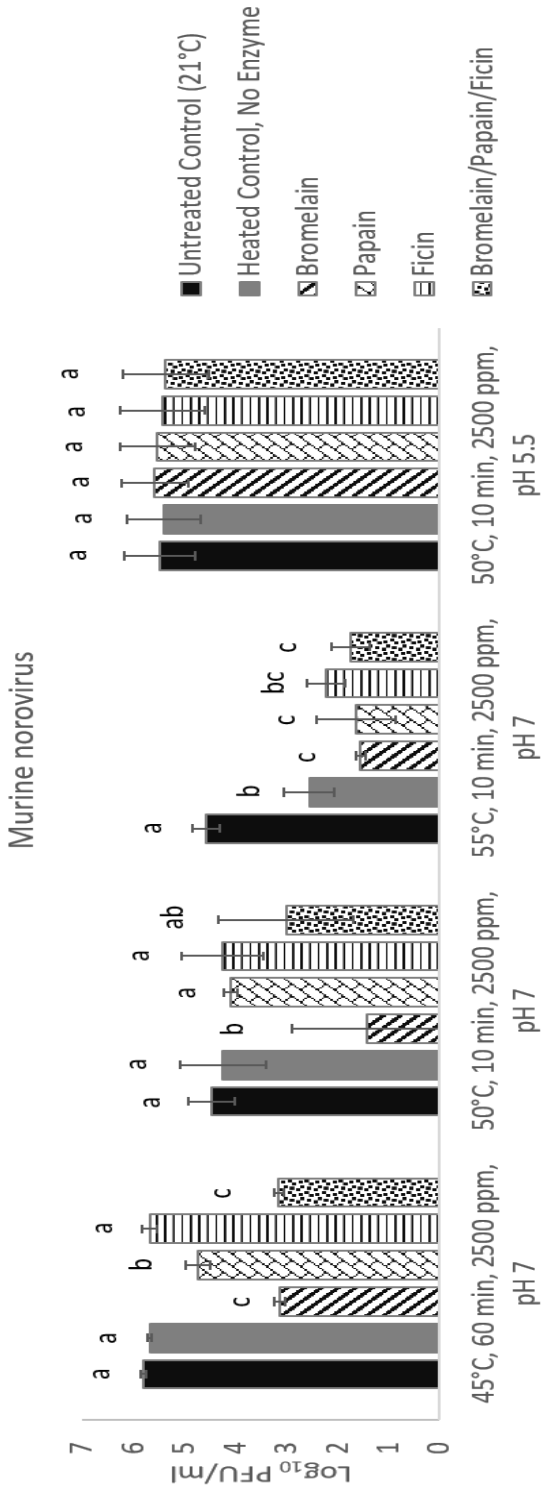
Table 3.3. Human norovirus detected by RT-qPCR after treatment with bromelain (2500 ppm) for 10 min at 50°C

Treatment	Trial 1 (RT-qPCRU)	Trial 2 (RT-qPCRU)
Untreated	1.5	None detected
Bromelain alone	2.0	None detected
Heat alone	2.0	None detected
Bromelain + Heat	1.5	None detected

A


Tulane virus
Log 10 PFU/ml
7
6
5
4
3
2
1
0
a a a a a a a a a a a a a a a a a a a a a a a a
45°C, 60 min, 2500 ppm
50°C, 10 min, 2500 ppm
55°C, 10 min, 2500 ppm
50°C, 10 min, 2500 ppm, citrate buffer
Treatment Conditions
Untreated Control (21°C)
Heated Control, No Enzyme
Bromelain
Papain
Ficin
Bromelain/Papain/Ficin

B


Murine norovirus
Log10 PFU/ml
7
6
5
4
3
2
1
0
a a
a
b
c
c
a
a
a
b
a
a
ab
a
b
c
c
bc
c
a a a a a a
Untreated Control (21°C)
Heated Control, No Enzyme
Bromelain
Papain
Ficin
Bromelain/Papain/Ficin
45°C, 60 min, 2500 ppm, pH 7
50°C, 10 min, 2500 ppm, pH 7
55°C, 10 min, 2500 ppm, pH 7
50°C, 10 min, 2500 ppm, pH 5.5
Treatment Conditions

C

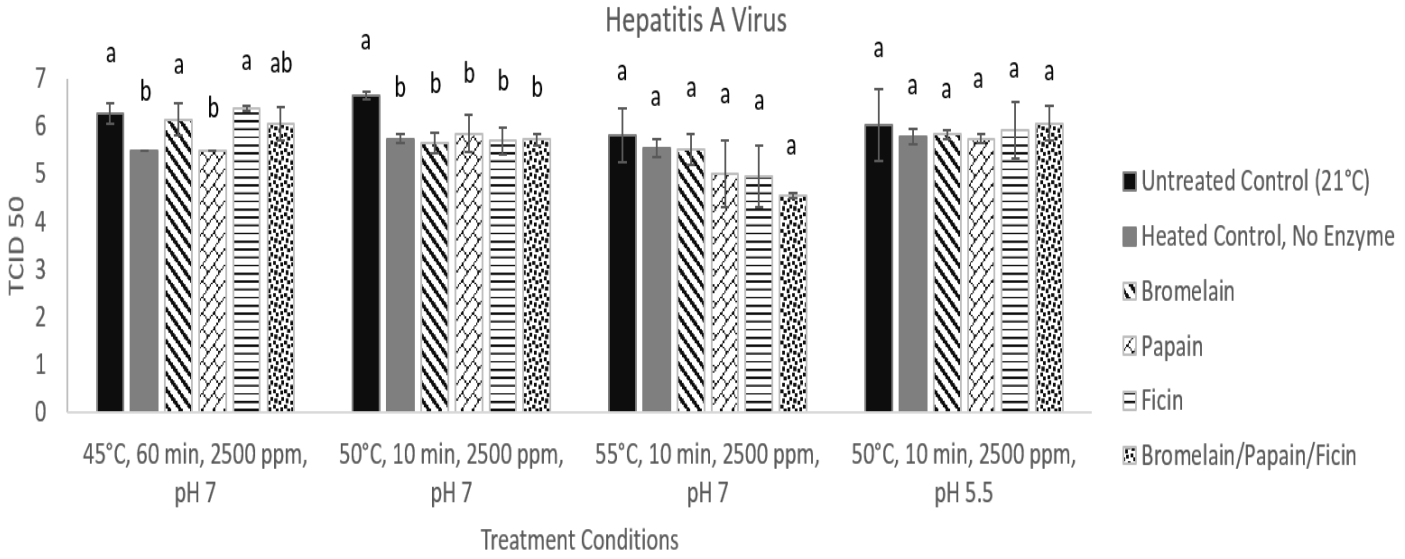


Figure 3.1. Effect of protease treatments on infectivity of A, TV; B, MNV; and C, HAV. Solid black bars represent untreated controls (21°C); all others were treated at indicated temperatures. Detection sensitivity was one $\log_{10}$ PFU/ml. Error bars represent standard deviation. Bars labeled with different letters were significantly different ($p<0.05$).

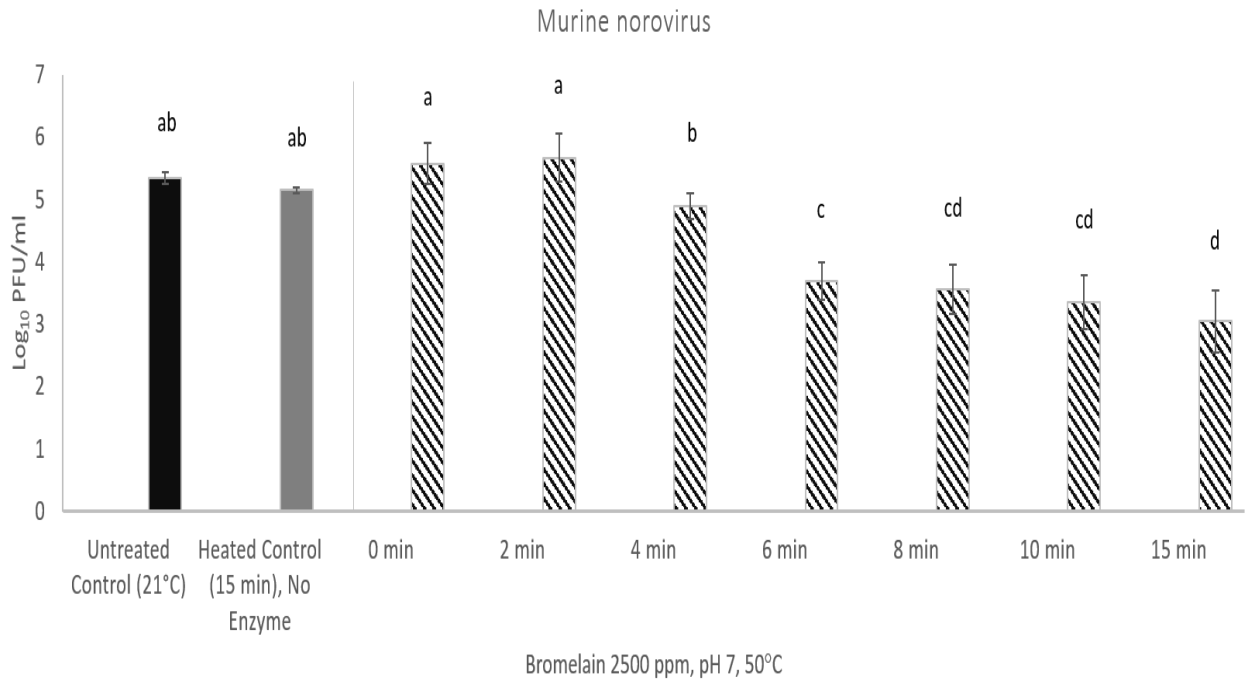


Figure 3.2. Effect of treatment time on inactivation of MNV by bromelain at 50°C as compared to untreated and heated controls. Detection sensitivity was one $\log_{10}$ PFU/ml. Error bars represent standard deviation. Bars labeled with different letters were significantly different ($p<0.05$).

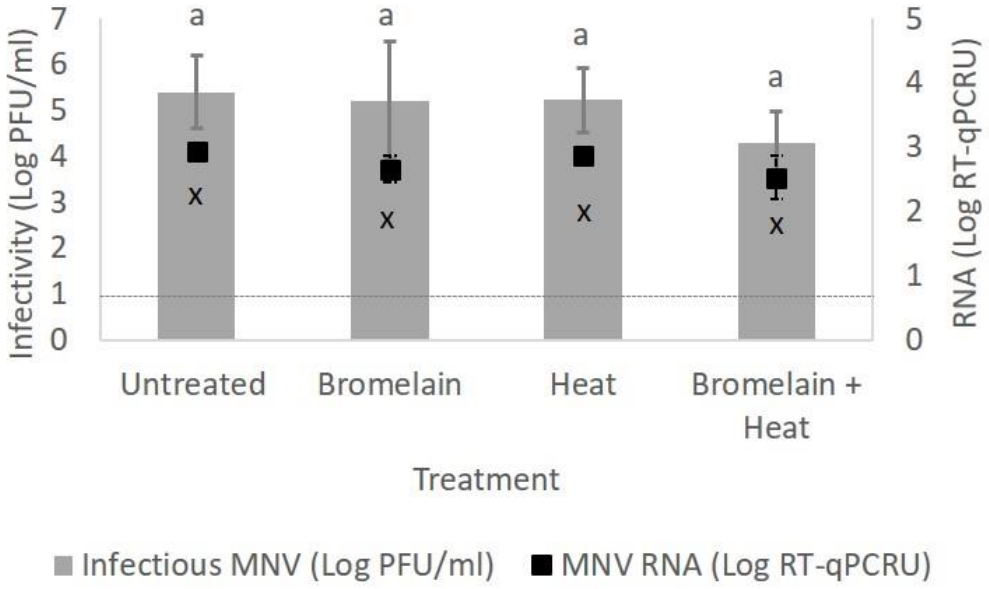


Figure 3.3. Detection of infectious MNV (gray bars) compared to MNV RNA (black squares) after treatment of MNV particles with bromelain (2500 ppm) for 10 min at 50°C. Detection sensitivity was one $\log_{10}$ PFU/ml and one RT-qPCRU. Error bars (gray for infectivity log PFU/ml, black for RNA log RT-qPCRU) represent standard deviation. Bars (a, b) and squares (x, y) labeled with different letters were significantly different ($p<0.05$).

