ABSTRACT

BAUGHER, JONATHAN LEWIS. Characterizing Human Norovirus Binding to Fresh Strawberries. (Under the direction of Dr. Lee-Ann Jaykus).

The consumption of fresh produce, specifically strawberries, has been associated with high profile human norovirus (HuNoV) outbreaks across the world. The dynamics of HuNoV binding to berries are unknown: viruses may attach to berry-specific ligands; bind non-specifically (electrostatic and hydrophobic interactions); or bind in a facilitated manner such as in association with native berry microflora. The purpose of this dissertation is elucidate the physiochemical forces and mechanisms of action that govern human norovirus binding interactions with fresh strawberries, then identify specific ligands such as histo-blood group antigens (HBGAs), the putative norovirus cellular receptor, that mediated these interactions.

HuNoV have been shown to bind to specific structures found on the extracellular polymeric substances (EPS) produced by biofilm-producing bacteria. To identify strong biofilm-producing bacterial species of the natural berry microflora that may significantly contribute to the binding and persistence of HuNoV on fresh berries, a general survey of the microbial populations present on fresh raspberries and strawberries was performed. A total of 172 cultivable (n=98 from raspberries; n=74 from strawberries) unique bacterial isolates were identified, with the microbial profiles differing by berry type. Selected isolates were tested for the capacity to form biofilms using a crystal violet-based assay. Biofilm production varied greatly among of isolates, but strains of *Pantoea agglomerans*, *Pseudomonas moraviensis*, *Klebsiella oxytoca*, and *Enterobacteriaceae spp*. were the strongest biofilm

producers (A_{405} > 2.2). Virtually all of the natural berry bacterial isolates showed minimal HuNoV binding.

The relative binding efficiency of select HuNoV [GI.6 and GII.4 (New Orleans)] strains to strawberries was characterized. Strawberry surface slices showed moderate HuNoV binding (84- 92%) across a broad pH range (3.5 to 7.0) using an RT-qPCR-based assay, suggesting electrostatic interactions alone do not strongly mediate attachment. HBGA-like moieties [A, B, H(O); Lewis A, B, Y] were absent on strawberry and raspberry varietals, as well as on the surface of native berry microflora, as determined by ELISA. Immunofluorescence confocal microscopy showed that HuNoV GII.4 (Houston) virus-like particles (VLPs) bound selectively to strawberry pistils and achenes, with even greater preference for sites of fracture and injury on the berry style. Preferential binding to these berry structures (achenes/pistils) was confirmed using RT-qPCR.

To elucidate the specific mechanisms governing the binding of HuNoV to strawberry surface structures, the roles of electrostatic and ligand-mediated interactions were evaluated. In initial studies, transmission electron microscopy (TEM) images confirmed that HuNoV virus-like particles (VLPs) bound and aggregated to the surface of pistils. A suspension-RT-qPCR method demonstrated that pH had little effect on HuNoV binding to pistils. Further, the surface zeta potential of the HuNoV GII.4 (Houston) VLP transitioned from electropositive to electronegative somewhere around pH 5.0, while that for the strawberry pistils remained consistently electronegative, suggesting that specific HuNoV-pistil binding interactions were able to overcome electrical repulsive forces. The results of compositional and linkage analysis of pistils [major carbohydrate constituents of glucose (39.1%), arabinose

(36.0%), and galactose (13.0%)] was utilized to systematically select lectins for competitive ELISA to determine if there were lectin-binding moieties associated with strawberry cell wall material (CWM). Several lectins were associated with moderate binding inhibition, i.e., lectin SNA, binding αNeuNAc(2→6)gal (no HBGA specificity); lectin BS-1, binding α-D-Gal >> GalNAc (HBGA specificity B>>A); lectin MOA, binding Galα1, 3Gal, 3Galβ1 (HBGA specificity B>>A); and lectin DBA GalNAc (HBGA specificity A1>>A2) at concentrations of 100 mM. Chemical oxidation (sodium periodate) and physical disruption (boiling) were shown to significantly inhibit NoV binding to CWM. These data support the hypothesis that strawberries contain multiple carbohydrate moieties that mediate specific HuNoV binding interactions. This increase in our understanding of how and why HuNoVs attach to strawberry surfaces can aid in the development of effective removal strategies such as targeted washes, as well as provide a strong basis for resolving the binding dynamics of HuNoVs to other commonly contaminated foods.

Characterizing Human Norovirus Binding to Fresh Strawberries

by
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DEDICATION

I am blessed to have so much support from so many amazing people. I dedicate this to my family (blood and otherwise) that truly made a difference in my life. Thank you so much.

BIOGRAPHY

Jonathan Baugher is an avid outdoorsman from New Bern, North Carolina. In 2005, he graduated from high school at Arendell Parrott Academy in Kinston, North Carolina. After high school, Jon enjoyed the Shenandoah Valley as he completed his Bachelor of Science in Biology at James Madison University. After completing his BS in 2009, Jon started his Master's in Food Science at NC State under the direction of Dr. Todd Klaenhammer. After completing his M.S. in Food Science in 2011, he started his PhD under the direction of Dr. Lee-Ann Jaykus. During this time, Jon was participated in many intramural sports, and took an active leadership role as food science club president. He served at Vice President of IFT Relations for one year, and was elected as national IFTSA President in 2012. He served 3 years as President and sat on both the IFTSA and IFT Board of Directors for that time period. The past two years he has served on the Board of Trustees of Feeding Tomorrow. Jon has made many close friends over the past 6 years, and looks forward to contributing to his FBNS department and mentoring future food scientists. He is a life-long member of the PACK.

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I want to thank my parents for your unwavering support, even when I faltered (which at times was often). I look up to you two as model of what a loving, supporting mom and dad should be. I want to thank the rest of my family and friends for all your help through this crazy past 6 years of my life. It took many twists and turns, and certainly wasn't dull! I would like thank Dr. Lee-Ann Jaykus for not making it easy. None of the best things that I have ever accomplished have been easy, and this project certainly wasn't easy! I have so much respect for you as a mentor and I appreciate you allowing me to grow as a professional.

To all my lab mates, post-docs, senior scientists (past and present) that I have had the pleasure to have worked with, you guys were not only a wealth of information and a source of support and counsel when research wasn't going well (which at times was quite often), you are amazing people that have become my life-long friends. The list of amazing people in Schaub Hall goes on and on, but if it weren't for Rosemary Dawes, I probably wouldn't have finished my M.S. To all my non-food science friends, you guys have always been there for me and I love you for it. I know I am ready for the next chapter in my life, but I would need a bigger book to properly describe to you all the fun and good times I had in this chapter.

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CHAPTER 1

Human Enteric Viruses and Berries: A Review

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Abstract

Enteric viruses, most importantly human norovirus (HuNoV) and hepatitis A virus (HAV), are significant contributors to the overall foodborne disease burden. These viruses can contaminate fresh produce items at any stage from pre- to post-harvest, usually in association with human fecal material in irrigation and wash waters, or present on the hands of harvesters or food workers. Despite a strong epidemiological link between enteric viruses and fresh berries, little is known about the tenacity with which human viruses attach to berry surfaces, or the mechanisms mediating this attachment. Most evidence on these phenomena is from work with leafy greens. The purpose of this review is to provide context for the association between human enteric viruses and fresh berries, with a focus on strawberries. Background information is provided, notable berry-associated outbreaks are described, and the body of evidence supporting the mechanisms of virus interaction with fresh produce in general is reviewed. Better understanding of enteric virus-food interactions will aid in the development of improved methods to prevent and/or inactivate these important pathogens from foods.

Introduction

From 2004 to 2015, the per capita annual consumption of fresh fruits and vegetables has increased from 130 to 150, and 175 to 183 servings, respectively (PBH.org, 2015). Increased consumption has been accompanied by increased attribution of foodborne disease to fresh produce. In fact, 48% of foodborne disease outbreaks in the U.S. are now associated with consumption of fresh produce (Painter, 2013). Likewise, the recognition of viruses as a cause of foodborne illness has emerged to the point that viruses are now recognized as the cause of 25-60% of the overall food-associated disease burden (Flint et al., 2005; Scallen et al., 2011). Although a number of commodities are commonly associated with foodborne viral disease outbreaks (e.g., molluscan shellfish and ready-to-eat foods for example), analysis of surveillance data of 364 single source outbreaks from 2001-2008 showed leafy vegetables (33%) and fruits/nuts (16%) were most commonly implicated (Hall, 2012). Human noroviruses (HuNoV) and hepatitis A (HAV) have been identified as the foodborne viruses with the highest priority and impact worldwide (FAO/WHO, 2008).

Foodborne viruses are part of a functional group called enteric viruses because the primary exposure route is through the gastrointestinal tract. This same location constitutes the initial site of infection, and in most cases, disease manifestation. The viruses associated with food-related disease are host (human)-specific, showing a high degree of tropism for specific cells. Since they require a live host cell in order to replicate, they are not able to grow or proliferate in or on food matrices; however, foods serve as vehicles for virus access to susceptible hosts. These viruses are extremely small (15-400 nm) (Koopmans, 2004), non-enveloped, usually having an RNA genome, environmentally persistent, and resistant to

commonly used food processes and preservation techniques (e.g., extremes of pH, drying, refrigeration). They are also highly resistant to most commonly used disinfectants and sanitizers at manufacturer-recommended concentrations. They are extremely difficult to study because they are unable to be cultured or are very challenging to culture in the laboratory. The cultivable surrogate or model viruses that are frequently used for survival, persistence, internalization, and removal studies can have significantly different characteristics than the enteric viruses they represent, so data arising from those studies are not always reliable.

With disease burden more fully characterized, significant increases in allocated resources by industry, academia, and government partners have been dedicated to developing methods to reduce viral contamination of foods and identify effective inactivation strategies. Because viral contamination of foods can occur in a number of ways, and there remain many barriers to research and evaluation of the efficacy of controls, prevention of disease continues to be challenging. One such challenge is improved understanding of the interactions between human viruses and fresh produce. For example, little is known about how and why enteric viruses bind to the surface of various produce items. Yet elucidating the biochemical and biophysical forces that govern virus attachment to fresh produce will provide the information needed to develop much better prevention and control strategies. The purpose of this review is to provide context for the association between human enteric viruses and fresh berries, with a focus on strawberries. Background information is provided, notable berry-associated outbreaks are described, and the body of evidence supporting the mechanisms of virus

interaction with fresh produce in general is reviewed. Implications of these findings to future work as well as emerging control measures will also be covered.

Strawberry Structure

The strawberry is botanically considered an aggregate fruit comprised of a fleshy, floral receptacle (enlarged flower stem) that supports clusters of the true fruiting bodies or achenes (Figure 1.) (Esau, 1977). The strawberry stem or peduncle (form of petiole) is the part of the growing berry that connects it to the rest of the plant. The peduncle is responsible for carrying water, minerals, and sugars to the leaves, stem, and roots (Poling, 2012).
The strawberry pith, located directly below the attachment of the peduncle to the berry, has almost no flavor and is usually removed prior to consumption. The vascular bundle is a string of lighter colored flesh that connects the achene to the pith. Pistils, the female organs that include the stigma (where pollen grains germinate) and the style (the pollen tube that delivers pollen to the ovary), are attached to the outer surface of each of the ~200 achenes on a strawberry receptacle (Poling, 2012).

Formed from the fusion of gametes, inside the dry ovary wall of each achene is an ovule that has the potential to develop into a unique strawberry seedling (Perkins-Veazie, 1995). The achenes have been shown to be directly involved in the ripening of the fruit by secreting auxins (plant hormones) that promote the thickening of the floral receptacle by increasing cell number and size. Although the offspring are unlikely to have desirable horticultural characteristics of the parent plant, proper fertilization and development of the

achenes is crucial for successful strawberry development (Mudge et al., 1981). Depending on weather and soil conditions, the development from open flower blossom to mature strawberry fruit is typically 20 to 30 days. To maintain the highest quality strawberry varietals for future growing seasons, strawberry plant propagation is performed by collecting runner plants that are identical to the "mother" plant, or divide and transplant the strawberry plant once multiple crowns (thickened stems) have grown (Poling, 2012).

Strawberry Production Practices

Strawberries are produced on more than 55,000 acres primarily in California, Florida, Oregon, North Carolina, Washington, Pennsylvania, Michigan, Wisconsin, New York, and Ohio. The U.S. ranks first in production, followed by China and Spain. Strawberries grow in most garden soils, but best yields occur in slightly acidified soil (pH 5.5 to 6.0) that is well-drained under full sunlight (Mid-Atlantic Berry Guide, 2010). The direction of the sun can influence the time of year the plants blossom can potentially leave plants growing in areas where late frosts commonly occur to be susceptible to late spring crop damage (Handley and Pritts, 1996). Strawberry plugs are planted in the fall to ensure proper plant set, and rooting occurs as long as the temperature is above 45°F. Because strawberry plants have a shallow root system (~6 inches) and their flower blossoms are susceptible to frost, irrigation systems are strongly recommended for most production systems (Lamont et al., 2001).

Frost damage just prior to or during strawberry flower blooming can result in significant crop losses. A black flower center is clear indication of frost damage to strawberry plants (Pritts, 2006). Mulch and other cover materials are commonly used to help insulate strawberries from frost, but have been shown to negatively impact the upcoming yield. Overhead irrigation systems are commonly used for frost control because flowers must be kept wet during a freeze in order to provide sufficient protection (Lamont et al., 2001). Flowers are not damaged until they reach a temperature of 28°F, so the energy release from ice melting to water can protect them. Row covers can modify the influence of wind and delay evaporative cooling of the ground. These row covers do not provide additional degrees of protection on a cold night, but they do significantly increase the amount of time it takes for the ground to cool down and reach equilibrium with the outside air. Also, water can be applied to the top of the row covers to provide additional protection to the flowers under frost conditions (Pritts, 2006).

The strawberry roots provide structural support, capture water and nutrients from the soil, and distribute the nutrients throughout the plant's vascular system. Macronutrients needed for proper strawberry growth and development include nitrogen, phosphorus, calcium, potassium, sulfur, and magnesium, while iron, manganese, boron, zinc, and copper are necessary micronutrients (Mid-Atlantic Berry Guide, 2010). It is common practice to provide supplemental nutrients to the soil via a drip irrigation system in early spring (Lamont et al., 2002). The majority of water taken up by the plant evaporates through the stomata of the leaves and stems, and strawberry plants must have adequate irrigation to replenish water that is taken up by the plant and subsequently lost. Because strawberry plants have large

numbers of stomata, water loss can be very significant high during warm months, and water consumption for overhead irrigation quite high (Mid-Atlantic Berry Guide, 2010).

Although automated strawberry pickers are being developed to ensure that only ripe fruits are harvested from the plants, the vast majority of strawberries are harvested by hand (Mid Atlantic Berry Guide, 2010). Ripe strawberries are highly susceptible to damage during harvest and must be handled with care. The berries are harvested a minimum of three times per week, and must be firm, well colored, and free from rot to ensure proper shelf life of the fruit. Harvesting the berries in the cool of the morning and post-harvest cooling is essential for lengthening their shelf life (Handley and Pritts, 1996).

Routes of Contamination for Fresh Produce

Foodborne viruses are generally spread by ingestion of foods contaminated with human fecal matter. In general, the viruses are introduced into the food chain via contaminated water or surfaces, or else unsanitary food handling by humans. Viruses can be transferred to fresh produce at any stage in the food production-processing-preparation chain by direct or indirect contact with vomitus or fecally-contaminated hands, food contact surfaces, or fomites such as equipment or food contact surfaces (Cliver, 1994). Unfortunately, during an epidemiological investigation of a foodborne outbreak, it can be challenging to determine at what point in the food chain the contamination event occurred, and the ultimate source of the viruses.

In the production and harvest stages, fresh produce can come into contact with virus-containing fecal material by several means. Improperly composted materials containing

human sewage, or even treated human sewage, may still contain infectious virus and should not be used as soil amendments or as fertilizers (Villar, 2007; Ueki, 2005). Enteric viruses have been shown to survive for months in sewage, and inadequately treated wastewater used for irrigation has been linked to virus contamination of fresh produce (Nasser et al., 1993; Bosch, 1995; Seymour and Appleton, 2001). The World Health Association (WHO) has stated that fresh fruits and vegetables intended for raw consumption must not be fertilized with any fecally-contaminated materials (Beuchat, 1998). While this is less of an issue in the developed world, many developing countries continue to utilize treated sewage sludge as supplemental nutrients for crop production. Importation of fresh produce from warm-weather climates has increased in the U.S. and Europe over the last two decades, and many of these products come from the developing world. In fact, nearly 50% of fresh fruit and 20% of fresh vegetables consumed in the U.S. are imported (Pollack, 2001).