Chapter 4

EFFECT OF BACTERIAL CELL-FREE SUPERNATANTS ON INFECTIVITY OF NOROVIRUS SURROGATES

4.1. Abstract

Bacterial metabolic products were evaluated for inhibitory effects on viral propagation in cell culture. Cell-free supernatants (CFS) were prepared from cultures of *Enterococcus faecalis* ATCC 19433, *Pseudomonas fluorescens* ATCC 13525, *Escherichia coli* 08, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* 168, *Bacillus coagulans* 185A, *B. coagulans* 7050, *Clostridium sporogenes* PA3679, and a commercial probiotic of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Lactobacillus salivarius, and Streptococcus thermophilus* in microbiological media or milk. Inhibitory effects of CFS on propagation of murine norovirus-1 (MNV) and Tulane virus (TV) in RAW 264.7 and LLCMK2 cells, respectively, were evaluated in the continuous presence of CFS or after exposure of host cells to CFS. Slight inhibition of viral propagation was observed for MNV and TV in the continuous presence of CFS of *B. subtilis* 168 and *E. faecalis* 19433, respectively. CFS cytotoxicity was also determined by microscopic examination. Virus persisted in the CFS that demonstrated cytotoxic effects suggesting a lack of direct effect of CFS on

virions. Viral propagation indicates a general lack of competitive inhibition by bacterial extracellular products and bears significance in understanding the persistence of virus in food and human systems shared by bacteria that are recognized for their colonization and competitive capabilities.

4.2. Introduction

Both cooperative and antagonistic interactions occur in microbial communities. Examples include quorum sensing among bacteria, bacteriocin production, protozoan internalization of bacteria, and infection of bacteria by viruses. Such interactions are often specific and exist among a narrow range of microorganisms where there is an apparent basis for self-preservation. The nature of interactions between bacteria and non-bacteriophage viruses is less well characterized. Bacterial biofilms have been reported to provide a structure within which virus may become entrapped and persist in an infectious state as has been demonstrated in laboratory-simulated water systems (*14, 16, 21*). Another study revealed interactions between virus-like particles of norovirus and crude protein extracts of microorganisms grown from wastewater sludge, raising speculation, though yet to be tested, that bacteria may possess or release proteins that could bear significance in viral transmission (*17*). Conversely, there have been several reports of antiviral effects of metabolic by-products of environmental and intestinal bacteria, including probiotic bacteria, on enveloped and non-enveloped viruses (*1, 2, 3, 4, 5, 6, 10, 15, 20, 24, 25, 26, 27*). Some of these reported antiviral effects were reduced by the addition of proteases or

protease inhibitors thereby suggesting bacterial proteolysis of virus (*5, 6*); whereas, some bacteria demonstrated antiviral effects that were proposed to be related to small molecular weight products of less than 1,000 daltons (*4, 6*). Other mechanisms of reported antiviral activity were speculated to involve the inhibition of infection by blocking cellular receptors, inhibition of some later stage of viral replication, viral aggregation, or crosstalk with host cells (*1, 10, 20, 24, 25, 26, 27*). A greater understanding of bacterial and viral interactions may aid in understanding viral persistence and point to potential control measures. In particular, a probiotic-derived antiviral compound could have significant benefit for clinical and/or safe food-processing applications depending on the nature of the antiviral effect, whether in the inhibition of interaction with the host or in direct effects on viral integrity. This study was undertaken to evaluate the effects of cell-free supernatants of bacteria important to food systems as the normal microbiota of soil, plant tissue, human digestive, and/or skin systems on the propagation of murine norovirus-1 (MNV) and Tulane virus (TV), viruses of the Caliciviridae genus which is shared by human norovirus.

4.3. Materials and Methods

4.3.1. *Cultures and cell lines*

4.3.1.1. *Viruses and host cells*. Murine norovirus (MNV-1) was propagated by inoculation in confluent 24-h RAW 264.7 cells incubated at 37ºC and 5% CO_2 for 48 h in Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech, Inc., Manassas, VA) containing 4.5 g l^{-1} of glucose, L-glutamine and sodium pyruvate

amended with fetal bovine serum (FBS, HyClone, Logan, UT) at 10% v/v, 4 mM glutamax (Invitrogen), sodium bicarbonate (Mediatech), and antibiotic/antimycotic (100 U penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml-1 amphotericin B, HyClone). Infected RAW cells were subjected to three freeze/thaw cycles (-80ºC/25 to 37ºC), collected, and pelleted by centrifugation (233 x *g*). The virus in the supernatant was collected, aliquoted and stored at -80ºC until further use. Tulane virus (TV) was propagated by inoculation of 24-h confluent LLCMK2 cells, incubated for 55 h at 37ºC with 5% CO_2 in Medium 199/EBSS (M199 with Earles Balanced Salts and L-glutamine, HyClone) amended with 10% FBS and antibiotic/antimycotic mix as stated above. TV was collected from LLCMK2 cells as previously described for MNV and stored at -80ºC until use.

4.3.1.2. *Bacteria*. *P. fluorescens* ATCC 13525, *E. coli* 08 (an avian isolate), *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 19433, *B. subtilis* 168, *B. coagulans* 185A, *B. coagulans* 7050, and *C. sporogenes* PA3679 were obtained from the culture collection of the University of Delaware, Department of Animal and Food Sciences. A commercial probiotic mixture of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Lactobacillus salivarius, and Streptococcus thermophilus* (Advanced CD Accuflora® Pro-biotic Acidophilus, Northwest Natural Products® Inc., Vancouver, WA) was purchased at a local pharmacy.

4.3.2. *Preparation of Cell-free Supernatants and Controls.* Bacteria were grown overnight under the conditions presented in Table 4.1. All were subcultured into

fresh media, except for the Accuflora® probiotic mix, and incubated approximately 17 h. Sporeforming bacteria were heat-shocked at 80ºC for 10 min prior to initial incubation. Gaspak jars with BD BasPak EZ Anaerobic Container System (Becton, Dickinson, and Co., Sparks, MD) were used to create anaerobic conditions. Cells of stationary-phase bacteria were pelleted by centrifugation at 2095 x *g* for 10 min, and the supernatant was withdrawn and filtered (0.2-µm pore size). The pH of the CFS and uninoculated media controls was measured at full concentration and at 10% v/v in DMEM and M199 cell culture media as used in viral propagation experiments with a Mettler Toledo FE20/EL20 pH meter (Columbus, OH). Cell-free supernatants were stored at -20ºC until use in antiviral activity assays.

4.3.3. *Assay for Antiviral Activity of CFS*

4.3.3.1. *Viral infection and propagation.* The effect of CFS on the inhibition of viral propagation in cell culture was evaluated by adaptation of the viral yield reduction assay described by Todorov *et al.* (*24, 25*). Host cells were identically inoculated into 25-cm^2 cell-culture flasks and the monolayer grown to approximately 90% confluency. Spent medium was aspirated and cells were rinsed with Hanks Balanced Salt Solution, 1x with calcium and magnesium (HBSS, Mediatech). Cells were exposed to CFS and virus by two methods: (1) Cells were overlaid with 10% CFS in 5 ml cell culture media and immediately inoculated with virus, and (2) cells were overlaid with 10% CFS in cell culture media for 3 h followed by aspiration of media, triplicate rinsing with HBSS to

remove CFS, and infection with virus in fresh cell culture media. The second method was conducted to test for potential infection inhibition as a result of direct irreversible effects of CFS on host cells. Virus was inoculated at approximately 10^2 to 10^3 PFU ml^{-1} and allowed to propagate for 44 h at 37ºC and 5% CO_2. Initial viral inoculum was determined by inoculation of an additional flask of cells with virus followed by immediate freezing at -80ºC and recovery as in test and control samples. Host cells exposed to 10% HBSS in cell culture media in the absence of viral infection served as negative controls. Host cells exposed to 10% v/v HBSS in cell culture media with infection by virus served as positive controls. The use of 10% HBSS was to account for the same reduction in concentration of cell culture media as was done with the addition of CFS. Additional control media included tryptic soy broth (TSB), TSB with 0.6% yeast extract (TSBYE), Lactobacilli MRS broth, UHT milk with 1% milkfat, UHT milk acidified with DL-lactic acid (Sigma, St. Louis, MO) (milk/LA) to the same pH of 4.81 as the milk after fermentation by Accuflora® probiotic mix, and the filtered (0.2-um pore size) supernatant of acidified milk (milk/LA/FS). Duplicate viral propagation trials were conducted.

4.3.3.2. *Recovery and quantification of viral propagation.* After the 44-h infection period, cells were observed microscopically for cytopathic effects (CPE), subjected to three freeze/thaw cycles, scraped from the flask, collected, vortexed, and pelleted as described in section 4.3.1.1 for virus propagation. Virus in the supernatant was enumerated by plaque assay (*9, 28*). Host cells were

grown to approximately 90% confluency in 12-well cell culture plates for 24 h in media as described in section 4.3.1.1. Virus was serially diluted in cell culture media and infected overnight under static conditions at 37ºC and 5% CO_2 with two wells inoculated per dilution. The inoculum was aspirated, and cells were overlaid with a 1:1 mixture of 3% SeaPlaque Agarose (Lonzo, Rockland, ME) and cell culture medium. For MNV plaque overlay, 2X MEM/EBSS (with L-glutamine, HyClone) containing 2% FBS and glutamax, sodium bicarbonate, and antibiotic/antimycotic as described in section 4.3.1.1 for DMEM was used. For TV plaque overlay, M199/EBSS cell culture medium containing 2% FBS and antibiotics/antimycotics was used. After solidification, agarose was overlaid with medium containing 2% FBS, and incubated for an additional 48 h to allow plaque formation. The fluid medium was aspirated and cells were stained for 6 to 8 h with 1% neutral red in medium containing 2% FBS prior to plaque enumeration.

4.3.4. *Assay for cytotoxicity.* The effect of CFS on uninfected cells was evaluated under the same conditions and proportions as infection in the 25-cm^2 flasks except scaled down to 12-well cell culture plates. After exposure to CFS, cells were rinsed with HBSS, overlaid with trypan blue, excess dye was aspirated, and cells viewed for cytotoxic effects as measured by dye uptake, sloughing, and/or morphological changes. Wells with uninfected and viral-infected cells served as controls. Two wells were tested per sample; duplicate trials were conducted.

4.3.5. *Data analysis.* Viral propagation was calculated by the difference in the initial viral inocula for each trial and the virus enumerated after infection in the presence of CFS (method 1) or in cells previously exposed to CFS (method 2). Statistical significance ($p<0.5$) in viral propagation among test samples and media controls were determined by analysis of variance and student's t-test with statistical software, JMP Pro Version 10.0.0 (SAS Institute Inc., Cary, NC) (*18*).