Unintentional sewage runoff that occurs as a consequence of flooding, or from leaky septic and waste holding tanks, can also cause virus contamination of fresh produce. In the fields, lack of adequate on-site toilet and hand-washing facilities can result in workers directly defecating onto the fields and/or handling fresh produce with improperly washed hands. Although the site of virus contamination was originally thought to be entirely on the surface of produce items, new studies are suggesting that certain cultivation methods such as hydroponic production may allow the internalization of enteric viruses into the vascular system of plants (DiCaprio, 2012; Hirneisen, 2013).

During the post-harvest stage, viral contamination of fresh produce mostly occurs during washing or preparation steps. If wash waters are contaminated, this can spread virus

to large batches of product, resulting in large foodborne outbreaks. Much more common is contamination due to poor personal hygiene of individuals directly handling produce items. This can occur during harvest, but also in packing, and most frequently, during food preparation. Vomiting episodes can result in the aerosolization of virus particles that can be deposited directly on product, or on surfaces that then come in contact with product (Marks et al., 2000; Lopman et al., 2012; Tung-Thompson et al., 2015).

Developing effective risk management strategies for fresh produce production, requires a complete understanding of all possible viral contamination routes. For berries in particular, contamination events occurred as a result of fecally-contaminated irrigation waters, HuNoV transfer from the hands of infected farm workers and/or during the processing and freezing by infected workers at the company level. The WHO recognizes that Good Agricultural Practices (GAPs), Good Hygiene Practices (GHPs) and Good Manufacturing practices (GMPs) must account for all foodborne pathogen risks, including viruses, from farm to fork. However, inconsistencies in global agricultural practices remain, such as the lack of international fresh produce pesticide/irrigation water guidelines (Pimentel et al., 2004).

Physical Characteristics of Foodborne Viruses

Enteric viruses are of simple structure, comprised of nucleic acid (DNA or RNA) surrounded by a non-enveloped, protein capsid. Requiring host machinery to replicate, these viruses have a defined host range based on one or more specific host cell receptors that interact with a region of the protein capsid. Although any virus that infects via the gastrointestinal tract and is consumed in contaminated food could theoretically be

"foodborne," there are predominant features of common foodborne viruses that make them adept at contaminating and persisting in foods.

One feature common to enteric viruses is that they are hardy. Specifically, they are resistant to desiccation and extremes of pH, and survive well on hands, surfaces, and in the environment (Green et al. 1998a; Barry-Murphy et al 2000; Clay, 2006; Dolin, 1972). Being non-enveloped, they are relatively resistant to detergents, organic solvents, and ethanol (Jimenez, 2006). In fact, these viruses resist virtually all commonly used sanitizers or disinfectant, at least when used at manufacturer recommended concentrations. They also tend to cause disease at very low infectious doses, and can be shed it high concentrations in the stool of infected individuals (Teunis et al., 2008; Atmar et al., 2008; Aoki et al., 2009).

Human Norovirus

Human norovirus (HuNoV), previously called "small round-structured viruses," are 27-38 nm in diameter and constitute two of the six genogroups of the Norovirus genus within the *Caliciviridae* family (CDC, 2011). The positive-sense single-stranded RNA of approximately 7.7 kilobases in length encodes three complete open reading frames (ORFs). The ORF1 encodes non-structural proteins p48, NTPase, p22, VPg, 3C-like protease, and RNA-dependent RNA polymerase (RdRp). The ORF2 encodes for the major capsid protein (VP1) and ORF3 encodes the minor capsid protein (VP2) (Hardy, 2005). Each non-enveloped virion consists of 90 dimers of VP1 and one or two copies of VP2 (Hardy, 2005).

With a high mutation rate estimated at 1.21×10^{-2} to 1.41×10^{-2} point substitutions per site (specific regions within the VP1 gene) per year (Victoria, 2009), the norovirus genus

displays tremendous strain diversity that results from an accumulation of point mutations due to error-prone RNA replication and recombination between viruses (Bull et al., 2007). In the major capsid protein (VP1), strains within a given HuNoV genogroup share a minimum amino acid sequence identity of 60% (Green, 2001). The highly variable capsid protruding domain, P2, binds the histo-blood group antigens (HBGAs) that serve as putative receptors and host susceptibility factors for infection for most HuNoV strains (Tan et al., 2004). Over the past two decades, there has been a marked evolution of the binding patterns of HuNoV to HBGAs, particularly amongst the epidemic GII. 4 strains. This genotype produces variants that replace previously dominant ones, with pandemic strains emerging every 3-4 years. This epochal evolution supports the hypothesis that genetic drift and evolution of norovirus, as driven by population immunity, is likely to be mediated by carbohydrate-protein interactions (Le Pendu et al., 2006). Additional studies must be performed to determine if the dominant GII.4 variants have unique infection characteristics such as enhanced person-to-person transmissibility and/or infectivity.

Norovirus can infect people of all ages, but most commonly young children (under age 5) (Vinjé et al., 2011). More severe manifestations, including hospitalization and death, occur in this population and the elderly (Patel et al., 2008). Individuals with a non-functional fucosyltransferase (FUT2), the enzyme responsible for the transfer of a fucose sugar to the end of the histo-blood group ABO(H) precursor in gastrointestinal cells and saliva glands, are largely protected from infection with the most common HuNoV GII.4 strains (Carlsson, 2009). People that are homozygous carriers of nonsense mutations in the FUT2 gene are classified as non-secretors, and do not produce ABH antigens (ABH antigens can still be

produced by FUT1 in erythrocytes). It is estimated that 20% of Caucasians are non-secretors who possess strong but not complete protection from HuNoV GII.4 (Rydell, 2009).

Approximately 18-100 infectious particles are required to cause norovirus infection in susceptible individuals (Teunis et al., 2008). After infecting a host, HuNoV is replicated within the small intestine; however, the exact mechanism of action for intestinal cell invasion and virus replication has not been resolved. The viruses are shed at high concentrations in vomitus, feces and other host fluids (10^5-10^{11} virus genome copies per gram) (Atmar et al., 2008; Aoki et al., 2009), and a person suffering from a HuNoV infection continues to shed virus for an average of 14.3 days. Young children, the elderly, immune compromised individuals, and individuals shedding high titers of HuNoV are prone to prolonged shedding of over 30 days (Aoki, 2009). In extreme cases, chronic shedding can occur, most notably in the immunocompromised (Sukhrie, 2010). Screening of all infected individuals at hospital locations showed that 26% of employees and 33% of patients were asymptomatic shedders of HuNoV (Gallimore, 2004). Based on this and other studies, it appears that asymptomatic shedding rates are similar to those for symptomatic shedding, with both showing significant variance in virus concentration shed and the duration of shedding (Teunis et al., 2015).

In the U.S., it is estimated that there are about 21 million symptomatic cases of HuNoV infection annually, 5.5 million of which are transmitted by foods (ranking 1st in foodborne illness causes; constituting 58% of all foodborne disease). Every year, food-associated norovirus infections result in over 15,000 hospitalizations (ranking 2nd) and 150 deaths (ranking 4th) in the U.S. (Scallen et al., 2011). There is winter seasonality of infection,

most likely from an increase in person-to-person contact and exposure to larger numbers of individuals confined indoors (Lopman, 2009).

Typically, the symptoms for acute norovirus gastroenteritis develop between 12 to 48 hours after exposure and last for 24 to 60 hours (CDC, 2006). The self-limiting disease is characterized by nausea, forceful vomiting, watery diarrhea, and abdominal pain. Additional HuNoV symptoms can include general lethargy, weakness, muscle aches, headache, cough, and low-grade fever. Although usually self-liming, severe cases may result in dehydration and electrolyte imbalance, particularly in the very young, the elderly, and immune-compromised (Goodgame, 2006). After infection, protective immunity to the specific HuNoV strain is considered incomplete and transient (Lindesmith, 2005; Zhu et al., 2013), although this has recently been debated.

Hepatitis A virus

Hepatitis A virus (HAV), previously known as infectious hepatitis, causes an acute illness affecting the liver. First isolated in stool specimens in 1973, HAV is a non-enveloped, positive-sense, single-stranded RNA virus within the genus *Hepatovirus* from the family *Picornaviridae* (Feinstone, 1973). Hepatitis A virus is small (diameter of 27-32 nm) and consists of 60 copies each of three different proteins (VP1, VP2, and VP3) in an icosahedral structure. The exposed amino acid residues of VP1 and VP3 establish the stable immune-dominant antigenic sites of the virion (Lemon, 1997).

The 7.5 kilobase genome contains a 5'-nontranslated region that spans over 10% of the total genome, resulting in complex secondary and tertiary structure (Hollinger, 2001).

Proteins are expressed via a single large polyprotein from a large ORF that extends through the majority of the genomic RNA (Koff, 1998; Lemon, 1994). The synthesized polyprotein is subsequently cleaved by a viral protease ($3C^{pro}$) to produce 3 (potentially 4) structural proteins and seven nonstructural proteins (Hollinger, 1996). The genome mutation rate is estimated at 1.73-9.76 x 10^{-4} (notably, 100-1,000 less frequent mutation than HuNoV) (Kulkarni, 2009) and highly conserved clusters of rare codons within the capsid genomic sequence restrict the antigenic variability of the capsid protein (Aragones, 2008). There is only one serotype of HAV and four different genotypes (I-III, VII) affecting humans (Cristina, 2007; Ching, 2002), with the majority of human isolates being of genotype I (subtype IA) (de Paula, 2002).

After ingestion, HAV enters the bloodstream through epithelial cells of the intestine, and it carried to its target organ, the liver. The virus replicates within the hepatocytes and Kupffer cells (liver macrophages) (Murray, 2012). Within 10-12 days after infection, HAV will be present in the blood but primarily detected in the feces of infected individuals. The typical incubation period before frank disease onset is 28 days (ranging 15-50 days), and peak virus shedding begins to decline soon after the appearance of symptoms (Connor, 2005). The HAV illness begins with a sudden onset of fever, malaise, anorexia, nausea, abdominal pain, dark urine, and jaundice (CDC, 2007). The clinical symptoms generally last no longer than two months; however, 10-15% of individuals experience prolonged or relapsing symptoms for up to six months. Although the majority of infected individuals cease shedding by the third symptomatic week, children have been shown to shed for greater

durations of time. Also, during symptomatic relapses, shedding of virus will continue (CDC, 2007).

The severity of HAV infection has been directly correlated with age, with the majority (70%) of children six years and younger being asymptomatic (CDC, 2007). In more than 80% of older children and adults, symptoms will manifest, the severity of which usually increases with age (Ciocca, 2000). Hepatitis A virus infection does not result in chronic disease, and immunity is life-long. Although severe symptoms such as fulminant hepatitis (liver failure) can occur, the overall mortality rate is less 0.3% (CDC, 2007). Worldwide, it is estimated that HAV causes 1.4 million symptomatic cases and tens of millions of asymptomatic infections annually. In 2010, acute hepatitis A was reported to cause 102,000 deaths worldwide (Global Burden of Disease Collab., 2013). In developing countries, approximately 90% of children over the age of 10 years have been infected with HAV and are immune by adulthood (Global Burden of Disease Collab., 2013). The first inactivated HAV vaccine (Havrix®, SmithKline Beecham) became available in Europe in 1991 and was approved in the U.S. in 1995. Later in 1995, a second inactivated vaccine was approved (Vaqta®, Merck). These vaccines are a whole-virus preparation produced by growth of attenuated HAV strains in cell culture, inactivated by formalin, and absorbed to an aluminum adjuvant (Maiwald et al., 1997). The vaccines are highly effective and provide seroconversion rates greater than 99.4% when administered as a single dose; a subsequent booster shot is given 6-12 months later (Viral Hepatitis Board, 1997). Residual antibodies against HAV are passively acquired from mother to infant and may interfere with vaccine

immunogenicity. The U.S. now recommends vaccination for protection from HAV infection for all children over the age of one (CDC.org, 2016).

Other Viruses

There are a variety of other enteric viruses that may be foodborne as well, although for the time being, they are thought to be transmitted predominantly by contaminated water or directly between people. Rotavirus, a member of the family *Reoviridae*, primarily infects children less than age five years, and is the single leading cause of severe diarrhea among infants (Dennehy, 2000). Rotavirus causes over 100 million cases of diarrhea in developing countries, nearly two million hospitalizations, and is attributed to as many as 350,000 to 600,000 deaths annually (UNICEF/WHO, 2009). Two approved live attenuated vaccines against rotavirus A, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck), are currently produced (Matson, 2006; O'Ryan, 2007). In 2009, the WHO recommended that all national vaccination programs incorporate the rotavirus vaccine into their schedules (Jiang et al., 2010). In 2008, Mexico saw a significant reduction (41%) in infant (children 11 months old or younger) diarrheal mortality rates after the vaccination program was implemented (Richardson et al., 2010).

Astrovirus (a member of the *Astroviridae* family) cause a diarrheal disease that is milder than rotavirus and also impacting predominantly young children (Koopmans et al., 1998). The same can be said for adenovirus types 40 and 41 (Roy et al., 2008). The majority of children acquire antibodies against astrovirus by age 5, and data suggests that antibodies

provide complete protection throughout adult life (Midthun et al., 1993). There is no current human vaccine against astrovirus.

An important emerging enteric virus is hepatitis E virus (HEV), reclassified recently into the genus Hepevirus of the *Hepeviridae* family (ICTV, 2009). Hepatitis E virus has a prolonged incubation period and causes clinical symptoms comparable to HAV. However, chronic HEV infections can occur in immuno-compromised individuals (Kamar, 2008). Pregnant women are particularly at risk of severe symptoms including fulminant hepatic failure; they have an elevated mortality rate of approximately 17-33% (Bonnet, 2012). Hepatitis E virus causes an estimated 20 million infections, 3 million symptomatic cases, and 57,000 deaths annually worldwide. Upon the cessation of symptoms, an individual acquires complete, life-long immunity (Lozano, 2013). In developing countries with poor wastewater treatment and a propensity for human sewage-contaminated irrigation systems, the WHO warns of higher risk of HEV contamination in raw or undercooked shellfish, raw or unpeeled fruits and vegetables, and drinking waters (WHO Factsheet, 2015)

Notable Recent Foodborne Virus Outbreaks

HAV in Green Onions

In the fall of 2003, a large HAV outbreak involving a single restaurant was identified in Pennsylvania. This outbreak caused 601 illnesses, at least 124 hospitalizations, and was responsible for the deaths of three people (Wheeler, 2005). Genome sequences obtained from RT-PCR amplification of stool samples originating from many infected individuals were identical. The molecular typing revealed genotype 1A, the most common U.S.

genotype. Interestingly, the HAV sequence identified from the Pennsylvania outbreak was identical to that implicated in foodborne outbreaks that occurred in Tennessee, Georgia, and North Carolina at approximately the same time (CDC, 2003). The sequence was also nearly identical to a strain associated with a similar green onion outbreak that occurred in 2001 (Dentinger et al., 2001). In a subsequent case-control study, the majority of cases (91%) had eaten mild salsa (odds ratio, 19.6; 95 percent confidence interval, 11.0 to 34.9), and 98% (odds ratio, 33.3; 95 percent confidence interval, 12.8 to 86.2) of cases had eaten green onions. These data provided compelling evidence that the primary source of contamination was the consumption of products containing green onions. Despite clinical screening of all the restaurant employees, it was concluded that none were the source of the outbreak (Wheeler, 2005).

An FDA trace-back investigation showed that the implicated green onions had been grown and harvested by hand in northern Mexico. Once harvested and packed, the raw product was distributed without any further repacking or processing. At least two farms in northern Mexico were identified as the probable source of the contaminated product. In November 2003, the FDA issued a ban on green onions harvested from four farms in Mexico. All were identified as contributing to various restaurant-associated HAV outbreaks that occurred within a 3 month period in the southeastern U.S. (CDC, 2003; FDA, 2005).

HAV in Pomegranates

In May 2013, a multistate outbreak of hepatitis A occurred in the U.S. A total of 165 individuals from 10 states became ill. Sixty-nine (42%) of the patients were hospitalized,

two developed severe fulminant hepatitis, and one required liver transplantation, although there were no mortalities. The median age of patients was 47 years [interquartile range (IQR) 35-58 years] and the majority (55%) were women (Collier et al., 2014). A frozen berry mix product that contained strawberries, red raspberries, blueberries, cherries, and pomegranate arils was implicated.