4.4. Results and Discussion

The bacteria utilized in the present study are widely associated with the environment, edible plants and animal tissues, human skin, and the gastrointestinal tract; many are among genera known for extracellular protease production (*11, 12*), probiotic potential and previously-reported antiviral effects (*1, 3, 15, 19, 20, 26, 27*). To the authors' knowledge, this is the first study to investigate the effects of bacterial metabolic products on viruses closely related to human norovirus in capsid structure and host receptor molecules.

Slight inhibition of viral propagation by approximately one-log PFU ml^{-1} in the absence of detectable cell cytotoxicity was observed for MNV in the continuous presence of the CFS of *B. subtilis* 168 and for TV in the continuous presence of CFS of *E. faecalis* 19433 as compared to uninoculated TSB, the medium in which these bacteria were grown (Table 4.2). Differences were not observed for these CFS when previously applied to cells and rinsed prior to the addition of virus (data not shown). These bacteria have previously been reported to have antiviral effects against poliovirus (*5*) and hepatitis A virus (*6*). In both of these

studies, antiviral effects were speculated to be due in part to bacterial proteolysis. Hotta *et al.* (*10*) reported antiviral effect of *E. faecalis* (previously known as *S. faecalis*) against herpes simplex virus-1 and adenovirus type 12. In the case of *E. faecalis*, inhibition was not attributed to direct effects on virus, but believed to be inhibitory to adsorption of virus to host cells. Further research is needed to characterize the inhibitory effects observed in the present study against MNV and TV.

Treatments that were cytotoxic to RAW cells at 10% v/v DMEM included MRS medium (pH 7.29), CFS of Accuflora probiotic grown in MRS medium (pH 6.75), CFS of *P. fluorescens* in TSB (pH 7.50), and the CFS of *E. coli* in TSB (pH 7.35). Reduced propagation of MNV in the presence of *P. fluorescens* CFS was observed (Table 4.2); however, while viral propagation was limited by host cell availability, the recovery of MNV was the same as the initial viral inoculum suggesting viral persistence in these samples and a lack of direct effect of CFS on the virus. The cytotoxic effect did not appear to be strictly a function of pH of the treatment as the pH range of controls and other CFS was 6.75 to 8.01. The cytotoxic effect of certain bacteriological media and CFS highlight potential challenges for application of cell culture assay for viral studies involving bacterial growth products even in the absence of live bacteria.

In contrast to some viral propagation data, reduced or no CPE was evident in RAW cells at the 44-h collection point after MNV infection in the continuous presence of acidified milk, filtered supernatant of acidified milk, CFS of the

probiotic mix grown in milk, and CFS of *B. coagulans* 185A, *B. coagulans* 7050, *S. epidermidis* 12228 and *C. sporogenes* PA3679 as determined by morphological changes and/or cell sloughing. Host cell adhesive properties, proliferation and immune response may be worthy of investigation as this was only observed in the RAW macrophage cell line. A time course study for viral collection, extended incubation, as well as a time-of-addition study for extracts may help elucidate if the treatments delayed some stage of viral multiplication or cell egress to help explain this apparent contradiction between the absence of observed CPE in spite of viral propagation. These data highlight the need to exercise caution in relying solely on CPE observations for assessment of antiviral activity.

This experiment was designed to mirror previous studies that evaluated the effect of individual bacteria culture products on viruses. The caliciviruses selected for this study are commonly-used surrogates for human norovirus (HuNoV) based on their infectivity in cell culture, genetic similarity to HuNoV, and, in the case of TV, interaction with histo-blood group antigens (*7*, *8, 22*). At the conditions tested, the bacterial extracts demonstrated slight or no antiviral activity, either directly on the virus or in inhibition of interaction with host cells. This study is limited to the conditions supportive of cell culture assays and pure culture of bacteria, and thus may not fully reflect exposures that could occur in natural environments including variable concentrations of metabolic products, temperatures, pHs, exposure times, other biological materials with which virus

and bacteria may interact in the gastrointestinal tract or environment, as well as various combinations and states of bacteria. For example, the production of bacterial proteolytic enzymes has been demonstrated to increase in a temperature-dependent manner when present in co-culture biofilms as compared to planktonic, single-culture bacteria (*23*). However, given that infectious MNV reportedly persists over a 20-day period in a fermented vegetable product with a final pH below 4.0 (*13*), direct effects of bacteria on virus in foods may also be absent as was observed in this study.

The data do not point to potential viral control strategies by most bacterial CFS, although it may be worthy to further study persistence of these viruses in the presence of live bacteria to evaluate the influence of cell-bound properties, as well as other CFS concentrations, duration of exposure, and retention of CFS treatment on cells prior to the addition of virus. Inasmuch as the tested viruses may represent similar noneveloped viruses of significance to humans, these findings may help in understanding the persistence of virus in environments shared by bacteria that are recognized for their colonization and competitive capabilities.

4.5. Acknowledgement

This project was funded in part by the USDA NoroCORE grant # 1111-2011-0494.

REFERENCES

1. Botić, T., T.D. Klingberg, H. Weingartle, and A. Cencic. 2007. A novel eukaryotic cell culture model to study antiviral activity of potential probiotic bacteria. *Int. J. Food Microbiol.* 12: 227-234.

2. Carver, D.H. and K. Naficy.1962. Inhibition of Arbor viruses (Group A) by a protein-like constituent of a *Corynebacterium*. *Proc. Soc. Exp. Biol. Med.* 111(2): 356-360.

3. Choi, H-J., J-H. Song, Y-J. Ahn, S-H. Baek, and D-H. Kwon. 2009. Antiviral activities of cell-free supernatants of yogurts metabolites against some RNA viruses. *Eur. Food Res. Technol.* 228: 945-950.

4. Cliver, D.O. and J.E. Herrmann. 1972. Proteolytic and microbial inactivation of enteroviruses. *Water Res.* 6: 797-805.

5. Deng, M.Y. and D.O. Cliver, 1992. Inactivatioon of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manure. *Appl. Environ. Microbiol.* 58(6): 2016-2021.

6. Deng, M.Y. and D.O. Cliver, 1995. Antiviral effects of bacteria isolated from manure. *Microbial. Ecol.* 30: 43-54.

7. Farkas, T., R.W. Cross, E. Hargit III, N.W. Lerche, A.L. Morrow, and K. Sestak. 2010. Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. *J. Virol.* 84(17): 8617-8625.

8. Farkas, T., K. Sestak, C. Wei, and X. Jiang. 2008. Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *J. Virol.* 82(11): 5408-5416.

9. Hirneisen, K.A. and K.E. Kniel, 2013. Comparing human norovirus surrogates: Murine norovirus and Tulane virus. *J. Food Prot.* 76(1): 139-143.

10. Hotta, S., M. Kojima, M. Fujisaki, S. Uchida, H. Kuroda, and C. Hamada, 1977. An antiviral substance extracted from *Streptococcus faecalis*. *Nature* 268: 733-734.

11. Lake, D.E., D.T. Bernard, and D.A. Kautter, 1992. Mesophilic anaerobic sporeformers, p. 275-289. *In* C. Vanderzant and D.F. Splittstoesser (eds.), Compendium of Methods for the Microbiological Examination of Foods, 3rd edition. American Public Health Association, Washington, D.C.

12. Lee, J.S. and A.A. Kraft, 1992. Proteolytic microorganisms, p. 193-198 *In* C. Vanderzant and D.F. Splittstoesser (eds.), Compendium of Methods for the Microbiological Examination of Foods, 3rd edition. American Public Health Association, Washington, D.C.

13. Lee, M.H., S.-H. Yoo, S.-D. Ha, and C. Choi.2012. Inactivation of feline calicivirus and murine norovirus during *dongchimi* fermentation. *Food Microbiol.* 31: 210-214.

14. Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkänen, L. Maunula, C-H. von Bonsdorff, P. J. Martikainen, S. A Wilks, C. W. Keevil, and I.T. Miettinen, 2007. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and Caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl. Environ. Microbiol.* 73(9): 2854-2859.

15. Moreno Muñoz, J.A., E. Chenoll, B. Casinos, E. Battler, D. Ramón, S. Genovés, R. Montava, J.M. Ribes, J. Buesa, Fábrega, and M. Rivero. 2011. Novel probiotic *Bifidobacterium longum* subsp. Infantis CECT 7210 strain active against rotavirus infections. *Appl. Environ. Microbiol.* 77(24): 8775-8783.

16. Quignon, F., M. Sardin, L. Kiene and L. Schwartzbrod. 1997. Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Appl. Environ. Microbiol.* 63(3): 978-982.

17. Sano, D., K. Wada, T. Imai, Y. Masago, and T. Omura. 2010. Norovirus-binding proteins recovered from activated sludge micro-organisms with an affinity to a noroviral capsid peptide. *J. Appl. Microbiol.* 109: 1923-1928.

18. SAS Institute Inc. 2012. JMP® Pro 10.0.0 64-bit Edition.

19. Seo, B.J., I.A.Rather, V.J.R. Kumar, , U.H. Choi, , M.R. Moon, , J.H. Lim, and Y.H. Park. 2012. Evaluation of *Leuconostoc mesenteroides* YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. *J. Appl. Microbiol.* 113: 163-171.

20. Serkedjieva, J., S. Danova, and I. Ivanova. 2000. Antiinfluenza virus activity of a bacteriocin produced by *Lactobacillus delbrueckii*. *Appl. Biochem. Biotechnol.* 88: 285-298.21.

21. Skraber, S., J. Schijven, C. Gantzer, and A.M.de Roda Husman. 2005. Pathogenic viruses in drinking-water biofilms: a public health risk? *Biofilms* 2: 105-117.

22. Taube, S., J.W. Perry, E. McGreevy, K. Yetming, C. Perkins. K. Henderson, and C.E. Wobus. 2012. Murine noroviruses bind glycolipid and glycoprotein attachment receptors in a strain-dependent manner. *J. Virol.* 86(10): 5584-5593.

23. Teh, K.H., S. Flint, J. Palmer, P. Andrewes, P. Bremer, and D. Lindsay,. 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. *Int. J. Food Microbiol.* 157: 28-34.

24. Todorov, S.D., M.B. Wachsman, H. Knoetze, M. Meincken, and L.M.T. Dicks. 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. *Int. J. Antimicrob. Agents* 25: 508-513.

25. Todorov, S.D., M. Wachsman, E. Tomé, X. Dousset, M.T. Destro, L.M.T. Dicks, B.D. Gombossy de Melo Franco, M. Vaz-Velho, and D. Drider. 2010. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. *Food Microbiol.* 27: 869-879.

26. Wachsman, M.B., M.E. Farías, E. Takeda, F. Sesma, A.P. de Ruiz Holgado, R.A. de Torres, and C.E. Coto. 1999. Antiviral activity of enterocin CRL35 against herpesviruses. *Int. J. Antimicrob. Agents* 12: 293-299.

27. Wachsman, M.B., V. Castilla, A.P. de Ruiz Holgado, R.A. de Torres, F. Sesma, and C.E. Coto. 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. *Antiviral. Res.* 58: 17-24.

28. Wei, J., Y. Jin, T. Sims, and K.E. Kniel. 2010. Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by Romaine lettuce. *Appl. Environ. Microbiol.* 76(2): 578-583.

Table 4.1. Bacterial growth conditions for CFS preparation

Bacterial culture	Growth medium [a]	Temperature (ºC)	Atmospheric Conditions
E. faecalis 19433	TSB	37	Aerobic
P. fluorescens 13525	TSB	Ambient (~22)	Aerobic
S. epidermidis 12228	TSB	37	Aerobic
B. subtilis 168	TSB	37	Aerobic
B. coagulans 185A	TSB	37	Aerobic
B. coagulans 7050	TSB	37	Aerobic
E. coli 08	TSB	37	Aerobic
C. sporogenes PA3679	TSBYE	37	Anaerobic
Accuflora® *L. acidophilus* *L. rhamnosus* *B. bifidum* *L. salivarius* *S. thermophilus*	MRS and milk (UHT, 1% milkfat)	Ambient (~22)	Anaerobic

[a] TSB, Tryptic soy broth, TSBYE (TSB amended with 0.6% yeast extract), MRS (Difco Lactobacilli MRS Broth), milk, commercially ultra-high temperature (UHT) processed and containing 1% milkfat.

Table 4.2. Viral propagation in continuous presence of bacterial cell-free supernatants

	Viral Propagation (PFU/ml) (st. dev.)*	
	MNV-1	TV
HBSS	5.15 (0.38)[a]	2.38 (0.1.) [x]
TSB	5.13 (0.05) [a]	2.37 (0.44) [x]
B. coagulans 185A	3.92 (0.02) [ab]	2.06 (0.06) [xy]
B. coagulans 7050	3.93 (0.08) [ab]	2.01 (0.15) [xyz]
B. subtilis 168	3.14 (0.99) [b]	2.09 (0.04) [xy]
E. coli 08	4.40 (0.43) [ab]	2.15 (0.37) [xy]
E. faecalis 19433	4.39 (0.17) [ab]	1.53 (0.38) [yz]
Ps. fluorescens 13525	2.81 (1.54) [b]	1.91 (0.13) [xyz]
S. epidermidis 12228	3.75 (0.19) [ab]	2.26 (0.81) [x]
TSBYE	4.10 (0.94) [ab]	2.41 (0.35) [x]
C. sporogenes PA 3679	4.11 (0.16) [ab]	1.82 (0.12) [xyz]
Milk	5.27 (0.04) [a]	2.43 (0.29) [x]
Milk acidified with lactic acid	3.98 (0.57) [ab]	2.45 (0.32) [x]
Milk acidified with lactic acid, filtered	4.41 (0.05) [ab]	2.35 (0.30) [x]
Accuflora® in Milk	4.95 (0.54) [a]	2.34 (0.37) [x]
Accuflora® in MRS	Not determined	2.37 (0.47) [x]
MRS	Not determined	2.43 (0.38) [x]

* Values within a column that are significantly different ($p<0.5$) are indicated by different superscripts.

Chapter 5

EFFECT OF BACTERIA AND BACTERIAL CONSTITUENTS ON RECOVERY AND RESISTANCE OF HUMAN NOROVIRUS AND TULANE VIRUS

5.1. Abstract

Norovirus encounters numerous and diverse bacterial populations in the host and environment, but the impact of bacteria on norovirus transmission, infection, detection and inactivation resistance are not well understood. Human norovirus (HuNov) and its surrogate, Tulane virus (TV), were investigated for interactions with viable bacteria, bacterial metabolic products, and bacterial cell constituents and evaluated for impact on viral recovery and inactivation resistance. TV was incubated with common soil, human intestinal, skin, and phyllospheric bacteria and recovered by centrifugation and filtration to detect unbound virus. The cell-free supernatant (CFS) of *Bifidobacterium bifidum* 35914, which produces glycan-modifying enzymes, was evaluated for recovery of HuNov from stool and *Enterobacter cloacae* 13047 and propagation of TV in LLC-MK2 cells. The impact of *Escherichia coli* O111.B4 lipopolysaccharide (LPS) and *Bacillus subtilis* peptidoglycan (PEP) was evaluated for impact on TV thermal and chlorine inactivation resistance. Incubation of TV with various bacteria did not impede viral recovery suggesting a lack of direct, stable binding between virus and

bacteria. The CFS of *B. bifidum* did not enhance recovery of HuNov from stool or suspension of *E. cloacae* nor did it reduce TV propagation. PEP increased TV thermal and chlorine inactivation resistance as compared to control TV in phosphate buffered saline (PBS). At 60°C, TV suspended in PBS and LPS was reduced by more than 3.7-$\log_{10}$ PFU/ml; whereas in PEP, TV reduction was approximately 2-$\log_{10}$ PFU/ml. Chlorine treatment at 200 ppm rendered TV undetectable (> 3-$\log_{10}$ PFU/ml reduction) in PBS and LPS ; however, TV was still detected in PEP, reduced by 2.9-$\log_{10}$ PFU/ml. Virus inactivation studies and food processing practices should account for this potential impact of bacteria on viral resistance.

5.2. Introduction

Norovirus has an estimated annual burden of 20 million illnesses in the United States (Hall *et al*., 2014) and 700 million illnesses globally (Belliot *et al*., 2014) and is the leading cause of foodborne gastroenteritis (Hall *et al*., 2014). Accordingly, the transmission, persistence, and resistance of human norovirus (HuNov) or its surrogates have been investigated (D'Souza *et al*., 2006; Escudero *et al*., 2012; Hirneisen and Kniel, 2013; Kotwal and Cannon, 2014; and Tuladhar *et al*., 2012). Some norovirus research has been conducted with virus alone in model systems, notably either in the absence of bacteria or without characterization of naturally-occurring bacteria, in spite of the vast quantity and diversity of bacteria that virus encounter in the environment (Lindow and Brandl, 2003; Wiken Dees *et al*., 2015) and host (Palmer *et al*., 2007). This approach

enhances the understanding of virus behavior with fewer confounding factors and minimizes methodology limitations encountered in isolating and enumerating virus from complex matrices; however, microbial communities have been demonstrated to impact the survival of microorganisms.

Interactions between virus and bacteria could have considerable implications for virus persistence, transmission, detection, and resistance to inactivation treatments. The potential for accumulation of viruses in bacterial biofilms in drinking water systems has been demonstrated for poliovirus-1 and bacteriophages B40-8 and MS2 (Lehtola *et al*., 2007; Quignon *et al*., 1997; Skraber *et al*., 2005). Virus may persist in these biofilms for days to months (Skraber *et al*., 2005), and are presumed to be afforded protection from environmental and chemical stresses as has been reported for bacteria embedded in a biofilm matrix. The presence of bacterial cell surface constituents, lipopolysaccharide and peptidoglycan, even in the absence of live bacteria, has been shown to enhance the thermal and chlorine resistance of the enteric virus, poliovirus (Robinson *et al*., 2014).

With particular regard to norovirus, several studies have demonstrated direct interaction between bacterial surface and viral capsid molecules. Bacterial proteins isolated from activated sludge have been demonstrated to bind ligands of conserved human norovirus (HuNov) amino acid capsid sequences (Sano *et al*., 2010). Direct associations between virus and several bacterial genera normally found in the human gastrointestinal tract have also been reported and

attributed to undefined, but potentially specific receptor-ligand interactions (Almand *et al*., 2017; Sano *et al*., 2010). The interaction of human norovirus with the exopolysaccharide of a bacterium closely-related to *Enterobacter cloacae* was attributed to moieties similar to histo-blood group antigens (HBGA) on the exopolysaccharide (Miura *et al*., 2013). Norovirus is known to bind HBGAs of host mucosal epithelial cells (Karst, 2010; Singh, *et al*., 2015; Tan and Jiang, 2005), and these molecules are recognized as one, although not necessarily the only, receptor on host cells (Karst, 2010; Murakami *et al*., 2013). The presence of bacteria with HBGA-like surface molecules or exogenous HBGA were reported to support norovirus infection of host B cells (Jones *et al*., 2014). Although the adherence of norovirus virus-like particles to HBGA-like moieties of plant and oyster tissue (Esseili *et al*., 2012; Gandhi *et al*., 2010; Tian *et al*., 2007) has been investigated, direct interactions between norovirus and the bacteria associated with these foods has not been reported (Deng and Gibson, 2017).

Whereas some bacterial surfaces bear HBGA-like moieties, other enteric bacteria produce enzymes capable of degrading blood group antigens. *Ruminococcus torques*, *Bifidobacterium bifidum*, and *B. infantis* have been demonstrated to constitutively produce extracellular glycosidases capable of degrading blood group A, B, and/or H-antigens among other mucin glycoproteins (Hoskins *et al*., 1985). Earlier studies demonstrated the production of extracellular and cell-bound blood-group degrading (BGD) enzymes by

pathogenic bacteria, *Clostridium perfringens* (Aminoff and Furukawa, 1970) and *Shigella dysenteriae* (Prizont, 1982), respectively.

Bacteria that produce glycan-modifying enzymes have been demonstrated to alter surfaces of some bacterial (Cromwell *et al*., 1977) and human intestinal cells (Varyukina et al., 2012). Degradation of B-like surface antigens of *Escherichia coli* 086 in the presence of fecal bacteria or their extracts derived from individuals with B secretor type has been demonstrated (Cromwell *et al*., 1977). Soluble factors with glycan-modifying properties derived from *Bacteriodes thetaiotaomicron* and *Lactobacillus casei* inhibited infection of rotavirus infection in human intestinal cells (Varyukhina *et al.*, 2012). The impact of BGD enzymes on norovirus interaction with either host cells or bacteria with HBGA-like surface moieties has not been evaluated.

The impact of these viral-bacterial interactions on virus survival and transmission between environment and host remains to be characterized. In consideration of the binding previously reported between norovirus and fecal bacteria, the interaction of virus and bacteria associated with plant foods commonly implicated as norovirus transmission vehicles warrants attention. The impact of mixed bacterial populations on norovirus binding, particularly the fecal bacteria that interact with norovirus and the fecal bacteria that produce glycosidases has not been investigated. The role of bacteria or bacterial constituents on viral resistance to treatments commonly used for food production also warrants investigation.

The objectives of this study were (1) to evaluate the effect of common soil, human intestinal, skin, and phyllospheric bacteria on the recovery of human norovirus surrogate, Tulane virus (TV), as an indicator of direct viral binding, (2) to evaluate the metabolic products of bacteria known to produce glycosidases for impact on binding of HuNov to bacteria and TV propagation in cell culture, and (3) to evaluate the effect of bacterial cell constituents on thermal and chlorine resistance of TV.

5.3. Materials and Methods

5.3.1. *Viruses.* HuNov GII was obtained from human stool samples provided by the Delaware Department of Public Health. TV (provided by Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was propagated in LLC-MK2 cells (ATCC CCL-7). LLC-MK2 cells were grown 24 h at 37ºC with 5% CO_2 to confluency in Medium 199/EBSS (M199 with Earles Balanced Salts and L-glutamine, HyClone, Logan, UT) amended with 10% fetal bovine serum (FBS, HyClone) and antibiotic/antimycotic (100 U penicillin, 100 μg ml^{-1} streptomycin, 0.25 μg ml-1 amphotericin B, HyClone). Confluent LLC-MK2 cells were inoculated with TV and incubated approximately 48 h at 37ºC with 5% CO_2. TV was collected from LLC-MK2 cells by three freeze/thaw cycles (-80ºC/25 to 37ºC) and centrifugation (233 x *g*) (Beckman Coulter, Indianapolis, IN, USA) to pellet cellular debris. The supernatant containing the virus was aliquoted and stored at -80ºC.