Further investigation supported that the pomegranate arils were the most likely component of the mix to serve as the source of contamination. Specifically, the workers producing the frozen mix routinely wore gloves; no workers reported any symptoms of HAV infection; and all frozen berry components except pomegranate arils were supplied in multiple lots and/or by multiple locations. The arils were, however, imported from Turkey as a single lot from a single supplier. Further, genotyping of clinical specimens revealed the HAV 1B genotype, uncommon in the Americas but endemic in the Middle East (Normann et al., 2008; Faber et al., 2009). HAV in the imported frozen pomegranate arils was condirmed by combining the epidemiology (with several sources), the genetic analysis from patients, and product tracing. A systematic review of methods or case-control study was not performed, because the specific food item was identified through the descriptive epidemiology alone. This was the first multistate foodborne HAV outbreak to utilize both vaccination and immunoglobulin post-exposure prophylaxis, as facilitated by public retailers (in-store pharmacies, vaccine administration staff, and automated calling systems), to efficiently contain the incident (Collier et al., 2014).

HuNoV in Strawberries

In late September and early October of 2012, Germany suffered the largest recorded foodborne outbreak in history (Bernard et al., 2014). Human norovirus was identified as the causative agent of acute gastroenteritis that affected 390 institutions with approximately 10,950 individuals suffering symptoms. The majority of institutions affected were schools (244/390, 62.6%) and childcare facilities (140/390, 35.9%), but also included three disability homes, two retirement homes, and one rehabilitation clinic. Mostly children and teenagers were affected by this outbreak, but staff members of each institution also became ill.

To determine the source and breadth of the outbreak, four analytical epidemiological investigations, two case-control studies, and two surveys were performed. Analytical epidemiological analysis pointed to dishes containing strawberries (strawberry compote and strawberry tart) as the most likely vehicles for transmission (odds ratio of 8.2, 95% confidence interval, 2.6 to 45.4) (Bernard et al., 2014; Task Force Gastroenteritis, 2012). Frozen strawberries had been used as a component of the implicated foods, which were produced by a regional catering kitchen. Originating from China, the strawberries were part of a 22 ton lot that was distributed to three catering agencies in Germany. Once the strawberries were identified, product was rapidly withdrawn from the market, leading to cessation of additional illnesses. Over 1,100 boxes (more than 11 tons) of contaminated product were prevented from reaching consumers (Bernard et al., 2014).

HuNoV in Frozen Raspberries

From May to early September of 2005, a total of six point source outbreaks (including a hospital, two nursing homes, a meals-on-wheels event, a restaurant, and a clothing company) of HuNoV associated with frozen raspberries occurred in Denmark (Korsager et al., 2005; Falkenhorst et al., 2005). The first five outbreaks, totaling over 1,000 cases, were all associated with frozen raspberries that originated from a large batch that was imported to Denmark from Poland. A five week delay in identifying the outbreak resulted in contaminated produce reaching an elderly meals-on-wheels service event. An estimated 400 people attending that event (median age 85 years old) were affected, with at least 23 subsequent hospitalizations. This latter outbreak occurred after consuming whole frozen raspberries that came from a different Danish importer and a different Polish producer (Falkenhorst et al., 2005).

Stool samples from five of the outbreaks were positive for HuNoV by RT-PCR. However, multiple strains (GII.4, GII.2, and GII.7) were identified in individual outbreaks. The number of different strains was explained by recognizing that the one large lot of Polish raspberries actually consisted for berries produced by a number of smaller-scale Polish farms in the same region. The raspberries batches were comingled, frozen, and packed into 2.5 kg packages for distribution throughout Europe. It was hypothesized that HuNoV contamination occurred as several independent farm-level contamination events resulting in heterogeneous HuNoV contamination in different shipments to Denmark (Falkenhorst et al., 2005). A definitive contamination event could not be identified,

Virus Attachment to Fresh Produce

Although many studies have sought to identify specific mechanisms of bacterial attachment to foods, a focal point for food virology is identification of significant and specific factors contributing to the interactions and adherence of viruses to foods (Berger, 2010). Interactions between HuNoV and molluscan shellfish have been the most widely studied to date. The shellfish work initially occurred in response to earlier reports that HuNoV selectively bind to histo-blood group antigens (HBGAs), which are now considered the putative host cell receptor/co-receptor for these viruses. HBGAs are distinct terminal polysaccharides of glycoproteins or glycolipids found in various cell types and tissues including the epithelial cells lining the gastrointestinal mucosa (Ravn and Dabelsteen, 2000). HBGAs include H type, A and B blood groups, and Lewis carbohydrates. A HBGA-specific binding pocket has been identified within the P2 region of the Norwalk (GI.I) virus capsid protein (Tan et al., 2004). Human norovirus strains representing different genotypes showed differential HBGA binding patterns, including strains that did not bind to any of the tested HBGA carbohydrates (Harrington et al., 2004). It turns out that oysters and other molluscan shellfish effectively bind HuNoV GI.I through HBGA A-like moieties found in the gastrointestinal lining of the animal (Tian et al., 2006). This binding specificity was later shown to facilitate HuNoV bioaccumulation and hinder efficient removal by depuration (Tian et al., 2007).

Virtually all of the initial studies to characterize binding of HuNoV to fresh produce have focused on leafy greens. Most of these have looked at mechanism of virus-produce interactions rather than actually visualizing the tissues to which the virus binds. Early

experiments were performed using butterhead lettuce. Based on the hypothesis HuNoV-lettuce interactions are mediated by electrostatic forces, Vega et al. (2005) used cultivable surrogate viruses having a similar pI to HuNoV [i.e., feline calicivirus (FCV), echovirus 11, and bacteriophages MS2 and ΦX174] in binding experiments at various pH values. Differential binding patterns were observed among the viruses, suggesting that electrostatic interactions are not the dominating factor dictating enteric virus attachment to the surface of lettuce. However, just the opposite conclusion was reached by the same investigators when they found that NaCl (electrostatically charged compound) was more effective in promoting desorption of virus from the butterhead lettuce surface than was Tween 80 (a hydrophobic interaction disruptor) (Vega et al., 2007). The importance of electrostatic interactions in virus binding to lettuce remains unclear, but it is likely both product and virus-specific. A reference system that includes representative surrogates has been produced in an effort to account for the known physiochemical impacts of electrostatic and hydrophobic interactions. The system was designed to predict virus attachment behaviors to produce surfaces such as Romaine lettuce and strawberries, although its usefulness appears limited (Deboosere et al., 2012).

The first studies to visualize that enteric viruses and/or their surrogates bound to fresh produce, and where this occurred, were actually done a little later than the work of Vega et al. (2005; 2007). For example, Gandhi et al. (2010) evaluated HuNoV attachment to Romaine lettuce using fluorescently-labeled recombinant Norwalk VLPs, showing by confocal microscopy that aggregates were not evenly distributed across the leaf surface, but rather were localized along the veins. This was confirmed by others using

immunofluorescence microscopy (Tian et al., 2011). Wei et al. (2010) used SYBR gold-labeled murine norovirus to demonstrate that the virus localized preferentially along cut edges of the leaves and the stomata of Romaine lettuce. Alexa Fluor-labeled HuNoV VLPs also attached to Romaine lettuce predominantly at cut edges, stomata, and minor veins (Esseili et al., 2012).

Tian et al. (2011), based on their evaluation of washing to remove HuNoV from Romaine lettuce leaves, set forth the hypothesis that tissue-specific ligands are responsible for differential binding patterns for HuNoV. More specifically, cell wall material (CWM) is probably the plant component mediating most virus-produce binding (Caffall and Mohnen, 2009; Ghandi et al., 2010). Ghandi et al. (2010) hypothesized that proteins were partially responsible for the virus-produce binding because oxidation of a lettuce extract improved binding, while heating the extract greatly diminished binding. In reality, virus-produce binding is likely to be complex and variable. For instance, Caffall and Mohnen (2009) showed that the binding of Romaine lettuce CWM carbohydrates to HuNoV VLPs differed by leaf age, with young leaves binding VLPs primarily through protein interactions, while older leaves bound through carbohydrates.

Of course, based on the previous work with molluscan shellfish, HBGA-like moieties or components thereof were deemed the most logical molecular candidate for promoting HuNoV attachment to fresh produce. Evidence supporting this hypothesis is mixed. For example, Gandhi et al. (2010) used a competitive binding assay with porcine gastric mucin (PGM) to determine the specificity of attachment between HuNoV VLPs and Romaine lettuce extracts. Because the extracts did not compete with PGM for VLP binding sites, they

concluded that lettuce-virus binding was not mediated by HBGA-like moieties. This was confirmed by Esseili et al. (2012) who did similar blocking experiments with Romaine lettuce using monoclonal antibodies (MAbs) against human HBGAs. They, too, failed to find evidence that HBGA-like molecules were responsible for these interactions. The same can be said for Hirneisen and Kniel (2013) in their work on homogenates of spinach and green onion.

However, recent investigations have caused us to rethink the hypothesis that HBGA-like carbohydrates in the CWM of lettuce mediate HuNoV binding. Esseili et al. (2012) used competitive ELISA with lectins that bound sugars to show that HuNoV attachment to Romaine lettuce was modulated at least in part by a number of carbohydrates known to occur in lettuce CWM. By digesting portions of the cell wall with degradative enzymes, effectively exposing more binding sites, Gao et al. (2016) observed significantly increased binding of GII.4 VLPs to Romaine lettuce. Using competitive ELISA, they found the presence of H type HBGA-like moieties in the lettuce tissues, and GII.4 VLPs bound more specifically to digested/exposed internal plant fucose molecules (Gao et al., 2016). These investigators concluded that HuNoV binding to fresh produce may be facilitated by numerous surface-mediated and internal binding ligands with some relationship to carbohydrates or at least monosaccharides.

As stated above, the bulk of the work looking at the attachment of HuNoV and the cultivable surrogate viruses to fresh produce has been done with leafy greens. Besides the obvious epidemiological findings that support HuNoV binding to fresh berries, there are few laboratory-based data characterizing this phenomenon. Le Guyader et al. (2004) were able,

after methodological optimization, to detect HuNoV by RT-qPCR from the raspberries associated with an acute gastroenteritis outbreak. Although Tian et al. (2011) observed (via immunofluorescent microscopy) that HuNoV VLPs bound to raspberry surfaces were randomly distributed, they hypothesized that differences in virus binding efficiency occurred because of the presence of produce-specific ligands. Other than these studies, there is no other information on HuNoV-berry interactions.

Conclusions

Foodborne viruses are extremely well adapted to persist among a diverse array of environmental conditions and readily bind to a variety of surfaces including fresh produce. Current virus removal and remediation strategies are insufficient, making prevention of contamination, and inactivation, difficult to achieve. The studies discussed above highlight the complexity in elucidating foodborne virus attachment to produce. While these studies focus almost entirely on leafy greens, there are a few consistent conclusions. Firstly, HuNoV appear to attach tenaciously to fresh produce. Such attachment is somewhat selective, with aggregation on or in structures such as veins and stomata, and in damaged locations. It has been hypothesized that selective attachment of HuNoV to fresh produce is mediated by HBGA-like moieties, and recent evidence is suggesting this may be the case, or at least key sugars commonly associated with these complex molecules. It appears that tissue damage may promote release and/or exposure of these molecules, which may result in stronger binding of virus. This is an important finding that suggests that the quality of fresh produce is directly correlated to the overall susceptibility of that produce to viral pathogen attachment.

Better understanding of the complex dynamics that govern HuNoV binding to fresh produce would provide valuable insight to determine if agricultural practices, human behaviors, and/or environmental changes can significantly contribute to enhanced or reduced virus attachment. This increased understanding of how and why HuNoV attach to produce surfaces would aid in the development of effective removal strategies such as targeted washes, as well as provide a strong basis for resolving the binding dynamics of HuNoV to other commonly contaminated foods.

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Tables

Table 1.1 Summary evidence characterizing the attachment of enteric viruses to fresh produce.

Produce Type	Virus	Hypothesis/Purpose	Methods	Major Findings	Reference
Butterhead lettuce	echovirus 11, feline calicivirus [FCV], MS2, and ϕX174)	Hydrophobic and electrostatic forces drive binding	Double layer plaque assays	NaCl greatly inluenced the attachment of virus to produce	Vega, E. et al., 2008
Butterhead lettuce	feline calicivirus (FCV), echovirus 11, phages fX174 and MS2	Dertemine if (pI) of viruses had any effect on attachment	Plaque assays	The adsorption of virus particles to the lettuce was variable	Vega, E. et al., 2005
Romaine lettuce	murine norovirus 1 (MNV)	Observe attachment of virus from biosolids/manure to produce	Plaque assays, confocal microscopy with SYBR gold	MNV in biosolids had higher levels of attachment than pure virus in manures	Wei, J., et al. 2010
Romaine lettuce	SaV, HuNoV, FCV, and MNV	SaV physiochemical resistence compared to HuNoV	TCID50, RT-PCR	SaV is a viable surrogate for testing the attachment to lettuce	Wang, Q, et al., 2012
Romaine/iceberg lettuce, cilantro, spinach, celery	rNVLP	Evaluated lettuce as potential HuNoV vehicle	ELISA, confocal microscopy (Alexa-Fluor 488)	Did not bind by HBGAs and did not compete with PGM for binding sites	Gandhi, K. et al., 2010
Romaine lettuce	NVLP (GII.4)	Examined role of carbohydrates in HuNoV attachment	ELISA, confocal microscopy (Alexa-Fluor 488)	Disrupting specific carbohydrate residues significantly reduced VLP binding	Esselli, M. A. et al., 2012
Romaine lettuce, raspberries	HuNoV, NVLP (GI.I and GII.4)	Effectiveness of HuNoV removal from produce	RT-qPCR, confocal microscopy (Alexa-Fluor 488)	Minimal to no gain in virus removal using electrolyzed water rinses	Tian, P. et al., 2011b
Romaine lettuce, spinach	HuNoV, MNV, SaV, and Tulane virus	Evaluate virus survival on stressed preharvest produce	TCID50, RT-PCR, and bacterial counts	Physical damage greatly enhanced viral persistence	Esselli, M. A., et al. 2016
Romaine lettuce, strawberries, and raspberries	HAV, HuNoV (GI.I and GII.4), MNV, phages (MS2, GA, and Qβ)	Investigate the physiochemical forces that define adhesion	RT-qPCR	MNV was the best surrogate in simulating an adhesion event	Deboosere, N. et al., 2012
Romaine lettuce, green onions	HuNoV (GII.4), MNV, Tulane virus	Investigated the attachment of viruses to fresh produce	RT-qPCR, confocal microscopy (Q.Dots)	Different viruses differ in localization patterns; virus aggregation on lettuce	DiCaprio, E. et al., 2015
Spinach	HuNoV (GII.4), MNV, Tulane virus	Assess the survival and stability of viruses on produce	RT-qPCR, plaque assays, Cryo-scanning EM	Virus innoculation location had largest impact on survival	Hirneisen, K. A., & Kniel, K. E. 2013

Table 1.2 Key features of important enteric viruses having potential foodborne transmission routes.