5.3.2. *Bacteria. Enterobacter cloacae* ATCC 13047, *Enterobacter faecalis* ATCC 19433, *Escherichia coli* 08 (an avian isolate), *Staphylococcus epidermidis* ATCC 12228, *S. aureus* AH1979, *Bacillus subtilis* 168, *B. coagulans* 7050, *Bifidobacterium bifidum* ATCC 35914, and *P. fluorescens* ATCC 13525 were obtained from the culture collection of the University of Delaware, Department of Animal and Food Sciences. *Pseudomonas* sp. and *Flavobacterium* sp. were isolated from processing equipment of a fresh produce processing facility. Bacteria were grown from frozen stocks in either tryptic soy broth (TSB, Acumedia, Neogen Corporation, Lansing, MI, USA) or Lactobacilli MRS Broth (Difco, BD, Sparks, MD) according to conditions outlined in Table I and subcultured prior to use in experiments. Anaerobic conditions were established with BD GasPak EZ Anaerobic container system (BD, Sparks, MD) in GasPak jars with CO_2 indicator (BD). Romaine lettuce was purchased at a local grocery store, and 25 g of leaves were suspended in 225 ml phosphate buffered saline (PBS), pH 7.4 (Mediatech, Inc. Manassas, VA, USA) and stomached for 60 sec. One ml of PBS was transferred to 9 ml tryptic soy broth and incubated at 21°C for approximately 17 h. Growth was subcultured and incubated 17 h in TSB at 21°C with gentle agitation.

5.3.3. *Bacterial cell-free supernatant. Bifidobacterium bifidum* 35914 was grown and subcultured anaerobically in MRS broth at 37°C. Cells of stationary-phase bacteria were pelleted by centrifugation at 2095 x *g* for 10 min, and the supernatant was filtered (0.2-µm pore size) to obtain cell-free supernatant (CFS).

The pH of the CFS was measured with a Mettler Toledo FE20/EL20 pH meter (Columbus, OH). CFS was stored at -20ºC until use in antiviral activity assays.

5.3.4. *Bacterial cell constituents.* Lyophilized preparations of lipopolysaccharide (LPS) of *Escherichia coli* O111.B4 and peptidoglycan (PEP) of *Bacillus subtilis* were obtained commercially (Sigma-Aldrich, St. Louis, MO, USA). The LPS and PEP were each resuspended in phosphate buffered saline, pH 7.4 to 1 mg/ml final concentration for treatment studies.

5.3.5. *Enumeration of infectious virus.* Infectious TV was enumerated by plaque assay (Shearer *et al*., 2014; Wang and Kniel, 2014). LLC-MK2 cells were seeded in 6-well cell culture plates and grown to at least 80% confluency at 37°C and 5% CO_2 with M199 as previously described. Cells were overlaid with samples serially diluted in Hanks Balanced Salt Solution, 1x with calcium and magnesium (HBSS, Mediatech) at 500 µl per duplicate well for each sample. Infected cells were incubated approximately 2.5 h at 37°C and 5% CO_2 after which inoculants were aspirated, and cells were overlaid with 2 ml of a 1:1 mixture of 3% peqGOLD Universal agarose (PEQ Lab, Erlangen, Germany) and M199 containing 2% FBS and antibiotic/antimycotic. Solidified agarose was overlaid with M199 medium containing 2% FBS and incubated for approximately 48 h to allow plaque formation. The fluid medium was aspirated, and cells were fixed for a minimum of 2 h 10% formaldehyde in phosphate buffered saline (PBS, pH 7.4, Mediatech, Inc., Manassas, VA, USA). The fixative was aspirated and agarose removed from the wells. Cells were overlaid with crystal violet (0.05% in

10% ethanol, Fisher Scientific, Kalamazoo, MI, USA) for a minimum of 30 min prior to visual enumeration of plaques.

5.3.6. *Enumeration of viral RNA.* HuNov RNA was extracted with the AllPrep® PowerViral® DNA/RNA Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. Reverse transcription (RT) was performed with the Sensiscript® RT Kit (Qiagen) according to manufacturer protocols at 37°C for 60 min in an Eppendorf thermocycler (Hamburg, Germany). HuNov primers (G2SK-F, 5'-CNTGGGAGGGCGATCGCAA and G2SK-R,5'-CCRCCNGCATRHCCRTTRTACAT, Sigma-Aldrich Corp.) (Kojima *et al*., 2002) targeted the shell domain of capsid protein VP1. Amplification of cDNA was carried out with the QuantiTect SYBR Green PCR kit (Qiagen) using the same primers as for reverse transcription, and real time PCR (qPCR) was performed in a Rotor-Gene Q (Qiagen). qPCR conditions included a hot start at 95°C for 15 min, 40 cycles of 94°C/15 sec denaturation, 60°C/30 sec annealing, and 72°C/30 sec extension with confirmation of amplicons by melt curves. HuNov RNA serially diluted in sterile, nuclease-free water was used to generate standard curves with the lowest dilution of detection designated 1 RT-qPCR unit (RT-qPCRU) (Almand *et al*., 2017, Tuladhar *et al*., 2012).

5.3.7. *Enumeration of bacteria.* Bacteria were serially diluted in buffered peptone water (BPW, Acumedia) and spread plated on tryptic soy agar (TSA, Difco, BD) or Lactobacilli MRS Agar (Difco, BD) and incubated according to conditions in Table I. Bacterial colonies were counted manually after 24 and 48h of incubation.

5.3.8. *TV association with bacteria.* Subcultured bacteria grown to stationary phase (Table I) were washed once with PBS by centrifugation at 1455 *x g* for 15 min and were resuspended in PBS. Cultures were diluted in PBS to obtain an initial inoculum of approximately 7 $\log_{10}$ CFU/ml for each bacterial culture and 5 $\log_{10}$ PFU/ml of TV. Initial bacteria inocula were enumerated as previously described. The suspensions of virus and bacteria were incubated aerobically for 2 h at 22°C with agitation (Clay Adams Nutator, BD) or at 37°C with agitation (100 rpm, Innova 44, New Brunswick Scientific, Enfield, CT, USA) as outlined in Table I. Controls included TV incubated 2 h without bacteria at 4, 22, and 37°C. After the 2-h attachment period, the TV-bacteria suspensions were centrifuged at 233 *x g* for 5 min to lightly pellet bacteria. The supernatant containing TV that did not pellet with the bacteria was filtered through a 13-mm diameter, cellulose acetate, EZFlow syringe filter (0.22 μm) (Foxx Life Sciences, Salem, NH, USA) to remove remaining bacteria for plaque assay enumeration of unbound virus. Two independent trials were conducted.

5.3.9. *HuNov recovery from stool in presence of B. bifidum 35914 CFS.* Human stool containing HuNov GII was mixed 1:1 with phosphate buffered saline, (PBS, pH 7.4) and vortexed vigorously at 30 sec. Aliquots of the diluted stool were diluted 10-fold (v/v) in phosphate buffered saline (PBS, pH 7.4), MRS broth unadjusted at pH 6.4, MRS broth adjusted to pH 4.6 with a 1:1 solution of acetic acid (1 M, Sigma) and L(+)-lactic acid (1 M, Acrōs Organics), or *B. bifidum* 35914 CFS at pH 4.6. All suspensions were vortexed vigorously for 30 seconds.

Duplicate tubes were tested per treatment condition per trial. One set of stool-containing PBS was centrifuged at 13,000 *x g* for 5 minutes to pellet debris. The supernatant was transferred to a fresh sterile tube tested for HuNov RNA. Another set of stool-containing PBS was treated with chloroform (10% v/v final) for 10 min at 21°C, centrifuged, and the supernatant tested for HuNov RNA. Remaining stool suspensions in PBS, MRS pH 6.4, MRS pH 4.6, and CFS were incubated for 6 h at 37°C, vortexed, centrifuged, and supernatants tested for HuNov RNA. Two independent trials were conducted.

5.3.10. *HuNov recovery from E. cloacae* 13047 *in presence of B. bifidum 35914 CFS.* Human stool containing HuNov GII was dispensed into sterile 1.5-ml centrifuge tubes and diluted 10-fold (m/v) with PBS, pH 7.4, MRS broth unadjusted at pH 6.4, MRS broth adjusted to pH 4.6 with a 1:1 solution of acetic acid (1 M, Sigma) and L(+)-lactic acid (1 M, Acrōs Organics), or *B. bifidum* 35914 CFS at pH 4.6. Stool suspensions were vortexed vigorously for 30 sec and centrifuged at 13,000 *x g* for 10 min. The supernatants were filtered to remove stool bacteria (0.22 μm, 13 mm EZ Flow Syringe filter, Foxx Life Sciences, NH, USA), and each supernatant was dispensed into two aliquots. To one aliquot of each medium, *E. cloacae* was added (initial inoculum 5.4-$\log_{10}$ CFU/ml); 10 ul of sterile PBS was added to the other aliquot. *E. cloacae* was grown in TSB, washed and resuspended in PBS prior to inoculation of HuNov-containing supernatants. The HuNov suspensions with and without *E. cloacae* were incubated for 6 h at 37°C and then centrifuged at 13,000 x g for 5 min to pellet *E.*

cloacae. The supernatant was tested for HuNov that did not pellet with *E. cloacae* as well as the *E. cloacae* remaining in the supernatant (3.2 $\log_{10}$ CFU/ml). Two independent trials were conducted.

5.3.11. *TV propagation in presence of B. bifidum 35914 CFS*. The effect of CFS of *B. bifidum* 35914 on the inhibition of viral propagation in cell culture was evaluated by adaptation of the viral yield reduction assay (Shearer *et al*., 2014; Todorov *et al.* 2005; Todorov *et al*., 2010). LLC-MK2 cells were identically-inoculated into 25-cm^2 cell culture flasks and the monolayer grown to approximately 90% confluency. Spent medium was aspirated and cells were rinsed with Hanks Balanced Salt Solution, 1x with calcium and magnesium (HBSS, Mediatech). LLC-MK2 cells were exposed to CFS and virus by two methods: (1) cells were overlaid with 10 or 25% CFS in 5 ml cell culture media and immediately inoculated with TV, and (2) cells were overlaid with 10% CFS in cell culture media for 3h at 37°C and 5% CO_2 followed by aspiration of media, triplicate rinsing with HBSS to remove CFS, and infection with TV in fresh cell culture media. Viral propagation was calculated by the difference in the initial virus inoculum (approximately 10^2 to 10^3 PFU ml^{-1}) and the virus enumerated after incubation for 48 h at 37°C and 5% CO_2. Initial viral inoculum was determined by inoculation of an additional flask of cells with TV followed by immediate freezing at -80ºC and recovery as in test and control samples. Host cells exposed to 10 or 25% HBSS in M199 without virus served as negative controls. Host cells exposed to 10 or 25% v/v HBSS in M199 with virus served as

positive controls. The use of 10% HBSS was to account for the same reduction in concentration of cell culture media as was done with the addition of CFS. Additional control media included MRS broth. Two independent trials were conducted.

5.3.12. *TV thermal resistance in presence of bacterial cell constituents.* TV (4.6 log_{10} PFU/ml) was suspended in PBS, LPS (1 mg/ml), or PEP (1 mg/ml). Aliquots (200 ul) were dispensed into 0.2-ml PCR tube strips (Eppendorf, Hamburg, Germany) and incubated for 2h at 37°C with agitation (100 rpm, Innova 44). After incubation, tubes were placed in a thermocycler (Vapo.Protect, Eppendorf) for 2 min at 20, 55, 60, or 65°C. Tubes were cooled in an ice bath immediately after treatment. Uninoculated PBS, LPS, and PEP were used as negative controls to evaluate cytotoxicity in LLC-MK2 cells. Treated samples were serially diluted in HBSS, and infectious TV was enumerated by plaque assay. The detection limit was 10 PFU/ml. Three independent trials were conducted.