Virus	Family	Diameter of Virion	Nucleic Acid	Genomic Length	Incubation Period	Hallmark Symptoms	Chronic Symptoms	Immunity
Norovirus	*Caliciviridae*	27-38 nm	Positive-Sense	7.7 kb	12-48 hours	Vomiting	Rarely, in Immuno-	Strain specific
			Single Stranded RNA			Acute gastroenteritis	Compromised	< 2 years
Hepatitis A	*Picornaviridae*	27-32 nm	Positive-Sense	7.5 kb	15-50 days	Fever, Malaise	None	Life-Long
			Single Stranded RNA			Jaundice		
Rotavirus A	*Reoviridae*	76.5 nm	11 RNA double helices	18.5 kb	< 48 hours	Vomiting	None	Life-Long
						Acute gastroenteritis		
Astrovirus	*Astroviridae*	28-35 nm	Positive-Sense	6.8-7.9 kb	3-4 days	watery diarrhea, fever	None	Life-Long
			Single Stranded RNA			abdominal pain		
Adenovirus	*Adenoviridae*	90-100 nm	Double Stranded DNA	26-48 kb	5-9 days	watery diarrhea	None	Life-Long to
			Positive-Sense			low-grade fever		Specific Genotypes
Hepatitis E	*Hepeviridae*	27-34 nm	Single Stranded RNA	7.3 kb	3-8 weeks	jaundice, vomiting	Yes, in Immuno-	Life-Long
						fatigue, diarrhea	Compromised	

Figures

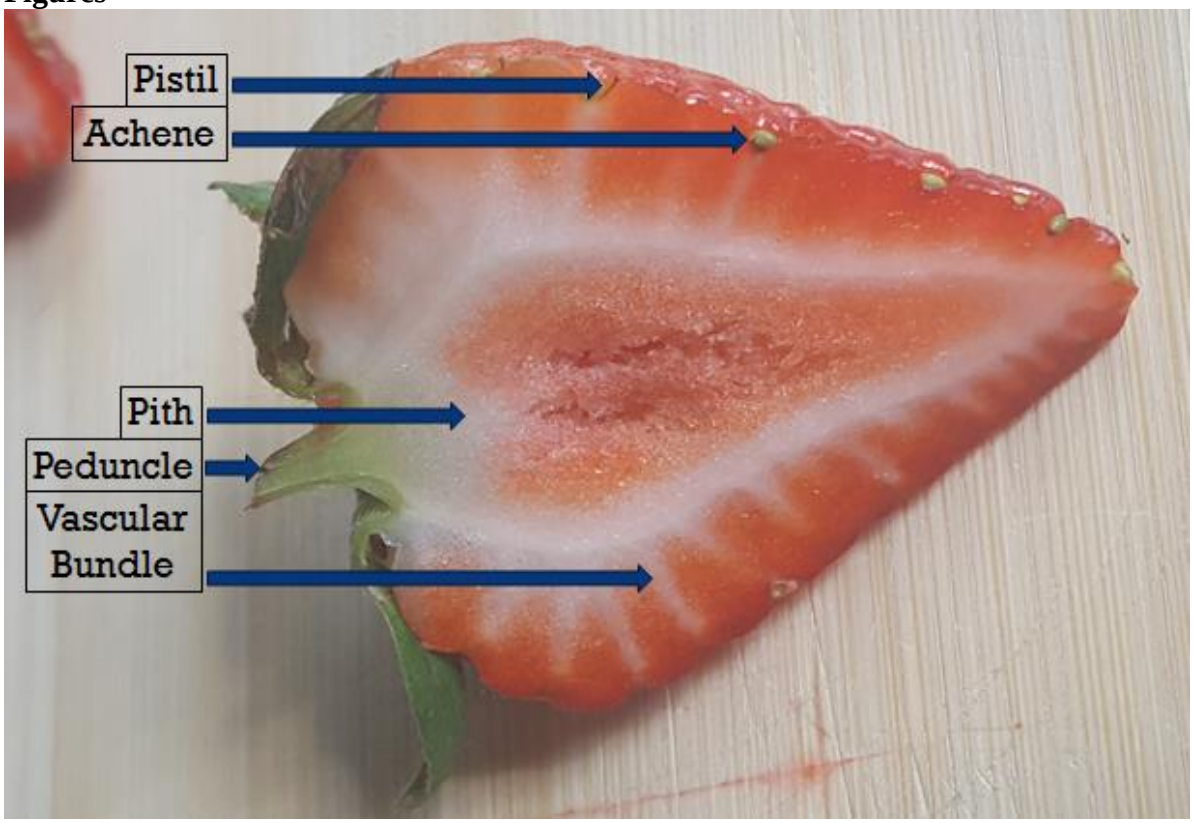

Figure 1.1 Major structural features of a mature strawberry.

CHAPTER 2

Natural Microbiota of Raspberries (*Rubus idaeus*) and Strawberries (*Fragaria* x *ananassa*): Microbial Survey, Bacterial Isolation and Identification, and Biofilm Characterization

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Abstract

Human norovirus (HuNoV) outbreaks have been associated with the consumption of contaminated fresh raspberries (*Rubus idaeus*) and strawberries (*Fragaria* x *ananassa*). The dynamics of HuNoV binding to berries are unknown: viruses may attach to berry-specific ligands; bind non-specifically (electrostatic and hydrophobic interactions); or bind in a facilitated manner such as in association with native berry microflora. In fact, HuNoV have been shown to bind to specific structures found on the extracellular polymeric substances (EPS) produced by biofilm-producing bacteria. A microbial survey was done on (*Rubus idaeus var. strigosus* 'Heritage') raspberries and 'Chandler' strawberries obtained from local U-Pick farms (Angier, NC and Raleigh, NC, respectively) by plating them on selective and differential media. A total of 172 (n=98 from raspberries; n=74 from strawberries) unique bacterial isolates were identified, with the microbial profiles differing by berry type. Selected isolates were tested for the capacity to form biofilms using a crystal violet-based assay. Biofilm production varied greatly among of isolates, but strains of *Pantoea agglomerans, Pseudomonas moraviensis, Klebsiella oxytoca,* and *Enterobacteriaceae spp*. were the strongest biofilm producers (A_{405} > 2.2). These strong biofilm-producing strains will be used in future studies to characterize the attachment of HuNoV to the natural berry microflora and

determine if adherence to specific microflora components (e.g., EPS) significantly contributes to the attachment and persistence of HuNoV on berries.

INTRODUCTION

Epidemiological studies have shown that human norovirus (HuNoV) is the leading causative agent for food-borne illnesses throughout the developed world (Widdowson et al., 2005). HuNoVs are small (28 -35 nm in diameter), non-enveloped, single-stranded RNA viruses that are primarily transmitted through the fecal-oral route. These viruses are shed by infected individuals at high levels (up to 10^{11} per g of feces) (Atmar et al., 2008), have a low infectious dose (~18-100 viral particles) (Teunis et al., 2008), and are environmentally persistent on a variety of fomites, surfaces, and foods. Causing acute gastroenteritis infections among all age and immune status groups, hallmark HuNoV symptoms include nausea, cramping, vomiting, and diarrhea, typically lasting for 48 to 72 h.

It is estimated that fruit/nut commodities account for ~14% of all food-borne illnesses (with known etiology) within the United States (Painter et al., 2013). In recent years, a number of large HuNoV outbreaks have been associated with fresh produce, specifically with raspberries and strawberries. These outbreaks have occurred in countries such Sweden, Finland, Poland, and Germany (Le Guyader et al., 2004; Falkenhorst et al., 2005; Sarvikivi et al., 2011; Bernard et al., 2014). Produce items become contaminated with HuNoV by contact with human fecal matter. This can occur when production or processing waters become contaminated with untreated human sewage, or by contact with the hands of an infected individual not practicing adequate personal hygiene (Tuan Xainazor et al., 2010). Unfortunately, washing methods have been determined to be an ineffective means of HuNoV removal (Tian, et al., 2011); therefore, minimizing the overall risk of contamination with HuNoV has been the primary focus of the agricultural and food industries.

Although many studies have identified specific mechanisms of bacterial attachment to fresh produce, few have determined any significant factors contributing to the interactions and adherence of food-borne viruses to produce (Berger, 2010). Recent studies showed that HuNoV effectively bound to the extracellular polymeric substances (EPS) of enteric bacteria isolated from fecal samples (Miura et al., 2013). EPS produced by bacteria is mainly composed of complex polysaccharides and serves as a scaffold for bacterial aggregation and biofilm formation (Costerton et al., 1987). Many bacterial species commonly associated with the natural microflora of produce are known biofilm producers. In this study, a general survey of the microbial populations present on fresh raspberries and strawberries was performed. Cultivable bacteria were isolated, sequenced, and their relative biofilm production capacity was quantified. Ultimately, the purpose of this study was to identify strong biofilm-producing bacterial species of the natural berry microflora that may significantly contribute to the binding and persistence of HuNoV on fresh berries.

MATERIALS AND METHODS

Collection, Isolation, and Identification of Natural Berry Microflora

Heritage raspberries and Chandler strawberries were harvested with disposable sterile gloves at Pick-Your-Own farms located in Angier, NC and Raleigh, NC, USA, respectively. One hundred g of berries were added to 100 ml of phosphate buffer saline (PBS) (Sigma, pH 7.2) and placed on a shaker (100 rpm) for 10 min at room temperature. The rinse buffer was collected and plated on a variety of media including plate count agar (PCA); potato dextrose acidified agar (PDAA); crystal violet tetrozolium (CVT) agar; phenyl ethyl alcohol (PEA)

agar; and de Manne, Rogosa and Sharpe (MRS) agar at 32°C for 48 h. Plates were counted to determine general microbial profile. Unique bacterial colonies were then selected (based on typical bacterial colony morphology) and restreaked for isolation. Pure bacterial colonies were stored in Cryocare tubes (Key Scientific, Stamford,TX) at -80°C. Duplicate colonies were sent to Genewiz (Research Triangle Park, NC) for whole colony PCR and sequencing performed using proprietary universal primers and analyzed using an ABI Prism 370*xl* DNA analyzer. Sequences were analyzed using CLC Sequence Viewer Version 6.8 and the BLAST Sequence Analysis Tool (http://blast.ncbi.nlm.nih.gov/).

Determining Bacterial Biofilm Production

Bacterial isolates identified as well-characterized genera and species commonly associated with biofilms were screened in triplicate for biofilm production. Bacterial cultures were grown in 10 ml of tryptic soy broth (TSB) at 32°C overnight, centrifuged for 8 min at 18,500 *x g* and resuspended in fresh TSB to an approximate OD_{600} of 0.1. In a non-treated, polystyrene 96-well plate (Costar, Corning, NY), 30 µl of resuspended culture and 170 µl of fresh TSB were added to each well, an EasyBreathe film (USA Scientific, Ocala, FL) was applied, and the plate was incubated at 32°C for 48 h. Wells were gently washed 3 times with 200 µl of PBS, and 200 µl of 1% crystal violet was added to wells for 1 min. Each well was washed with 300 µl of deionized water, tapped dry, and 200 µl of 95% ethanol was added. The 96-well plate was immediately read at OD_{600} using an infinite M200 Pro NanoQuant microplate reader (Tecan, Morrisville, NC).

RESULTS

Overall, strawberries contained a higher total microbial load (1.0×10^6 cfu/g) than did raspberries at (6.3×10^5 cfu/g) (Table 1)**.** The Gram-negative bacteria were the most abundant microbes on the surface of both raspberries and strawberries. Yeasts and molds were the second most abundant microbes found on the raspberries and strawberries at 3.3×10^4 cfu/g and 2.1×10^5 cfu/g, respectively. The raspberries showed similar abundance of yeasts and molds, lactic acid bacteria, and *Enterobacteriaceae*. Although there was almost one $\log_{10}$ more lactic acid bacteria present on the raspberries than that of the surface of strawberries, there were greater than 2 $\log_{10}$ more Gram-positive bacteria present on the strawberries.

Unique bacterial colonies were isolated from the selective and differential media based on differences in colony morphology, and 204 plates (n=101 from raspberries; n=103 from strawberries) were sent off for whole colony PCR sequencing. A total of 172 (n=98 from raspberries; n=74 from strawberries) unique bacterial isolates were successfully sequenced. Microbial profiles were substantially different when comparing the raspberry and strawberry isolates (Table 2). Gram-negative bacterial genera including *Erwinia* and *Xanthomonas* were found among the raspberry isolates but were absent in the strawberry isolates (Table 2). Compared to the strawberry isolates, raspberries had an abundance of unique Gram-positive isolates with 32 from the genus *Curtobacterium* and five lactic acid bacterial isolates from the genus *Lactococcus.* While raspberries had over three times more isolates from the genus *Sphingomonas*, both raspberries and strawberries had high numbers of *Pantoea* isolates (13 and 20 isolates, respectively), moderate numbers of *Pseudomonas*

isolates, and both berry types had a few isolates from the family *Enterobacteriaceae* (unable to be further differentiated by species). However, strawberries had 17 isolates from the Gram-positive genus *Bacillus* compared to one isolated from raspberries. Strawberries also had several unique Gram-negative genera including *Gluconobacter*, *Klebsiella*, and *Tatumella*.

The crystal violet biofilm assay showed that there were significant differences in the biofilm formation capacity between isolates of different genera, species, and strain (Figure 1). Under these assay conditions, isolates from the genus *Bacillus* were observed to be low biofilm-producing strains with an average $ABS_{405} < 0.44$. Isolates form the *family Enterobacteriaceae*, *Klebsiella oxytoca*, *Pantoea agglomerans*, and *Pseudomonas moraviensis* were all shown to be strong biofilm producers with average $ABS_{405} > 2.2$, however great heterogeneity and variability were observed in the biofilm production among isolates of the family *Enterobacteriaceae* and genus *Erwinia*. Statistically significant differences ($P \leq 0.05$) in biofilm production were determined within isolates of the same species such as *Pantoea agglomerans* and *Klebsiella oxytoca*.

DISCUSSION

The microbial counts for both raspberries and strawberries were relatively high, but within the expected cultivable microbial density for freshly harvested produce items (Babic, 1995). Despite growing through plastic mulch row covers, strawberries lie on the ground surface or are elevated just above ground level; therefore, strawberries come into contact with a higher number of microbes than raspberries that are suspended in the bushes. A high

concentration of yeasts and molds was expected in both produce items because their spores are ubiquitously found in abundance throughout the fruit growing season and they are most resistant to the acidic berry conditions (Eckert, 1988). The bacterial sequencing results identified genera and species that corresponded well to the specific micro-environment of each berry. While many of the bacteria are ubiquitously found throughout the anatomical micro-environments of a plant (roots, stems, leaves, flowers/fruit), certain bacterial genera and species have evolved in a niche micro-environment and are characteristic of a specific location on the plant (Ottesen et al., 2013). The sequencing results for the raspberry isolates showed many bacteria from the genus *Curtobacterium* that are associated with the elevated leafy, bushy region of the plant that does not come in immediate contact with the ground (Dunleavy, 1989). The sequencing results of the strawberry isolates identified many *Bacillus* isolates that are indicative of the roots and plant parts that were in constant contact with the ground surface or soil (Garbeva et al., 2008). As advancements in isolation and sequencing technologies continue, farming operations will be able to efficiently monitor microbial pest and spoilage species within their fields and apply the optimal therapeutic treatments to maximize pre-harvest crop health and yields, as well as provide post-harvest benefits such as reduced spoilage and extended shelf-life produce.

It was not surprising to identify a number of bacterial isolates with strong biofilm-producing capabilities derived from these raspberries and strawberries. Microbial biofilms are ubiquitous, complex polysaccharide matrices that provide protection from environmental stresses and enable additional microorganisms to colonize the surfaces of fresh produce items (Wolfaardt, 1994). Common biofilm-producing bacteria such *Bacillus* spp. *Erwinia* spp., and

Pseudomonas spp. contain pectinolytic and proteolytic enzymes that contribute to soft rot and ultimately produce spoilage (Hao et al., 1994). Produce biofilms have been demonstrated to develop extensively after various washing treatments, and throughout modified atmosphere and refrigerated storage (Poulsen, 1999). These natural berry biofilm-producing strains may contain similar moieties to those of host cell receptors and co-receptors for HuNoV, facilitating HuNoV binding with high affinity via specific structures or ligands (Tan et al., 2011).

The surface microbiota of fresh produce can be exposed to HuNoV throughout the stages of pre-harvest and postharvest production; these surface microbes are potentially "biological shuttles" that incidentally facilitate the transfer of HuNoV to human consumers of fresh produce items. If the natural microbiota does not produce specific HuNoV binding ligands, it does not preclude HuNoV from binding bacteria non-specifically (electrostatic and hydrophobic interactions) or enhancing the direct attachment of HuNoV to the produce surface via a reduction in charge repulsion or steric hindrance. Performing this microbial survey has provided a better understanding of the overall surface microbiota of raspberries and strawberries. Identifying and characterizing the biofilm-producing bacterial isolates will allow future studies to determine if the natural berry microflora significantly contributes to the binding and persistence of HuNoV on fresh berries. This should provide valuable insight for targeted strategies to prevent HuNoV contamination, or remove HuNoV from the surface of berries.

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Table 2.1. Microbial survey (reported as cfu/g) based on duplicate selective media plating of raspberries and strawberries collected in Pick-Your-Own fields in Angier, NC and Raleigh, NC, USA, respectively.

Media (targeted organisms*)	**Raspberry (cfu/g)**	**Strawberry (cfu/g)**
PCA (All Aerobes Microbes)	$6.3x10^5$	$1.0x10^6$
CVT (Gram-Negatives BAC)	$1.5x10^5$	$5.1x10^5$
PDA A (Yeasts/Molds)	$3.3x10^4$	$2.1x10^5$
MRS (Lactic Acid BAC)	$2.3x10^4$	$2.5x10^3$
LMBA (Enterobacteriaceae)	$1.3x10^4$	$7.4x10^4$
PEA (Gram-Positive BAC)	$1.8x10^2$	$4.8x10^4$

*Based on growth/presence on selective media agents

Table 2.2. Bacterial genera profiles of raspberries and strawberries based on BLAST results from 16S sequencing.