5.3.13. *TV chlorine resistance in presence of bacterial cell constituents.* TV (4 log_{10} PFU/ml) was suspended in PBS, LPS (1 mg/ml), or PEP (1 mg/ml). Aliquots (180 µl) were dispensed into 0.2-ml PCR strip tubes and incubated for 2 h at 37°C with agitation (100 rpm, Innova44). After incubation, 20 µl of the appropriate chlorine stock solution freshly-prepared from sodium hypochlorite in household bleach (Clorox Bleach Concentrate, 6% sodium hypochlorite with 5.7% available chlorine) in PBS was added to obtain final concentrations of 0 (PBS alone), 2, 20, and 200 ppm chlorine for treatment. Chlorine levels of stock solutions were

verified with a chlorine test kit (Hach Company, Loveland, CO, USA). TV was treated for 5 min at 20°C. Uninoculated PBS, LPS, and PEP served as negative controls and for cytotoxicity determination. After treatment, chlorine was quenched with 20 µl each of sodium thiosulfate (5%, Fisher Scientific) and M199 medium amended with 10% FBS). Samples were serially diluted in HBSS, and infectious TV was enumerated by plaque assay. The detection limit was determined to be 10 PFU/ml. Three independent trials were conducted.

5.3.14. *Data analysis.* Statistical significance ($p<0.05$) of treatments was determined by analysis of variance and Student's *t*-test with statistical software JMP Pro, version 13.0.0 (SAS Institute Inc., 2016).

5.4. Results and Discussion

Recent studies have revealed direct interactions between human norovirus and human fecal bacteria (Almand *et al*., 2017) as well as the potential importance of bacteria for norovirus infection (Jones *et al*., 2014). Studies on interactions between other enteric viruses and bacteria suggest contradictory potential impacts on virus depending on the bacteria, their constituents, or metabolic products. Bacteria may aid (Jones *et al*., 2014) or limit (Cliver and Herrmann, 1972; Kandasamy *et al*., 2016; Pant *et al*., 2007) viral infection. Bacteria may enhance viral resistance (Robinson et al., 2014) to disinfection or exhibit antiviral properties (Aboubakr *et al*., 2014; Choi *et al*., 2009). The studies presented herein investigated the impact of various bacteria or their constituents

on binding, recovery, infectivity, and inactivation resistance of HuNov or its surrogate, Tulane virus.

Virus have the potential to be transmitted from fecal contamination to foods throughout production and handling. The potential role of bacteria to serve as carriers of virus to enhance transmission and persistence is unknown. Previous studies demonstrated diminished recovery of human norovirus after incubation with enteric bacteria as an indication of direct binding between bacteria and norovirus (Almand *et al*., 2017). The present study investigated the binding of TV to bacteria associated with the human intestinal tract, human skin, soil, or phyllosphere in consideration of the variety of bacteria that virus may encounter from host to soil to plant surfaces. After incubation of virus and bacteria together, bacteria were pelleted by centrifugation to avoid underestimation of viral recovery due to subsequent blocking of filters, and the supernatant was filtered to remove unpelleted bacteria to avoid overestimation of unbound viral particles. TV incubated with bacteria was recovered at comparable levels as TV incubated without bacteria (Figure 1) for all bacteria studied. These data suggest that direct interactions between TV and bacteria were limited or unstable. These findings do not mirror those reported with human norovirus and various fecal bacteria (Almand *et al*. 2017). Differences in HuNov and TV association with fecal bacteria were reported in previous studies (Almand *et al*., 2017); therefore, in spite of the similarity in interactions with HBGAs, differences in capsid surfaces and receptor binding may account for the lack of binding observed with TV in this

study. The exclusive role of HBGA-like molecules for TV or HuNov binding has not been established nor have all bacteria that bind HuNov been characterized for surface molecules, and thus, other interactions may account for some binding reported in previous studies. Bacterial surfaces were not characterized in the present study. Simultaneous study of HuNov and TV interactions with various bacteria characterized for surface properties would be helpful to better elucidate the role of bacteria for viral environmental transmission and persistence as well as methods for viral detection in complex matrices.

The second set of studies investigated HuNov recovery and TV infectivity in the presence of cell-free growth products by *B. bifidum* 35914. *B. bifidum* has been demonstrated to constitutively produce extracellular glycosidases capable of degrading mucin glycoproteins (Hoskins *et al*., 1985). These include sialidase, β-galactosidase, β-N(Ac)glucosaminidase, and β-N(Ac)galactosaminidase as well as enzymes with activity against blood group H-antigens (Hoskins *et al*., 1985). The impact of these glycosidases to degrade bacterial or host cell surfaces with presumed HBGA-like molecules and the subsequent impact on viral binding and infectivity are unknown, but could bear significance for viral pathogenesis, transmission and detection. The cell-free supernatant of *B. bifidum* was investigated for impact on recovery of HuNov from a stool matrix and from direct interaction with *E. cloacae*. *B. bifidum* CFS was also investigated for impact on cell-culture propagation of TV.

The recovery of HuNov GII from a clinical stool sample using *B. bifidum* CFS as compared to buffer or media was investigated (Figure 5.2). Extraction and detection of HuNov from stool often involves resuspension of stool in PBS. This method was compared to recovery of HuNov GII by PBS subsequently treated with chloroform to inactivate bacteria. HuNov recovery from stool was also investigated by PBS, *B. bifidum* CFS, and bacterial growth media, MRS, at normal pH of 6.4 and adjusted pH of 4.6 to match the pH of the CFS. HuNov was naturally-occurring in these stool samples and not inoculated to a known concentration, so recovery quantification in each medium is relative to the other media. There was greater detection of HuNov GII genomic material from all media as compared to PBS at room temperature; however, HuNov recovery was on the same log scale among the treatments (Figure 5.2). The CFS did not provide any greater recovery of HuNov from the fecal sample suggesting a lack of impact of extracellular metabolic products of *B. bifidum* on viral-bacterial associations in this matrix. The enzymatic composition of the CFS was not characterized in this study; future studies could include direct addition of glycosidases to stool matrices or bacterial suspensions to evaluate this strategy for enhanced recovery of HuNov.

HuNov was previously reported to directly interact with *E. cloacae* potentially through HBGA-like molecules found on the bacteria surface (Almand *et al*., 2017). In the present study, HuNov recovery after co-inoculation with *E. cloacae* in the absence of other stool bacteria was evaluated in PBS, MRS at pH 6.4 and

4.6, and the CFS of *B. bifidum*. Bacteria were pelleted after exposure to HuNov as was done in binding studies with TV; however, the supernatant was not filtered due to the low level of HuNov in the inoculum and consequent likelihood HuNov would decrease below detection with filtration. *E. cloacae* remained in the supernatant at approximately 3.2 $\log_{10}$ CFU/ml as determined with a control treated identically as the samples; therefore, some HuNov detected in the supernatant could be associated with the *E. cloacae* that did not pellet. Detection of HuNov GII genomic material in the supernatant was not significantly different among the media evaluated regardless of the presence or absence of *E. cloacae* (Figure 5.3). The CFS of *B. bifidum* did not enhance recovery of HuNov from interaction with *E. cloacae* greater than was observed with PBS (Figure 5.3).

In the final study with *B. bifidum* CFS, no significant differences were observed in the propagation of TV in positive control media or test samples with 10% *B. bifidum* CFS (Table 5.2). This was observed for both methods of CFS exposure, during the infection period in the absence of FBS or throughout propagation in amended cell culture media. TV propagation in LLC-MK2 cells was inhibited in the presence of 25% CFS in M199; however, 25% CFS was also cytotoxic to LLC-MK2 as evidenced by morphological changes and cell detachment. Although there was a reduction in TV recovered after exposure to 25% CFS as compared to the initial inoculum, the difference was not statistically significant, suggesting the CFS also does not have a direct degradative effect on TV at the levels tested. These data suggest that extracellular metabolic products

of resident intestinal bacteria with reported glycosidase production did not interfere with attachment to host cells *in vitro* as evidenced by equivalent TV propagation in 10% CFS as compared to control media. The impact of mixed bacterial products on viral attachment *in vivo* is unknown.

The final studies addressed the thermal and chlorine resistance of TV suspended in commercial preparations of *Escherichia coli* O111.B4 lipopolysaccharide and *Bacillus subtilis* peptidoglycan. These studies followed a modified experimental design previously reported in which LPS and PEP were demonstrated to increase poliovirus thermal resistance by 3°C as measured by delayed RNA release over a thermal gradient and increased chlorine resistance up to 10^3-fold in PEP as compared to PBS (Robinson *et al*., 2014). The temperatures and chlorine levels selected for the present study were derived from conditions previously reported for TV resistance (Hirneisen and Kniel, 2013). This study builds on those data to evaluate the influence of bacterial cell constituents on viral inactivation resistance as measured by viral infectivity. This would not be possible in cell culture with live bacteria or HuNov.

Both thermal (Figure 5.4) and chlorine (Figure 5.5) resistance of TV increased in the PEP suspension as compared to PBS alone or in suspension with LPS. Notably, the PEP was not fully soluble in buffer. TV infectivity was reduced by less than 2 log PFU/ml in PBS, LPS, and PEP at 55°C as compared to the control at 20°C; these findings are consistent with previously reported TV thermal resistance (Hirneisen and Kniel, 2013). TV was not detected ($>$ 3.7-log PFU/ml

reduction) after 60°C treatment when suspended in PBS and LPS, but was reduced by only 1.99 log PFU/ml in PEP ($p<0.05$). More subtle changes may have been detected if tested over multiple time points at each temperature. With chlorine exposure, TV was reduced by ≤ one log PFU/ml by 2 and 20 ppm chlorine in PBS, LPS, and PEP. Chlorine treatment of 200 ppm rendered TV undetectable (> 3-log PFU/ml reduction) in PBS and LPS; however, TV was still detected in PEP, reduced by 2.86 PFU/ml. The increased chlorine resistance observed with TV in PEP may be due to reduced efficacy of chlorine as a result of increased organic load, although this was not observed at the conditions tested with LPS. Alternatively, the increased resistance may be due to some direct association with PEP that provides protection to TV.

In conclusion, full recovery of TV from suspensions of fecal, skin, soil, and phyllospheric bacteria imply individual bacteria of these strains may not specifically bind and serve as direct carriers for TV; however, the correlation to HuNov and role of mixed bacterial communities and their products for viral protection and transmission remain to be evaluated. Similarly, the extracellular products of a probiotic bacterium known to produce glycan-modifying enzymes did not alter HuNov recovery from fecal suspension, binding to *E. cloacae*, or TV propagation in these model systems; however, more basic study of the CFS, individual glycosidases, and specific viral-bacterial and viral-host interactions may be worthy of investigation. Virus in suspension of bacterial cell constituents

had increased inactivation resistance; and thus, inactivation studies and food processing practices should account for this potential impact.

5.5. Acknowledgement

This material is based upon work that is supported in part by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 1111-2011-0494.

REFERENCES

1. Aboubakr, H. A., A. A. El-Banna, M. M. Youssef, S. A. A. Al-Sohaimy, and S. M. Goyal. 2014. Antiviral effects of *Lactococcus lactis* on feline calicivirus, a human norovirus surrogate. *Food Environ Virol*. 6: 282-289.

2. Almand EA, M. D. Moore, J. Outlaw, and L. Jaykus. 2017. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS One* 12(3): e0173124.doi:10.1371/journal.pone.0173124.

3. Aminoff, D. and K. Furukawa. 1970. Enzymes that destroy blood group specificity. *J. Biol.Chem.* 245: 1659-1669.

4. Belliot, G., B. A. Lopman, P. Pothier, K. Ambert-Balay, and P. Pothier. 2014. The burden of norovirus gastroenteritis: an important foodborne and healthcare-related infection. *Clin. Microbiol. Infect*. 20, 724–730. http://dx.doi.org/10.1111/1469-0691.12722

5. Choi, H-J., J-H. Song, Y-J. Ahn, S-H. Baek, and D-H. Kwon. 2009. Antiviral activities of cell-free supernatants of yogurts metabolites against some RNA viruses. *Eur. Food Res. Technol.* 228: 945-950.