Genera*	# Raspberry Isolates	# Strawberry Isolates
Bacillus	1	17
Curtobacterium	32	0
family Enterobacteriacae	3	5
Erwinia	5	0
Gluconobacter	0	5
Klebsiella	0	4
Lactococcus	5	0
Pantoea	13	20
Pseudomonas	7	11
Sphingomonas	17	5
Tatumella	0	2
Xanthomonas	6	0

*Based on highest sequence identity of 16S BLAST results

Figures

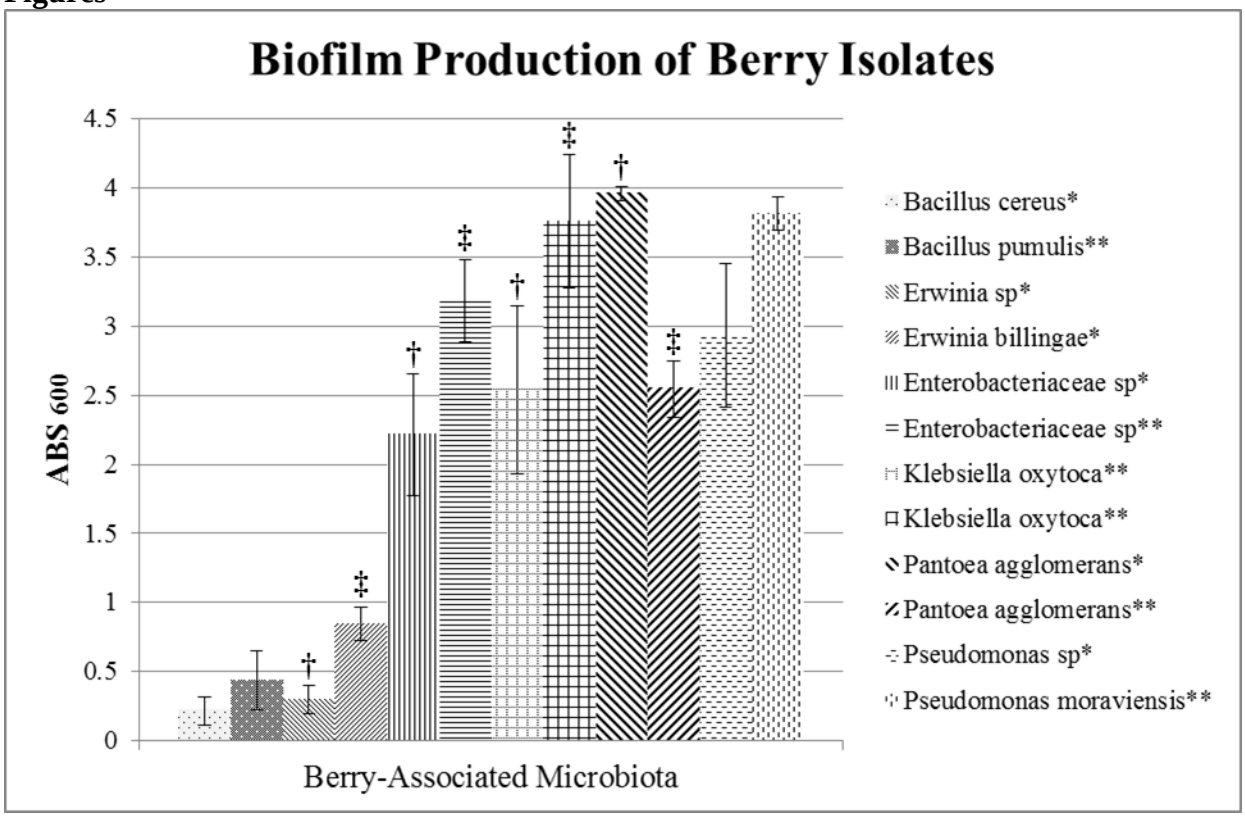

Figure 2.1. Graph showing the average biofilm production of natural isolates from different genus and species (raspberries* and strawberries**). † and ‡ indicates statistical differences ($P \leq 0.05$) in biofilm production among isolates within the same family (Enterobacteriaceae), genus, and/or species.

CHAPTER 3

Characterization of Human Norovirus Attachment to Fresh Strawberries

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Abstract

Fresh produce, specifically berries, has been associated with high profile human norovirus (HuNoV) outbreaks, however little is known about how the virus binds to berries. The purpose of this study was to elucidate the binding dynamics of HuNoV to fresh strawberries. The relative binding efficiency of select HuNoV [GI.6 and GII.4 (New Orleans)] strains was first characterized. This was followed by determination if specific binding ligands (histo-blood group antigens, or HBGA-like moieties) could be identified in fresh strawberries/strawberry components, and in berry-associated bacterial isolates. Strawberry surface slices showed moderate HuNoV binding (84- 92%) across a broad pH range (3.5 to 7.0) using an RT-qPCR-based assay, suggesting electrostatic interactions alone do not strongly mediate attachment. Virtually all of the natural berry bacterial isolates showed minimal HuNoV binding. HBGA-like moieties [A, B, H(O); Lewis A, B, Y] were absent on strawberry and raspberry varietals, as well as on the surface of native berry microflora, as determined by ELISA. Immunofluorescence confocal microscopy showed that HuNoV GII.4 (Houston) virus-like particles (VLPs) bound selectively to strawberry pistils and achenes, with even greater preference for sites of fracture and injury on the berry style. Preferential binding to these berry structures (achenes/pistils) was confirmed using a suspension assay followed by RT-qPCR. This study provides evidence of efficient HuNoV

binding to fresh strawberries and suggests that the mechanism of that binding is both selective and complex.

Introduction

Based on recent epidemiological evidence, enteric viruses are now recognized as the leading cause of foodborne illness worldwide, with human norovirus (HuNoV) being the major culprit (Scallen et al., 2011;Widdowson et al., 2005). In the United States, alone, norovirus-contaminated foods are estimated to cause 5.5 million illnesses (ranked 1^{st}), 15, 000 hospitalizations (ranked 2^{nd}), and 150 deaths (ranked 4^{th}) annually (Scallen et al. 2011). Although a number of key commodities are commonly associated with foodborne viral outbreaks (molluscan shellfish and ready-to-eat foods for example), analysis of surveillance data of 364 single source outbreaks from 2001-2008 showed that leafy vegetables (33%) and fruits/nuts (16%) were most commonly implicated (Hall, 2012).

Human norovirus contamination of foods most often happens due to contact with human fecal matter. The virus is shed by infected individuals at high concentrations (up to 10^{11} per g of feces) (Atmar et al., 2008), has a low infectious dose (~18-100 viral particles) (Teunis et al., 2008), and is environmentally persistent in foods and on surfaces (D'Souza et al., 2006). The virus is also very difficult to inactivate, especially when on foodstuffs. Fresh produce can come into contact with human fecal matter in a variety of ways, including the application of virus-contaminated fertilizers and amendments, waters (irrigation systems, processing water), and direct hand contact with infected individuals at pre- and/or post-harvest phases (Tuan Xainazor et al., 2010).

While fresh produce items produced in any region can become contaminated with enteric viruses, the relatively poorer access in developing countries to advanced measures to manage human sewage and personal hygiene increases the likelihood of contamination in

these parts of the world. This is of concern even in countries such as the U.S., where nearly fifty percent of fresh fruit and twenty percent of fresh vegetables are imported (FDA, 2013). Indeed, recent high profile outbreaks in Sweden, Finland, Poland, and Germany were linked to raspberries and strawberries grown in developing countries (Le Guyader et al., 2004; Falkenhorst et al., 2005; Sarvikivi et al., 2011; Bernard et al., 2013). For many of these outbreaks, the direct mode of product contamination was never identified.

It is well documented that human pathogens adhere to the tissue of fresh produce items and many studies have identified specific mechanisms of bacterial interaction and attachment to a variety of foods (reviewed by Berger et al., 2010). There have been far less such studies on virus-food interactions. It is possible that virus-produce interactions could be mediated by electrostatic interactions, although this has not been comprehensively studied. It is known that the tenacious binding of HuNoV to molluscan shellfish tissue is mediated by histo-blood group antigen (HBGA)-like moieties found in the oyster gastrointestinal lining (Tian et al., 2006). Histo-blood group antigens are the putative host cell receptor(s)/co-receptor(s) for HuNoV. It has been hypothesized that HBGA-like components also facilitate virus binding to fresh produce items (Gandhi et al., 2010; Esseili et al., 2012), but only recently has compelling scientific evidence of this been reported (Gao et al., 2016). It is also possible that native enteric microflora having HBGA-like moieties on their outer surfaces and/or in extracellular polymeric substances bind HuNoV, promoting attachment (Miura et al., 2013). Biofilms formed by these bacteria may also bind to viruses (Baugher and Jaykus, 2015).

Most studies on virus-produce interactions have focused on leafy greens. Although recent evidence demonstrated that HuNoV bind to the surface of raspberries (Tian et al., 2011), the elucidation of the biochemical and biophysical forces that govern this attachment to berry surfaces remains unclear. The purpose of this study was to elucidate the binding dynamics of HuNoV to fresh strawberries. Three different binding mechanisms (electrostatic, HBGA-mediated, and bacteria-mediated) were evaluated. Immunofluorescent confocal microscopy was used to visualize the attachment of VLPs to specific berry structures. The HuNoV binding efficiency to strawberries and strawberry components throughout berry development phases was also investigated.

Materials and Methods

Viruses, Virus-like Particles (VLPs), and bacteria

Berry-associated surface bacteria were isolated and identified as previously described (Baugher and Jaykus, 2015) and included raspberry isolates [*Pseudomonas moraviensis* and *Klebsiella oxytoca*], and strawberry isolates [*Pantoea agglomerans* and *Enterobacteriacae* spp.]. Other bacterial isolates used as experimental controls were obtained from ATCC (Manassas, VA) and included *Enterobacter cloacae* ATCC 13047, *Staphylococcus epidermidis* ATCC 35984, and *Lactobacillus gasseri* ATCC 33323. Stool samples originating from outbreaks and previously confirmed positive for HuNoV GII.4 (New Orleans) and GI.6 HuNoV were obtained courtesy of S.R. Greene (North Carolina Department of Health and Human Services, Raleigh, NC). Human norovirus GII.4 Houston

virus like particles (VLPs) were provided courtesy of Dr. Robert Atmar (Baylor College of Medicine, Houston, TX).

Berries

For binding studies as a function of berry variety, homogenized strawberry varietals (Camarosa, Chandler, Galletta, B1033, Totem, NCC02-05), and raspberry varietals (Autumn Britten, Caroline, Nantahala) were obtained courtesy of Dr. Gina Fernandez (NCSU Horticulture Department) and stored at -20°C until use. For all HuNoV binding studies, Camarosa and Chandler strawberries were obtained from the Central Crops Research Station (NC Department of Agriculture and Consumer Services, Clayton, NC) and stored at 4°C until processed on the day of harvest. Using a culinary mandolin, 1.5 mm of strawberry surface slices were collected immediately prior to experiments. Strawberry achenes and pistils were removed from berry surfaces by hand using electron microscopy tweezers and stored at -80°C.

HuNoV binding to whole strawberries and strawberry components by suspension assay

Camarosa strawberries were collected during the three major berry developmental stages (strawberry flowers, unripened berries, and ripened berries). To eliminate RNA extraction inhibitors prior to virus exposure, 4 g of intact 1.5 mm strawberry surface slices were soaked by immersion in 10 ml phosphate buffered saline (PBS pH 7.2, Life technologies, Carlsbad, CA) for 10 min at room temperature (RT), followed by centrifugation for 2 min at 2,000 *x g* and removal of the supernatant. This process was

repeated two more times. Washed berry surface slices were then mixed with PBS and pH adjusted (using 0.1 M hydrochloric acid and sodium hydroxide) to 3.5, 5.5 and 7.0 in a total of 9.8 ml. To each pH adjusted treatment, 200 µl of GI.6 and GII.4 (New Orleans) HuNoV-positive stool sample [20% suspension; total input virus per experiment of ~10^6 and ~10^7 genome equivalent copies (GEC) for GI and GII.4 (New Orleans), respectively.] Similar experiments were performed in which 12 mg of strawberry pistils were mixed with 500 µl of a 1:5 dilution of GI.6 and GII.4 (New Orleans) HuNoV-positive stool sample [total input virus per experiment of ~10^5 and ~10^6 GEC for GI and GII.4 (New Orleans), respectively.] After rotating the berry tissue-virus suspensions at RT for 2 h, the tissue was sedimented by centrifugation at 8,200 *x g* or 5 min, and 1 ml and 100 µl of the supernatant was collected for the berry and pistil experiments, respectively. These supernatants were retained for RNA extraction and RT-qPCR as described below.

Human norovirus binding to bacteria by suspension assay

Bacterial cultures were propagated overnight under the following conditions: *Enterobacter cloacae* ATCC 13047 *Pseudomonas moraviensis, Pantoea agglomerans, Klebsiella oxytoca, Enterobacteriacae sp*., were grown in trypticase soy broth (TSB, Becton Dickinson, Sparks, MD) at 30°C; *Staphylococcus epidermidis* ATCC 35984 was grown in TSB at 37°C; and *Lactobacillus gasseri* ATCC 33323) was grown in de Man Rogosa Sharpe (MRS) broth (Sigma Aldrich, St. Louis, MO) at 37°C. The bacteria in 10 ml of each culture (~10^8 cfu/ml) were pelleted by centrifugation at 8,200 *x g* for 8 min and resuspended in PBS. One hundred microliters of each resuspended culture was mixed with 100 µl of GI.6 or GII.4

(New Orleans) HuNoV-positive stool sample (total input virus per experiment of ~10^5 GI.6 GEC and ~10^6 GII.4 GEC) and the solution brought up to a volume of 500 µl using PBS. After rotation at RT for 2 h, the solutions were centrifuged at 13,000 *x g* and 100 µl of supernatant was collected. The supernatant was held for RNA extraction and RT-qPCR as described below.

RNA extraction and RT-qPCR amplification for HuNoV

RNA extraction was performed on the supernatants using the Nuclisens easyMAG system (Biomerieux, Durham, NC) as per manufacturer's instruction, with reconstitution volume of 50 µl. For GII.4 (New Orleans) RNA, the RT-qPCR reactions were carried out using primers JJV2F, COG2R, and probe RING2-TP [JJV2F (5'-CAAGAGTCAATGTTTAGGTGGATGAG-3'); COG2R (5-'TCGACGCCATCTTCATTCA CA-3'); and probe RING2-TP (5'-56-FAM TGGGAGGGCGA TCGCAATCT-3BHQ-3')] (Jothikumar et al., 2005). For GI.6 RNA, the reactions were carried out using primers COG1F (5'-CGYTGGATGCGNTTYCATGA-3') and COG1R (5'-CTTAGACGCCATCATCATTYAC-3') and probes RING1(A) (5'-56-FAM-AGATYGCGATCYCCTGTCCA-3BHQ-3') and RING1(B) (5'-56-FAM-AGATCGCGGTCTCCTGTCCA-3BHQ-3') (Kageyama et al., 2003). RT-qPCR was done using the Superscript III Platinum One-Step RT-qPCR system (Invitrogen, Carlsbad, California) according to manufacturer's instructions. Briefly, 25 µl RT-qPCR reactions consisted of 12.5 µl of 2x reaction mix, 8.0 µl DNase-RNase free water, 200 nM of each primer, 0.5 µl of the enzyme mix (SuperScript III RT/Platinum® Taq Mix), and 2.5 µl of the

RNA template. Amplification was done under the following conditions: 50^0C for 15 min, 95^0C for 2 min, followed by 45 cycles of 95^0C for 15 s, 54^0C for 30 s and 72^0C for 30 s in a SmartCycler (Cepheid, Sunnyvale, CA). The RNA copy number was extrapolated from a standard curve based on Ct values obtained by RT-qPCR amplification of serially diluted RNA obtained from each stool sample used as initial inoculum. Percent binding efficiency was calculated based on loss to supernatant by subtracting the RNA copy number (estimated GEC) of the supernatant from the input copy number, dividing by the input copy number, and multiplying the quotient by 100 (Papafragkou et al., 2008).

Determining the presence of HBGA-like moieties in berries and bacteria

To determine the presence/absence of HBGA-like moieties in berries and bacterial isolates, a modification of the Tian et al. (2007) ELISA protocol was used. One hundred microliters of homogenized raspberry and strawberry varietals were added to medium-binding polystyrene 96-well plates (Costar, Corning, NY) and incubated overnight at 4° C. The inoculum was removed from the wells, then the wells were immediately blocked with 200µl 10% skim milk (Oxoid Ltd, Basingstoke, UK) by overnight incubation at 4° C. The wells were washed three times with 200 µl of PBS 0.05% Tween (PBST) for 5 min each wash, then twice with 200 µl of PBS for 5 min each wash. A 100 µl volume of primary antibody [Abcam, Cambridge, UK; A, B, H (Ab2523), Lewis A (Ab3967), Lewis B (Ab3968), Lewis Y (Ab3359)] diluted 1:45 in PBS was added to each well, followed by incubation at RT for 1 h. The wells were again washed thrice with PBST and twice with PBS. The anti-mouse IgG- alkaline phosphatase secondary antibody (Sigma-Aldrich) diluted

1:3000 in PBS was applied to each well with incubation at RT for 1 h. The wells were washed again as described above. One hundred microliters of substrate (p-nitro phenyl phosphate; Sigma Aldrich) was applied to each well, incubated for 15 min, and the plate read at OD_{450} using an Infinite M200 Pro Tecan instrument (Mannedorf, Switzerland). Data were expressed as the ratio of the OD_{450} of an individual treatment divided by the OD_{450} of the negative control. Values were considered significant if the positive/negative ratio was greater than 2.0 (Tian et al., 2006; Almond and Jaykus, 2016). Positive controls were 0.1% porcine gastric mucin type II (Sigma Aldrich) for A, B, and H antibodies; and purified Lewis carbohydrates (Carbosynth, Berkshire, UK) for the Lewis antibodies. The presence of HBGA-like moieties in bacterial isolates was performed as described above with minor modifications. Specifically, 10 ml of each overnight culture (~10^8 cfu/ml) were pelleted by centrifugation at 8,200 *x g* for 8 min and resuspended in PBS. 100 µl of the resuspended culture was applied to wells of Nunc high-binding plates (Thermo-Fisher Scientific, Waltham, MA) and the blocking was performed with SuperBlock (Thermo-Fisher) for 2 h at 4° C. Negative controls included (i) wells coated with berries or bacteria but having no VLPs added; and (ii) SuperBlock-coated wells with VLPs added.

Evaluation of HuNoV VLP attachment to strawberries using immunofluorescence confocal microscopy

Visualization of HuNoV VLP attachment to strawberry tissue was done by confocal microscopy using a modification of the Esseili et al. (2012) protocol. Primary antibody NS14 (courtesy of R, Atmar, Baylor College of Medicine) was complexed with Zenon Alexa Fluor

488 using a mouse IgG labeling kit (Thermo Scientific) at a ratio of 2:1 (antibody: Alexa Fluor) by optimizing manufacturer's instructions. Individual whole ripe Camarosa strawberries (14-20 g per berry) were mixed with 2 ml of VLP suspension (10µg/ml in PBS, pH 7.2) in 250 ml glass beakers for 1 h at RT under gentle shaking. The berries were washed three times, 5 min each with PBS under shaking conditions. Berries were fixed with 4% paraformaldehyde (Sigma Aldrich) in PBS for 1 h at RT, followed by washing 3 times, 5 min each with PBS, and then blocked with 2 ml of SuperBlock three times for 15 min each. Two ml of Alexa Fluor 488-labeled primary antibody NS14 (2 µg/ml in PBS, pH 7.2) was added to each beaker, followed by incubation for 1 h at RT, under gentle shaking. The berries were washed three times with PBST, berries surface slices (1.5 mm thickness) were taken and placed on cell imaging dishes (Eppendorf, Hamburg, Germany). Confocal microscopy (Zeiss LSM 710; Leica Microsystems,Wetzler, Germany) was used to visualize the Alexa Fluor 488 labeled VLPs using the 488 wavelength of the Argon excitation laser. Achenes were observed directly from the surface of the whole strawberry, while individual pistils were removed from the surface prior to imaging. The experiment was performed in triplicate at the Cellular and Molecular Imaging Facility (North Carolina State University, Raleigh, NC). Controls included strawberries treated as above but without VLPs or the primary antibody-Alexa Fluor 488 conjugate.

Statistical analysis

Data were expressed as mean ± standard deviation of three independent experimental replicates. The data were analyzed by unpaired T test and one-way Analysis of Variance

(ANOVA) with the Tukey's multiple comparison test using GraphPad Prism ver. 5.0 d (San Diego, CA). Values of $p \leq 0.05$ were considered statistically significant.

Results

HuNoVs bound to whole strawberry slices

Suspension assays followed by RT-qPCR were done to assess the binding efficiency of two different HuNoV strains [GI.6 and GII.4 (New Orleans)] to strawberry surfaces at three different pH values (3.5, 5.5, and 7.0) (Figure 1). Moderate to high mean binding efficiency (84-92%) was observed for both viruses across the pH range tested. In general, lower binding efficiency was observed at pH 3.5; however, the only statistically significant differences ($p<0.05$) were between the means of pH 3.5 and 7.0 treatments for GI.6. There was no statistically significant difference ($p>0.05$) is percent binding efficiency when comparing GI.6 and GII.4 (New Orleans) HuNoV strains to each other at any given pH tested.

Bacterial isolates moderately bound HuNoV

The binding efficiency of bacterial isolates to HuNoV GI.6 and GII.4 (New Orleans) was also determined by suspension assay followed by RT-PCR detection (Figure 2). All bacterial isolates bound GII.4 (New Orleans) with efficiency ranging from 70-82%, with the exception of *L. gasseri*, which bound significantly less virus (~52%, $p<0.05$). The data for GI.6 was similar to that of GII.4 (New Orleans) for most bacterial strains, although a little more variable. *Pseudomonas moraviensis*, a raspberry isolate, and *S. epidermidis* ATCC

35984 showed significantly greater capture efficiency for the GI.6 strain (mean of 92% and 87%, respectively, $p \leq 0.05$) compared to the other bacteria. On the other hand, *L. gasseri* ATCC 33323 showed the lowest mean percent binding (48%), which was also statistically significant ($p \leq 0.05$). *Pantoea agglomerans* had the greatest variability HuNoV GI.6 binding.

Minimal to no HBGA-like moieties were detected on berries

The presence of HBGA-like moieties on berries was evaluated using ELISA (Figure 3). Overall, strawberry and raspberry varietals showed no evidence of appreciable concentrations of the HBGA-like moieties A, B, H(O), Lewis B, and Lewis Y. This was evidenced by minimal, although sometimes slightly statistically significant, differences in sample absorbance values in relation to negative controls. These differences were more notable for the Lewis A-like antigen, as there were statistically significant ($p > 0.05$) differences in absorbance between test samples and the negative control. However, no one particular varietal stood out as having particularly strong binding to Lewis A antibody.

Minimal to no HBGA-like moieties were detected on berry-associated microflora.

Pseudomonas moraviensis was the only bacterial strain of the seven screened that showed significant ELISA signals, suggesting the presence of the HBGA-like moieties, A, B, H(O) (Figure 4). In fact, the mean treatment/negative control ratios for *P. moraviensis* were identical to that of the positive control. None of the seven strains showed appreciable evidence of the presence of Lewis A, B, and Y-like moieties.

HuNoV VLPs attached to the surface of strawberries

Human norovirus VLPs (GII.4 Houston) were shown to attach to the surface of strawberries by immunofluorescence confocal microscopy. Virus-like particle binding appeared to be localized in association with specific strawberry structures and not randomly dispersed across the surface of the berry. For instance, HuNoV VLPs selectively aggregated on the surface of strawberry achenes (commonly referred to as the "seed" but in actuality, one individual "fruit" with a fertilized ovary) (Figure 5A). The VLPs also had affinity for strawberry pistils (commonly referred to as "hairs" but which are actually comprised of remnants of the stigma and style). In Figure 5C, binding of HuNoV VLP aggregates can be seen dispersed among the golden pollen grains affixed to the surface of the pistils (Figure 5C). Sites of fracture and injury on the surface of the pistils (specifically the style) were also a common location for VLP aggregation (Figure 5E).

HuNoV selectively bind to strawberry achenes and pistils

Based on the confocal microscopy results, studies were undertaken to further elucidate HuNoV binding as a function of specific fruit tissues. The binding efficiency for HuNoV to Camarosa strawberries throughout the three main berry developmental stages (strawberry flowers, unripened berries, and ripened berries) was determined by suspension assay-RT-PCR (Figure 5). Whole berry surface slices bound over 90% of GI.6 and GII.4 (New Orleans) at the unripened and ripened berry stages. Interestingly, when the achenes/pistils were removed from the berries, which were then assessed for virus binding by suspension assay-RT-qPCR, a statistically significant reduction in virus binding efficiency

(<83%) was observed. When strawberry pistils were isolated and tested similarly, they bound > 94% of the virus. The pistils were shown to bind significantly more GII.4 (New Orleans) (98.2%) than strawberry slices with and without achenes/pistils (94.9% and 81.8%, respectively) ($p \leq 0.05$). For GI.6, the pistils bound significantly more virus as compared to strawberry slices without achenes/pistils ($p \leq 0.05$).

Discussion

Strawberry is botanically classified as an aggregate fruit comprised of a fleshy, floral receptacle that supports clusters of the true fruiting bodies or achenes. Inside the dry ovary wall of each achene is an ovule that has the potential to develop into a unique strawberry seedling (Perkins-Veazie, 1995). The achenes are directly involved in the ripening of the fruit by secreting auxins (plant hormones) that promote the thickening of the floral receptacle by increasing cell number and size (Mudge et al., 1981). Pistils, the remnant female organs that include the stigma (where pollen grains germinate), and the style (the pollen tube that delivers pollen to the ovary) are attached to the outer surface of each of the ~200 achenes on a strawberry receptacle (Poling, 2012). The fleshy receptacle is comprised of an internal pith, a cortex layer, and an epidermal layer (Suutarinen et al., 1998). The receptacle serves to hold the achenes and provide water and nutrients to the achenes and surrounding parenchyma cells (Fait et al., 2008).

Although it has previously been shown that HuNoV bind to the surface of raspberries (Tian et al., 2011), the biochemical and biophysical forces that govern virus attachment to berry surfaces remains unclear. The forces that can contribute to the virus-berry binding

dynamics include non-specific interactions (electrostatic and/or hydrophobic); specific ligand-mediated binding (HBGA-like and/or non-HBGA-like moieties); and facilitated binding via the native microflora of the berry and/or associated biofilms. In the initial studies described here, we showed that HuNoV GI.6 and GII.4 (New Orleans) demonstrated moderate to high binding efficiency (84-92%) to the surface of fresh strawberries, and that this degree of binding was only modestly influenced by pH (3.5 vs. 5.5 vs. 7.0). These data suggest that electrostatic interactions have a nominal role in mediating HuNoV-berry interactions, and provided the impetus to investigate more specific interactions in subsequent experiments.

Histo-blood group antigens are considered the putative HuNoV host cell receptors/co-receptors for most strains (Huang et al., 2003; Marionneau, et al., 2002), and synthetic HBGAs are used widely for virus capture (Tian et al., 2012). *Enterobacter cloacae* and other bacteria have been shown to have the capacity to bind several HuNoV genotypes, with suggestion that this phenomenon is mediated by HBGA-like compounds on the bacteria (Miura et al., 2013). In a previous study, Baugher and Jaykus (2015) identified unique bacterial isolates derived from fresh raspberries and strawberries and characterized them for biofilm production. Based on the hypothesis that these bacteria may mediate HuNoV biding, high biofilm producing strains and ATCC control cultures were screened for HuNoV binding. For the isolates derived from berries, HuNoV binding efficiency ranged from a low of 69.3% to a high of 91.7% (Figure 2). However, none of the isolates had detectable HBGA-like Lewis A, B, or Y-like moieties, nor did they have A, B, and H(O)-like moieties

when evaluated by ELISA. These results suggest that the native berry microflora has little specific impact on the ability of HuNoV to bind to the surface of fresh strawberries.

Although HuNoV clearly bind to leafy greens, the mechanism(s) responsible for that binding have remained elusive (Gandhi et al., 2010; Esseili et al., 2012). Only recently has the presence of H type HBGA-like carbohydrates in the cell wall of Romaine lettuce been demonstrated, but only after enzymatic digestion of cell wall material (Gao et al., 2016). It was hypothesized that the process of digestion exposed binding sites, likely H-type HBGA-like moieties and fucose residues, which increased binding efficiency for GII.4 VLPs (Gao et al., 2016). This was confirmed using competitive assays with monoclonal antibodies (recognizing H-type HBGA) and plant lectins (recognizing α-L-fucose in the H type). That study provided the first definitive evidence for HBGA-like moieties in Romaine lettuce tissue, with fucose originating from the plant hemi-cellulose as the likely molecule responsible for virus-lettuce interactions (Gao et al., 2016). It is possible that that surface and/or interior berry moieties such as fucose residues may provide specific binding sites for HuNoV attachment as well.

Using immunofluorescent confocal microscopy, we observed that HuNoV (Houston) VLPs selectively attached to the surface of strawberries via localized specific structures, i.e., the achenes and pistils (Figure 5). Similar to data have been reported for lettuce leaves, on which predominant surface structures such as the major veins and the stomata were sites for virus aggregation (Ghandi et al., 2010; Esseili et al., 2012). We also found that VLPs had a propensity to bind to areas of fracture and injury, perhaps because such tissue damage

exposes internal plant sugar moieties to which HuNoV have affinity. This hypothesis is similar to that proposed for HuNoV binding to damaged areas of Romaine lettuce leaves (Wei et al., 2010; Esseili et al., 2012; Gao et al., 2016).

In an effort to confirm selective binding to strawberry pistils, suspension-RT-qPCR assays were performed on fruit with and without achenes/pistils. Interestingly, HuNoV binding was significantly less efficient on fruit in which the achenes/pistils were removed, compared to whole fruit. Also, isolated strawberry pistils assayed similarly showed this highest preferential binding efficiency for both GI.6 and GII. 4 (New Orleans) (Figure 6). These data support preferential binding of virus to the achenes. Unfortunately, little is known of the composition and chemical structure of the achenes and pistils of strawberries, but a hypothesis that the carbohydrate composition of these structures differs from that of the berry itself seems reasonable. It may be that the achenes possess higher concentration and/or density of sugars or other molecules that selectively facilitate HuNoV binding. However, because HuNoV binding occurred even in the absence of achenes, it is likely that other binding factors are involved. In the only other relevant study, HuNoV binding on raspberries was observed to be sporadically dispersed between the drupelets and not aggregating to the surface of the berry (Tian et al., 2011).

Differential binding of HuNoV to specific produce tissue types has implications for control. For instance, preferential binding to damaged locations means that special attention to growth and harvest conditions would be critical to minimizing the likelihood of tenacious virus binding to the fruit. In short, berry quality may have a direct correlation to product safety. Current washing methods are largely ineffective at HuNoV removal from produce

items (Baert et al., 2008; Casteel et al., 2009). These methods include electrolyzed water and hydrophobic rinses, both designed to disrupt non-specific electrostatic and hydrophobic interactions between microorganisms (Tian et al., 2011), which may not be the best approach to removing HuNoV contamination. Further investigation into ligand-associated virus attachment may lead to the development of more effective washing and disinfection strategies, or the production of strawberry varietals bred to minimize the concentration of virus-binding molecules.

There are a number of methodological limitations to this study. For instance, the results from the ELISA used for detecting the presence of HBGA-like moieties [A, B, and H (O)] in berries (Figure3A) and on bacterial isolates (Figure 4A) had relatively poor positive control binding ratios. Similar issues with using PGM as a positive control were encountered when investigating HBGA-like moieties on the surface of fecal bacterial isolates (Almond and Jaykus, 2016). The PGM does not show a strong signal with the monoclonal antibodies designed to bind specifically to human HBGA-like moieties. Low ELISA absorbance readings (A_{450} <0.6) were also observed for HuNoV VLP capture assays, and no appreciable HBGA-like moieties were observed throughout Romaine lettuce experimentation by Esseili et al. (2012). It is possible that this assay was unreliable. A high degree of variability was observed for the suspension assay used to evaluate the binding of HuNoV to bacterial isolates (Figure 2). Very recently, it has been shown that growth conditions significantly influence the expression of HBGA-like moieties on human gut-derived bacteria (Almand and Jaykus, 2016), and it is possible that a similar phenomenon exists for plant-derived microflora. However, this was not investigated here. The use of berry slices and the dissection of achenes

and pistils from the fruit for microscopy and binding assays may have exposed internal carbohydrate residues that contributed to higher HuNoV binding. These might not be exposed if the fruit were in its intact, healthy state. Unfortunately, processing the berries in this manner is necessary for these types of experiments.

HuNoV are extremely well adapted to persist through a diverse array of environmental conditions and readily bind to many surfaces, including fresh strawberries. There are no known methods currently available to remove or inactivate HuNoV from the surface of berries. Consistently documented in the literature is the vulnerability of fresh cut produce items and specific plant structures to serve as virus attachment sites (Wei et al., 2010; Esseili et al., 2012). Better characterization of this phenomenon will provide valuable insight into ways in which HuNoV bind to berries, data that can be used to establish if agricultural practices and environmental changes can significantly contribute to enhanced or reduced HuNoV attachment. It may also aid in the development of more effective removal strategies, such as targeted washes, as well as provide a strong basis for better understanding the binding dynamics of HuNoV to other food commodities. We contend that this research that may reduce the burden of HuNoV disease associated with strawberries and other related fresh produce items.