6. Cliver, D.O. and J.E. Herrmann. 1972. Proteolytic and microbial inactivation of enteroviruses. *Water Res.* 6: 797-805.

7. Cromwell, C. L. and L. C. Hoskins. 1977. Antigen degradation in human colon ecosystems. Host's ABO blood type influences enteric bacterial degradation of a cell surface antigen on *Escherichia coli* 086. *Gastroenterology* 73: 37-41.

8. Deng, W. and K. E. Gibson. 2017. Interaction of microorganisms within leafy green phyllospheres: Where do human norovirus fit it? *Int. J. Food Microbiol.* 258: 28-37.

9. D'Souza, D.H., A. Sair, K. Williams, E. Papafragkou, J. Jean, C. Moore, and L. Jaykus, L. 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. *Int. J. Food Microbiol.* 108: 84-91.

10. Escudero, B.I., H. Rawsthorne, C. Gensel, and L. A. Jaykus. 2012. Persistence and transferability of noroviruses on and between common surfaces and foods. *J. Food Prot.* 75(5): 927-935.

11. Esseili, M.A., X. Gao, S. Tegtmeier, L. J. Saif, and Q. Wang. 2016. Abiotic stress and phyllosphere bacteria influence the survival of human norovirus and its surrogates on preharvest leafy greens. *Appl. Environ. Microbiol.* 82: 352-363.

12. Esseili, M.A., Q. Wang, and I.J. Saif. 2012. Binding of human GII.4 norovirus virus-like particles to carbohydrates of Romaine lettuce leaf cell wall materials. *Appl. Environ. Microbiol.* 78(3): 786-794.

13. Gandhi, K.M., R. E. Mandrell, and P. Tian. 2010. Binding of virus-like particles of Norwalk virus to Romaine lettuce veins. *Appl. Env. Microbiol.* 76(24): 7997-8003.

14. Hall, A. J., M. E. Wikswo, K. Pringle, L. H. Gould, and U. D. Parashar. 2014. Vital signs: Foodborne norovirus outbreaks – United States, 2009-2012. *MMWR* 63:1-6.

15. Hirneisen, K.A. and K. E. Kniel. 2013. Comparing human norovirus surrogates: murine norovirus and Tulane virus. *J. Food Prot.* 76(1): 139-143.

16. Hoskins, L.C., M. Agustines, W. B. McKee, E. T. Boulding, M. Kriaris, and G. Niedermeyer. 1985. Mucin degradation in human colon ecosystems, Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J. Clin. Invest.* 75: 944-953.

17. Jones, M.K., M. Watanabe, S. Zhu, C. L. Graves, L. R. Keyes, K.R. Grau, M. B. Gonzalez-Hernandes, M. N. Lovine, C. E. Wobus, J. Vinjé, S. A. Tibbetts, S. M. Wallet, and S. M. Karst. 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science*: 346: 755-759.

18. Kandasamy, S. A. N. Vlasova, D. Fischer, A. Kumar, K. S. Chattha, A. Rauf, L. Shao, S. N. Langel, G. Rajashekara, and L. J. Saif. 2016. Differential effects of *Escherichia coli* Nissle and *Lactobacillus rhamnosus* Strain GG on human rotavirus binding, infection, and B cell immunity. *J. Immunol*. 196: 1780-1789.

19. Karst, S.M. 2010. Pathogenesis of noroviruses, emerging RNA viruses. *Viruses* 2: 748-781.

20. Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* 100: 107-114.

21. Kotwal, G. and J. L. Cannon. 2014. Environmental persistence and transfer of enteric viruses. *Curr. Opin. Virol.* 4: 37-43.

22. Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkänen, L. Maunula, C-H. von Bonsdorff, P. J. Martikainen, S. A Wilks, C. W. Keevil, and I. T. Miettinen. 2007. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and Caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl. Environ. Microbiol.* 73(9): 2854-2859.

23. Lindow, S.E. and M. T. Brandl. 2003. Microbiology of the Phyllosphere. *Appl. Env. Microbiol.* 69(4): 1875-1883.

24. Miura, T., D. Sano, A. Suenaga, T. Yoshimura, M. Fuzawa, T. Nakagomi, O. Nakagomi, and S. Okabe. 2013. Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses. *J. Virol.* 87(17): 9441-9451.

25. Murakami, K., C. Kurihara, T. Oka, T. Shimoike, Y. Fujii, R. Takai-Todaka, Y. Park, T. Wakita, T. Matsuda, R. Hokari, S. Miura, and K. Katayama. 2013. Norovirus binding to intestinal epithelial cells is independent of histo-blood group antigens. *PLoS One* 8(6): e66534.

26. Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown. 2007. Development of the human infant intestinal microbiota. *PLoS Biology* 5, 1556-1572.

27. Pant, N., H. Marcotte, H. Brüssow, L. Svensson, and L. Hammarström. 2007. Effective prophylaxis against rotavirus diarrhea using a combination of *Lactobacillus rhamnosus* GG and antibodies. *BMC Microbiol*. 7: 86. doi:10.1186/1471-2180-7-86.

28. Prizont, R. 1982. Degradation of intestinal glycoproteins by pathogenic *Shigella flexneri*. *Inf. Immun*. 36: 615-620.

29. Quignon, F., M. Sardin, L. Kiene, and L. Schwartzbrod. 1997. Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Appl. Environ. Microbiol.* 63(3): 978-982.

30. Robinson, C. M., P. R. Jesudhasan, and J. K. Pfeiffer. 2014. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe* 15: 36-46.

31. Sano, D., K. Wada, T. Imai, Y. Masago, and T. Omura. 2010. Norovirus-binding proteins recovered from activated sludge micro-organisms with an affinity to a noroviral capsid peptide. *J. Appl. Microbiol.* 109: 1923-1928.

32. SAS Institute Inc. 2016. JMP® Pro 13.0.0 64-bit Edition.

33. Shearer, A. E. H., D. G. Hoover, and K. E. Kniel. 2014. Effect of bacterial cell-free supernatants on infectivity of norovirus surrogates. *J. Food Prot.* 77:145-149.

34. Singh, B.K., M. M. Leuthold, and G. S. Hansman. 2015. Human noroviruses' fondness for histo-blood group antigens. *J. Virol.* 89(4): 2024-2040.

35. Skraber, S., J. Schijven, C. Gantzer, and A. M. de Roda Husman. 2005. Pathogenic viruses in drinking-water biofilms: a public health risk? *Biofilms* 2: 105-117.

36. Tan, M., and X. Jiang, X. 2005. The P domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors. *J. Virol.* 79(22): 14017-14030.

37. Tian, P., A. L. Engelbrektson, X. Jiang, W. Zhong, and R. E. Mandrell. 2007. Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. *J. Food Prot.* 70(9): 2140-2147.

38. Todorov, S.D., M.B. Wachsman, H. Knoetze, M. Meincken, and L.M.T. Dicks. 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. *Int. J. Antimicrob. Agents* 25: 508-513.

39. Todorov, S.D., M. Wachsman, E. Tomé, X. Dousset, M.T. Destro, L.M.T. Dicks, B.D. Gombossy de Melo Franco, M. Vaz-Velho, and D. Drider. 2010. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. *Food Microbiol.* 27: 869-879.

40. Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R Beumer, and E. Duizer. 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl. Environ. Microbiol.* 78(21): 7769-7775.

41. Varyukhina, S., M. Freitas, S. Bardin, E. Robillard, E. Tavan, C. Sapin, J.-P. Grill, and G. Trugnan. 2012. Glycan-modifying bacteria-derived soluble factors from *Bacteroides thetaiotaomicron* and *Lactobacillus casei* inhibit rotavirus infection in human intestinal cells. *Microbes Infect*. 14: 273-278.

42. Wang, Q. and K. E. Kniel. 2014. Effectiveness of calcium hypochlorite on viral and bacterial contamination of alfalfa seeds. *Foodborne Pathog. Dis*. 11:759-768.

43. Wiken Dees, M., E. Lysøe, B. Nordskog, and M. Bente Bruerber. 2015. Bacterial communities associated with surfaces of leafy greens: Shift in composition and decrease in richness over time. *Appl. Env. Microbiol*. 81(4): 1530-1539.

Table 5.1. Bacterial growth conditions.

Bacteria	Growth Medium	Growth Temperature (°C)	Growth Atmosphere	Growth Conditions
E. cloacae ATCC 13047	TSB	37	Aerobic	Static
E. faecalis 19433	TSB	37	Aerobic	Static
E. coli 08	TSB	37	Aerobic	Static
S. epidermidis 12228	TSB	37	Aerobic	Static
S. aureus AH1979	TSB	37	Aerobic	Static
B. subtilis 168	TSB	37	Aerobic	Static
B. coagulans 7050	TSB	37	Aerobic	Static
B. bifidum ATCC 35914	MRS	37	Anaerobic	Static
Ps. fluorescens 13525	TSB	22	Aerobic	Agitated
Pseudomonas sp.	TSB	22	Aerobic	Agitated
Flavobacterium sp.	TSB	22	Aerobic	Agitated
Lettuce bacteria	TSB	22	Aerobic	Agitated

TSB, tryptic soy broth; MRS, Lactobacilli MRS Broth

Table 5.2. Tulane virus propagation in presence of *Bifidobacterium bifidum* cell-free supernatant*

	pH of Media at 24°C (st. dev.)	TV Propagation (PFU/ml) CFS – Infection (st. dev.)**	TV Propagation (PFU/ml) CFS – Propagation (st. dev.) **
Positive control, 10% M199	7.42 (0.02)	3.41 (0.07) [a]	3.43 (0.35) [a]
Positive control, 10% HBSS	7.60 (0.09)	3.41 (0.01) [a]	3.61 (0.48) [a]
Positive control, 10% MRS	7.21 (0.00)	3.48 (0.04) [a]	3.70 (0.46) [a]
Positive control, 25% HBSS	7.50 (0.01)	ND	3.61 (0.68) [a]
Positive control, 25% MRS	6.99 (0.03)	ND	2.57 (0.33) [a]
Test, 10% CFS	6.70 (0.04)	3.30 (0.10) [a]	3.73 (0.68) [a]
Test, 25% CFS	5.88 (0.24)	ND	-0.70 (0.47) [b]
Test, pretreat cells/rinse, 10% CFS	6.70 (0.04)	ND	3.81 (0.83) [a]
Test, pretreat cells/rinse, 25% CFS	5.88 (0.24)	ND	2.82 (2.05) [a]

* n=2
** Values within a column that are significantly different ($p<0.5$) are indicated by different superscripts.
ND, not determined.