ACKNOWLEDGEMENTS

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Figures

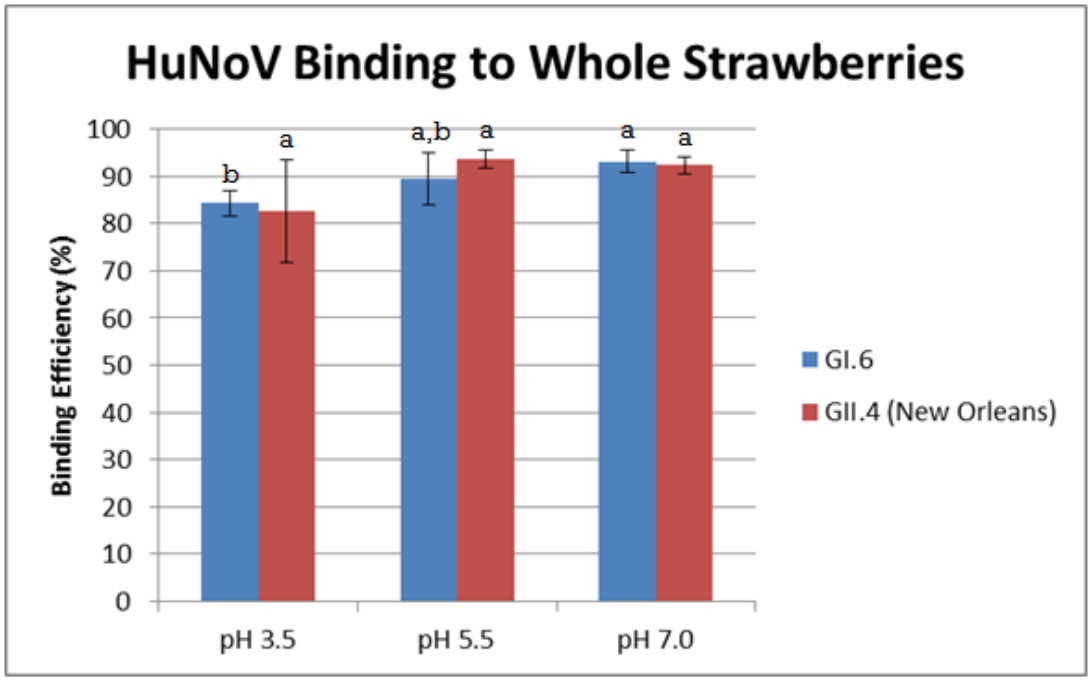

Figure 3.1. Suspension assay followed by RT-qPCR for evaluation of the binding efficiency of HuNoV GI.6 and GII.4 (New Orleans) to whole strawberries across a range of pH values (3.5, 5.5, 7.0). Different letters indicate statistically significant differences ($p \leq 0.05$) between sample means of each HuNoV strain across the pH range. No statistically significant difference was observed between HuNoV strains at a given pH treatment.

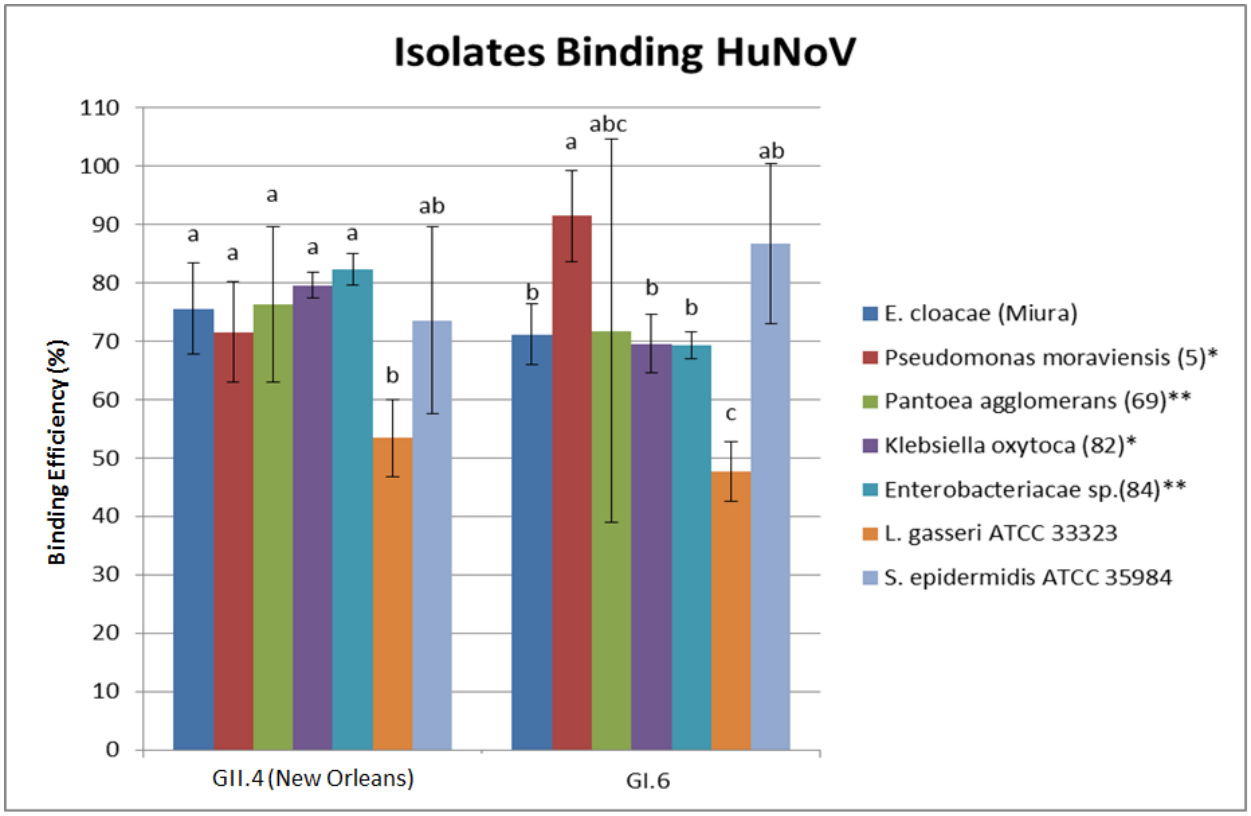

Figure 3.2. Suspension assay followed by RT-qPCR for evaluation of the binding efficiency of HuNoV GI.6 and GII.4 (New Orleans) to native berry bacterial isolates and control strains. Different letters indicate statistically significant differences ($p \leq 0.05$) between sample means when comparing bacterial binding to any one HuNoV strain. * raspberry isolates; ** strawberry isolates.

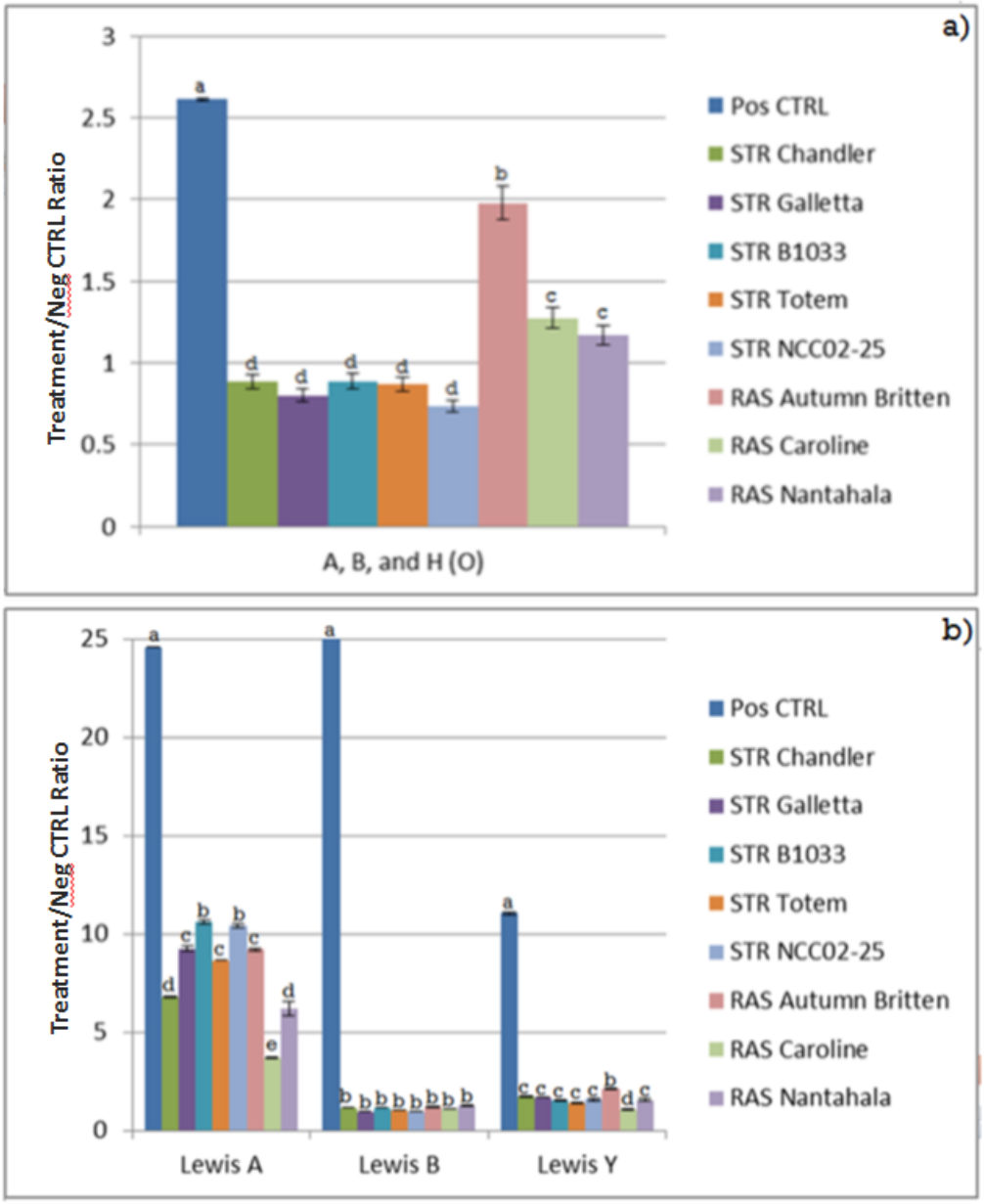

Figure 3.3. ELISA results for determination of the presence of HBGA-like moieties [Panel A: Types A, B, and H (O)] and [Panel B: Lewis A, B, and Y] in different raspberry and strawberry varietals. Different letters indicate statistically significant differences ($p \leq 0.05$) between sample means by varietal for any one HBGA type.

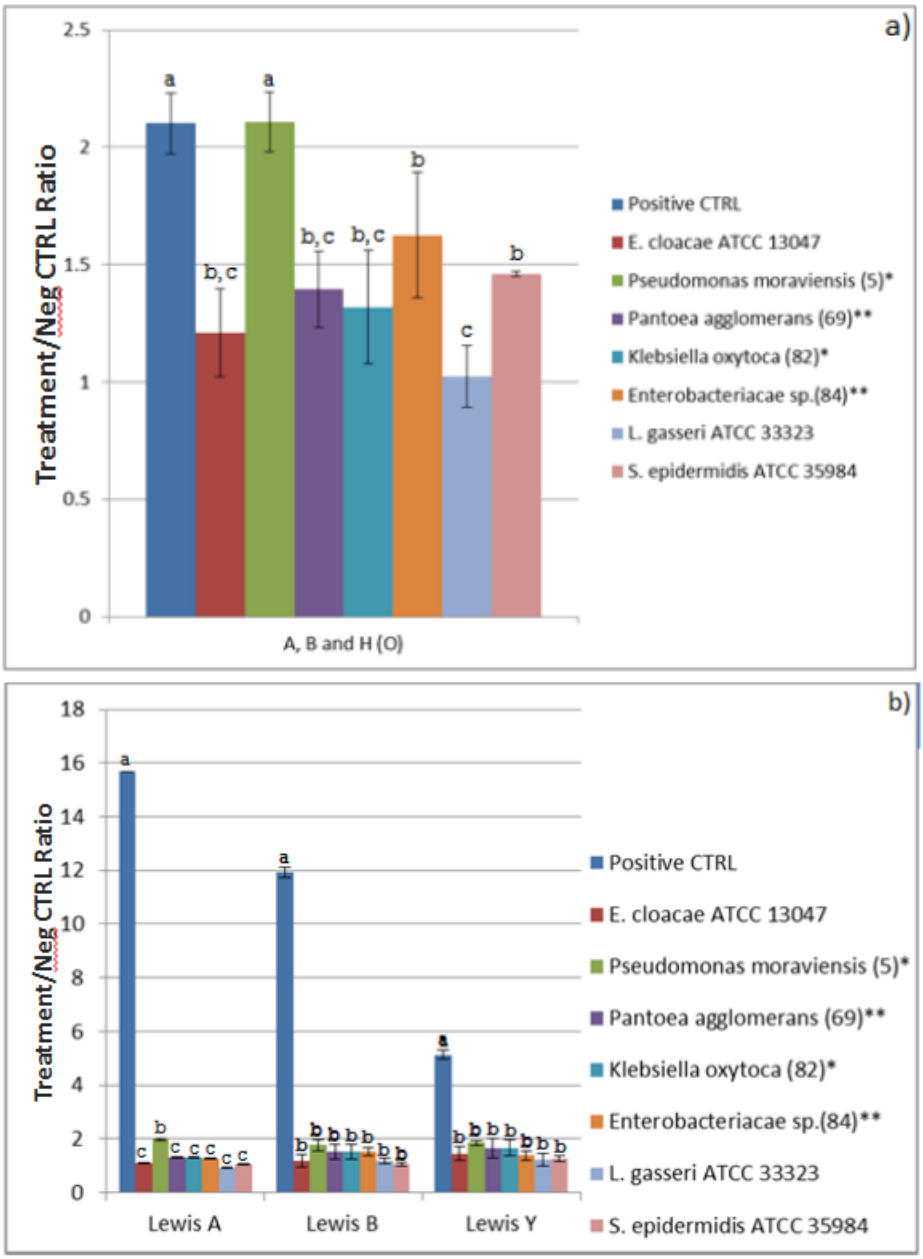

Figure 3.4. ELISA results for determination of the presence of HBGA-like moieties [Panel A: Types A, B, and H (O)] and [Panel B: Lewis A, B, and Y] in bacterial isolates derived from raspberries and strawberries, and several references strains. Different letters indicate statistically significant differences ($p \leq 0.05$) between sample means across bacterial strains for any one HBGA type. * raspberry isolates; ** strawberry isolates.

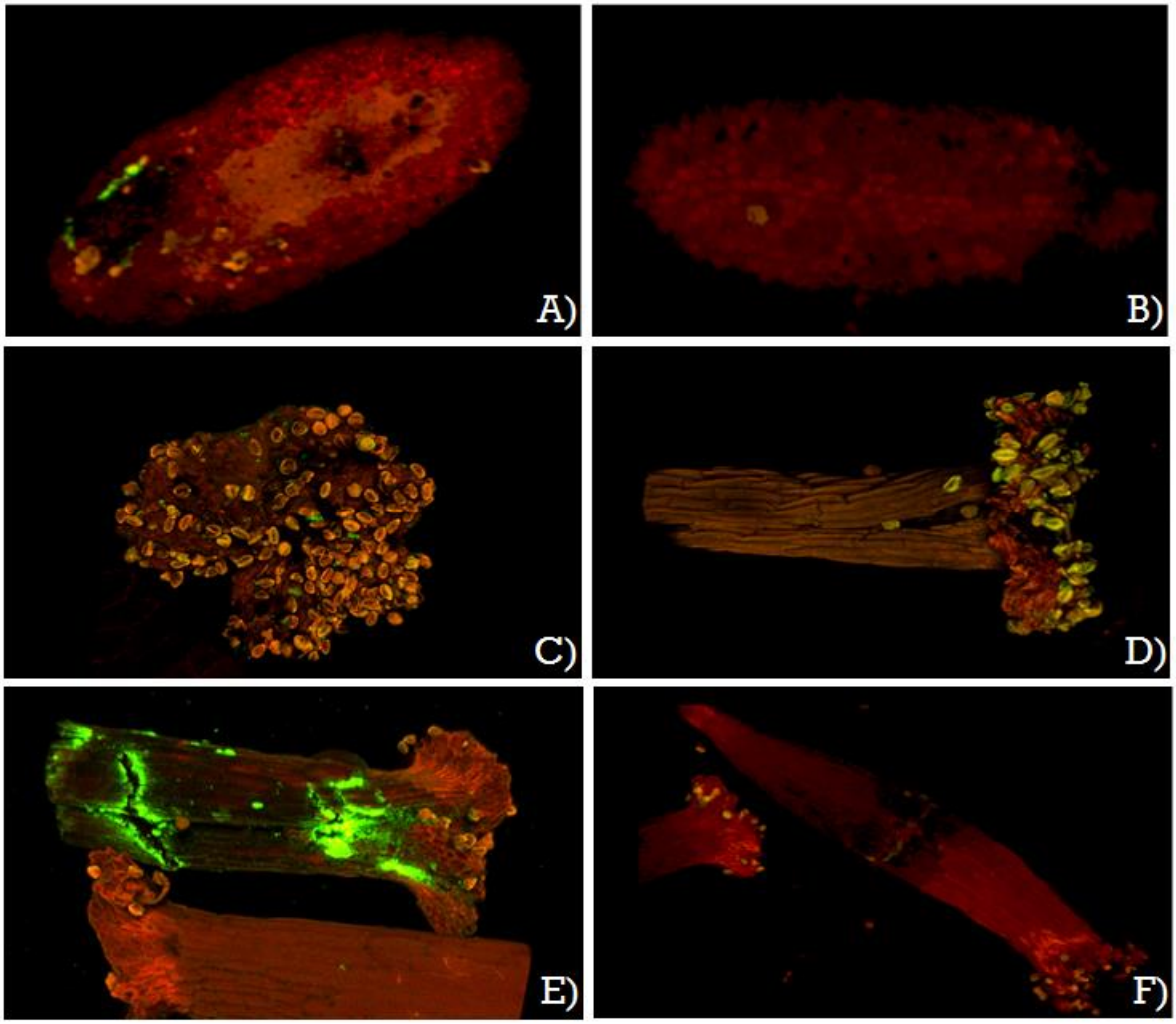

Figure 3.5. Confocal microscopy images of HuNoV GII.4 (Houston) VLPs bound to the surface of Camarosa strawberries. Green regions are VLPs bound to achene (Panel A), pistil surface (Panel C), and fractured portions of the pistil (Panel E). Experimental controls (without VLP exposure) shown in panels B, D, F.