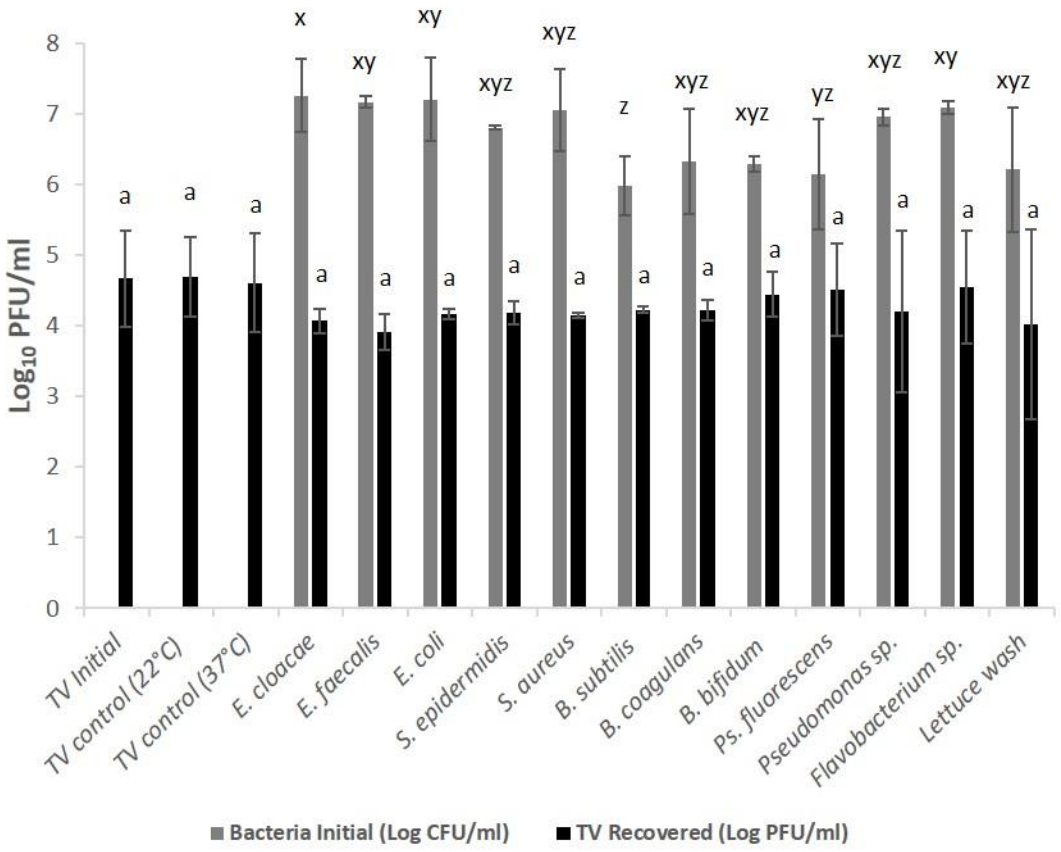


Figure 5.1. Binding of TV to bacteria as measured by recovery of infectious TV (black bars) after exposure to bacteria (initial bacterial populations, gray bars) in PBS for 2 h at 22 or 37°C. Error bars represent standard deviation (n=2). Bars labeled with different letters within a set, TV recovered (*a, b*) or initial bacteria (*x, y, z*), are significantly different ($p<0.05$).

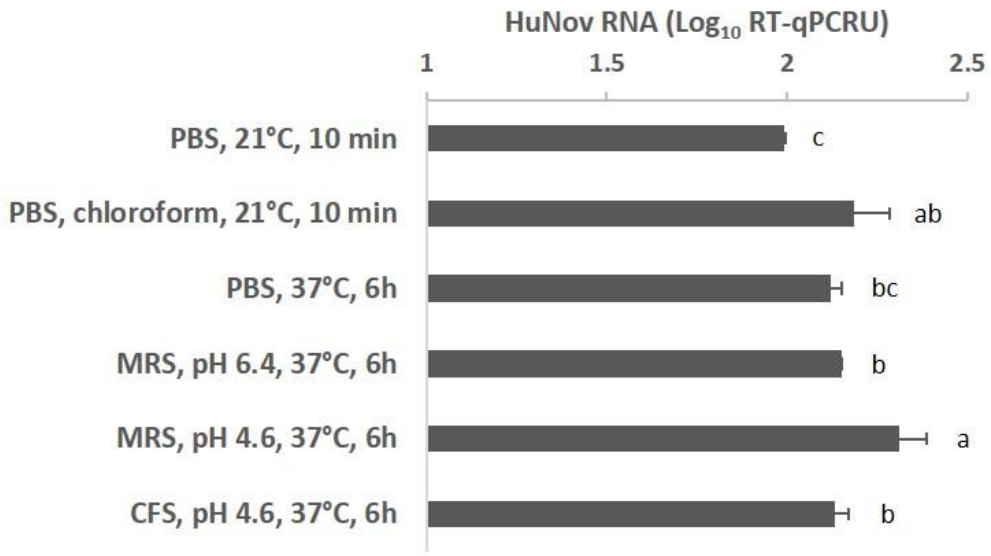


Figure 5.2. Recovery of HuNov GII from a clinical stool sample in PBS, MRS broth, or CFS (cell-free supernatant) of *B. bifidum* 35914. Error bars represent standard deviation (n=4; two replicates each per two independent trials). Bars labeled with different letters are significantly different ($p<0.05$).

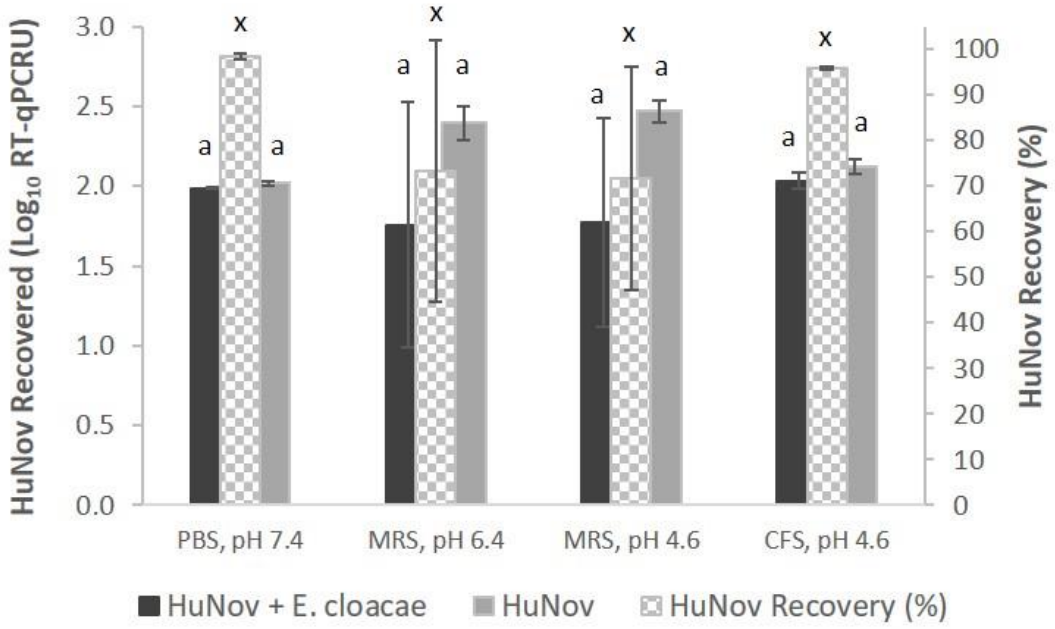


Figure 5.3. HuNov GII recovered after incubation with (black bars) or without *E. cloacae* (gray bars) in PBS, MRS broth, or *B. bifidum* CFS (left axis) and percent recovery of HuNov GII (patterned bars) after incubation with *E. cloacae* relative to control HuNov incubated without *E. cloacae* (right axis). Error bars represent standard deviation (n=2). Bars labeled with different letters are significantly different ($p<0.05$): HuNov recovered (log_{10} RT-qPCRU) with or without *E. cloacae* (*a, b*); HuNov recovery (%) (*x,y*).

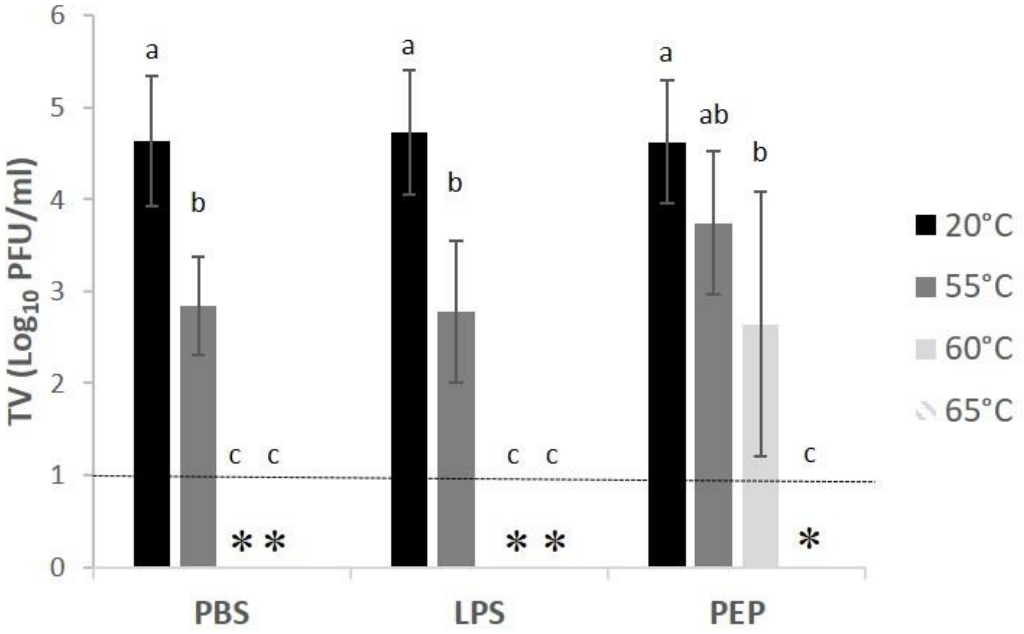


Figure 5.4. Thermal resistance of TV suspended in phosphate buffered saline (PBS), *E. coli* O111.B4 lipopolysaccharide (LPS), or *B. subtilis* peptidoglycan (PEP). Error bars represent standard deviation (n=3). Asterisks indicate TV was below the detection limit of one log PFU/ml (dashed line). Bars labeled with different letters are significantly different ($p < 0.05$).

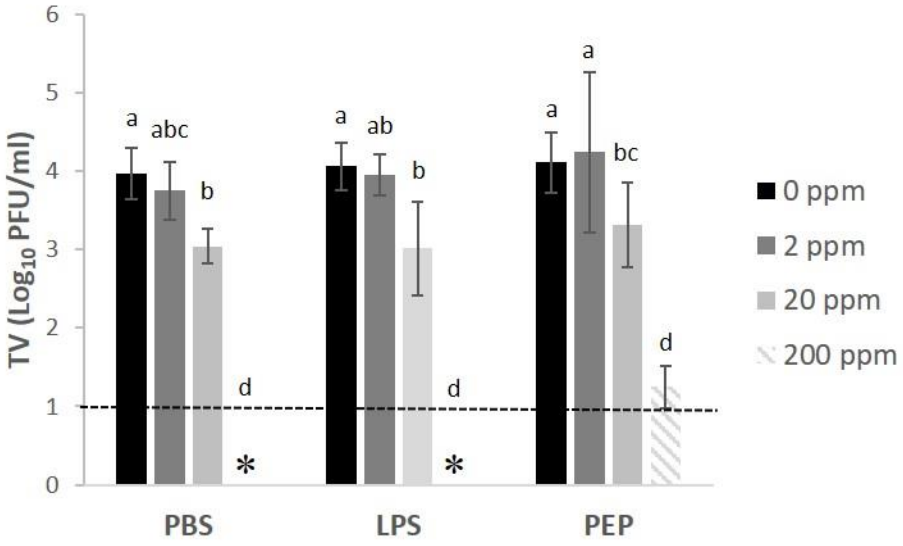


Figure 5.5. Chlorine resistance of TV suspended in phosphate buffered saline (PBS), *E. coli* O111.B4 lipopolysaccharide (LPS), or *B. subtilis* peptidoglycan (PEP). Error bars represent standard deviation (n=3). Asterisks indicate TV was below the detection limit of one log PFU/ml (dashed line). Bars labeled with different letters are significantly different ($p < 0.05$).

APPENDIX

REPRINT PERMISSION POLICY FOR DISSERTATION CHAPTERS 2 AND 4

Journal of Food Protection and *Food Protection Trends*
Copyright, Open Access and Permissions Policy

Revised October 20, 2016