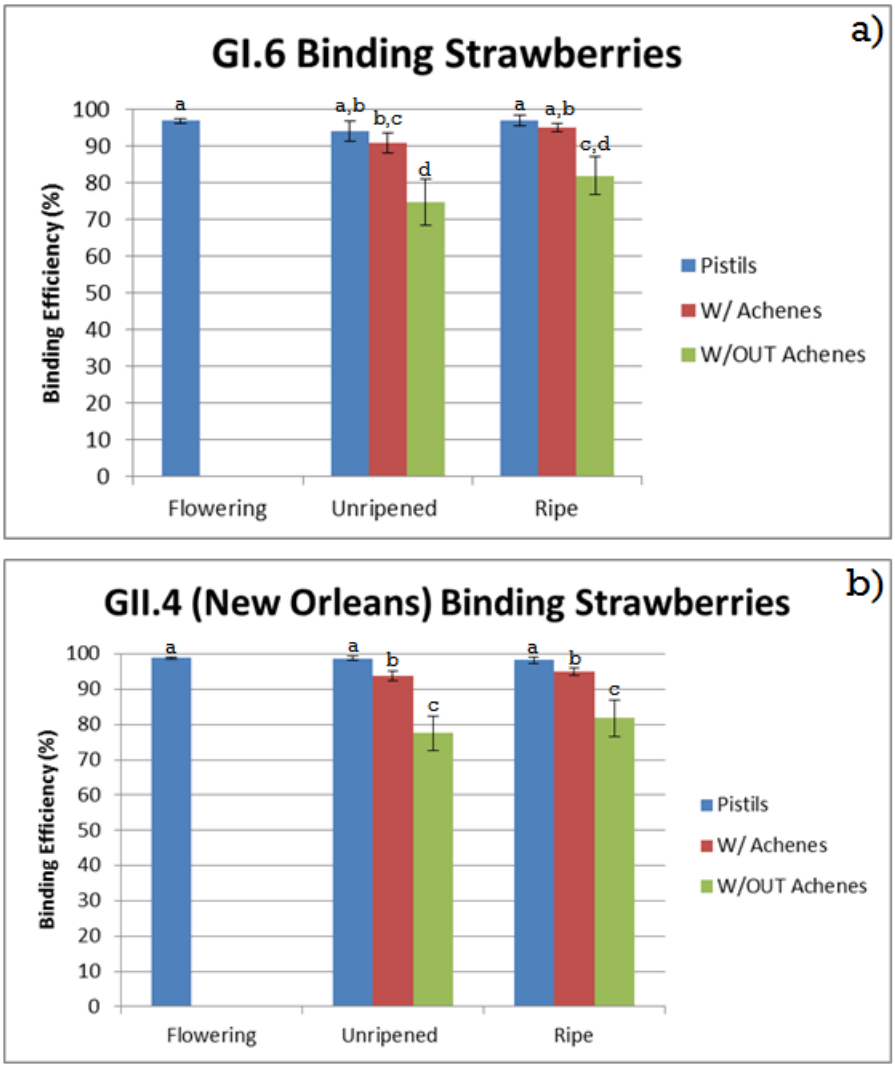

Figure 3.6. Suspension assay followed by RT-qPCR for evaluation of the binding efficiency of HuNoV GI.6 (Panel A) and GII.4 (New Orleans) (Panel B) to pistils alone, berry slices with achenes, and berry slices without achenes throughout berry development. Different letters indicate statistically significant differences ($p \leq 0.05$) between sample means of each HuNoV strain throughout strawberry development.

CHAPTER 4

Characterization of the Binding Mechanisms of Human Norovirus to Strawberries

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Abstract

Fresh berries are a significant contributor to human norovirus (HuNoV) foodborne outbreaks. In the previous study, HuNoV was shown to bind tenaciously to fresh strawberries, a phenomenon that was mediated by specific virus-binding surface structures, namely pistils/achenes. The purpose of this study was to elucidate the mechanisms governing that binding, i.e., the role of electrostatic and ligand-mediated interactions. Transmission electron microscopy (TEM) images confirmed that HuNoV virus-like particles (VLPs) bound and aggregated to the surface of pistils. A suspension-RT-qPCR method demonstrated that pH had little effect on HuNoV binding to pistils. Further, the surface zeta potential of the HuNoV GII.4 (Houston) VLP transitioned from electropositive to electronegative somewhere around pH 5.0, while that for the strawberry pistils remained consistently electronegative between pH 3.5-9.3, suggesting that specific HuNoV-pistil binding interactions were able to overcome electrical repulsive forces. Compositional and linkage analysis of pistils [major carbohydrate constituents of glucose (39.1%), arabinose (36.0%), galactose (13.0%), and rhamnose (5.3%)] was utilized to systematically select lectins for competitive ELISA. Several lectins were associated with moderate inhibition of VLP binding to strawberry cell wall material (CWM), i.e., lectins SNA, binding

αNeuNAc(2→6)gal (no HBGA specificity); BS-1, binding α-D-Gal >> GalNAc (HBGA specificity B>>A); MOA, binding Galα1, 3Gal, 3Galβ1 (HBGA specificity B>>A); and DBA GalNAc (HBGA specificity A1>>A2). These data support the hypothesis that strawberries contain multiple carbohydrate moieties that mediate specific HuNoV binding interactions, although the exact nature of that specificity has yet to be determined.

Introduction

Foodborne illnesses are caused by a variety of agents including viruses, bacteria, parasites, toxins, metals, and prions (Mead et al., 1999). Epidemiological studies have shown that human norovirus (HuNoV) is the leading causative agent of foodborne illness in the developed world (Widdowson et al., 2005). Of the 9.4 million cases of food-associated disease in the U.S. annually, it is estimated that 5.5 million (58%) of these are caused by HuNoV (Scallen et al., 2011). Economic modeling has estimated the aggregated annual cost of these illnesses to range from $51 to $78 billion every year (Sharff, 2012).

Norovirus is a genus within the *Caliciviridae* family, a group of small (28 -35 nm in diameter), non-enveloped, genetically diverse viruses having a single-stranded RNA genome of ~7.3-8.3 kb in length. Transmitted primarily by the fecal-oral route, human norovirus (HuNoV) is shed by infected individuals at high concentrations (up to 10^{11} per g of feces) (Atmar et al., 2008), has a low infectious dose (~18-100 viral particles) (Teunis et al., 2008), and is environmentally persistent (D'Souza et al., 2006). Hallmark HuNoV symptoms include nausea, cramping, vomiting, and diarrhea, typically lasting for 48 to 72 h and the virus causes illness in all age groups, Recovery is usually complete but disease can be life-threatening, particularly in the elderly or in regions of the world not having access to reliable medical care. Because there are so many HuNoV strains that are antigenically diverse, and immunity is relatively short-lived, the typical person will experience five norovirus infections in a 79-year lifespan (Hall et al., 2013).

A number of food commodities are most often associated with HuNoV outbreaks, including molluscan shellfish, ready-to-eat (RTE) and prepared foods, and fresh produce

items such as leafy greens, scallions, and berries (Hall et al., 2012). Internationally, a number of large HuNoV outbreaks have been associated with fresh produce, including raspberries and strawberries in countries such Sweden, Finland, Poland, and Germany (Le Guyader et al., 2004; Falkenhorst et al., 2005; Sarvikivi et al., 2011; Bernard et al., 2013). Produce items can become contaminated by HuNoV through a variety of sources including the application of virus-contaminated organic fertilizers and amendments, waters (irrigation systems, processing waters), or by direct contact with human fecal material during pre- and/or post-harvest phases (Tuan Zainazor et al., 2010). Both current and emerging washing methods are largely ineffective in removal or inactivation of HuNoV from contaminated fresh produce (Baert et al., 2008; Casteel et al., 2009). This includes electrolyzed water and hydrophobic rinses that are designed to disrupt non-specific electrostatic and hydrophobic interactions between microorganisms and produce items, without resulting in deleterious effects on product quality (Tian et al., 2011).

Although studies have identified specific mechanisms of bacterial attachment to foods, a focal point for food virology research is the identification of the means by which virus-produce interactions occur (Berger, 2010). A number of studies have suggested that HuNoV binding to fresh produce is at least in part mediated by non-specific electrostatic and hydrophobic forces (Tian et al., 2007; Vega et al., 2008; Da Silva et al., 2011). However, given the fact that molluscan shellfish have been shown to effectively bind HuNoV through specific interactions with histo-blood group (HBGA)-like antigens found in the oyster gastrointestinal lining (Tian et al., 2006), there has been interest in whether such molecules contribute to virus binding with fresh produce. Although not yet proven for berries, HBGA-

like antigens recently have been identified in the cell wall of Romaine lettuce (Gao et al., 2016).

In the previous chapter of this dissertation, effective binding of HuNoV to strawberries was demonstrated, and preliminary evidence suggested that the berry pistils had increased affinity for the virus. The purpose of this study was to further elucidate the mechanism(s) governing the binding of HuNoV to strawberry tissue, with the underlying hypothesis that such binding is structurally and compositionally specific. The general approach included combinations of suspension-RT-qPCR assay, electrokinetic potential methods, transmission electron microscopy, compositional analysis, and competitive ELISA to further refine our knowledge of the mechanisms behind HuNoV-strawberry binding interactions.

Materials and Methods

Berries, virus, and virus-like particles (VLPs)

For all HuNoV binding studies, fresh Camarosa and Chandler strawberries were harvested from the Central Crops Research Station (NC Department of Agriculture and Consumer Services, Clayton, NC) and stored at 4°C until further processed that day. Strawberry pistils were removed from whole strawberries by hand using electron microscopy tweezers and stored at -80°C until use in assays. Stool samples originating from outbreaks and previously confirmed positive for HuNoV GII.4 (New Orleans) and GI.6 HuNoV were obtained courtesy of S.R. Greene (NC Department of Health and Human Services, Raleigh,

NC). Human norovirus GII.4 Houston (HOV) and Grimbsy (GRV) virus-like particles (VLPs) were provided courtesy of Dr. Robert Atmar (Baylor College of Medicine, Houston, TX).

HuNoV binding to pistils by suspension assay

Each suspension assay was performed by mixing 12 mg of Camarosa pistils with 500 µl of a 1:5 solution of phosphate buffered saline (PBS), pH 7.2 (Life Technologies, Carlsbad, CA) (pH adjusted using 0.1 M hydrochloric acid and sodium hydroxide to 3.5, 5.5 and 7.0) to which had been added 100 µl of GI.6 or GII.4 (New Orleans) HuNoV-positive stool sample [total input virus of ~10^5 and ~10^6 genome equivalent copy (GEC) for GI.6 and GII.4, respectively)] in a 1.5 ml Lo-Bind centrifuge tube (Eppendorf, Hauppage, NY). After rotating the berry pistil-virus suspension at room temperature (RT) for 2 h, the pistils were pelleted by centrifugation at 8,200 *x g* for 5 min, and 100 µl of the supernatant was collected and stored at -80°C before assay by nucleic acid amplification.

RNA extraction and RT-qPCR

RNA extraction was performed on these supernatants using the Nuclisens easyMAG system (Biomerieux, Durham, NC) as per manufacturer's instructions, with a reconstitution volume of 50 µl. For GII.4 (New Orleans) RNA, RT-qPCR reactions were carried out using the primers JJV2F (5'-CAAGAGTCAATGTTTAGGTGGATGAG-3'and COG2R (5-'TCGACGCCATCTTCATTCA CA-3'),, and probe [RING2-TP (5'-56-FAM TGGGAGGGCGATCGCAATCT-3BHQ-3'] (Jothikumar et al., 2005). For GI.6 RNA, the

reactions were carried out using primers COG1F (5'-CGYTGGATGCGNTTYCATGA-3') and COG1R (5'-CTTAGACGCCATCATCATTYAC-3') and probes RING1(A) (5'-56-FAM-AGATYGCGATCYCCTGTCCA-3BHQ-3') and RING1(B) (5'-56-FAM-AGATCGCGGTCTCCTGTCCA-3BHQ-3') (Kageyama et al., 2003). RT-qPCR was carried out using the Superscript III Platinum One-Step RT-qPCR system (Invitrogen, Carlsbad, California) according to manufacturer's instructions. Briefly, 25 µl RT-qPCR reactions consisted of 12.5 µl of 2x reaction mix, 8.0 µl DNase-RNase free water, 200 nM of each primer and probe, 0.5 µl of the enzyme mix (SuperScript III RT/Platinum® Taq Mix) and 2.5 µl of the RNA template. Amplification was done under the following conditions: 50^0C for 15 min, 95^0C for 2 min, followed by 45 cycles of 95^0C for 15 s, 54^0C for 30 s and 72^0C for 30 s in a SmartCycler (Cepheid, Sunnyvale, CA). The RNA copy number was extrapolated from a standard curve based on Ct values obtained by RT-qPCR amplification of serially diluted RNA obtained from each stool sample used as inoculum. Percent binding efficiency was calculated based on loss to supernatant by subtracting the RNA copy number of the supernatant from the input copy number (estimated GEC), dividing by the input copy number, and multiplying the quotient by 100 (Papafragkou et al., 2008).

Zeta potential of VLPs and strawberry pistils

Electrophoretic mobility of the purified VLP suspensions was determined using a Zetasizer Nano ZSP (Malvern Instruments, Malvern, UK), with a 10 mW HeNe laser at 633 nm and a photodiode located 173° form the incident laser beam (courtesy of Velev lab group, NCSU Department of Chemical and Biomolecular Engineering). Zetasizer software

calculated the zeta potential from electrophoretic mobility using the Smoluchowski formula (Smoluchowski, 1941). VLPs were placed in a glass cuvette containing PBS solutions of varying pH (pH 2.1, 3.2, 3.6, 4.3, 5.2, 6.5, and 7.3) and immediately measured to determine zeta potential before significant aggregation occurred. Solutions of 0.1 M hydrochloric acid and sodium hydroxide were used for pH adjustments. Each VLP treatment was performed in triplicate.

To prepare the pistils for electrophoretic mobility assays, 10 mg were washed in 500 µl PBS, and centrifuged at 8,200 *x g* for 5 min three times to remove residual sugars and debris that could significantly impact the electrophoretic readings. The pistils were then affixed to the sample stage of a surface zeta potential cell (Malvern Instruments, Malvern, UK). Standard latex particles (Malvern Instruments) were pH-adjusted (pH 3.5, 4.7, 5.5, 7.2, 9.2) and used as tracer particles. The zeta potential of the tracer particles was measured three times at 125 µm, 250 µm, 375 µm, and 500 µm from the sample surface. Zetasizer software used the slope of a line fit to the average zeta potential measured at each distance and the zeta potential of the tracer particles (measured at 1000 µm from the sample surface) to calculate the zeta potential of the pistil layer. For all zeta potential studies, experiments were performed in triplicate at each pH.

Transmission electron microscopy (TEM) of HuNoV VLP-pistil binding

The suspension assay was performed as described above using GII.4 (Houston) VLPs (1.4 ng/µl) with 12 mg of Camarosa strawberry pistils. The supernatant was removed and the pistils were placed in 1 ml of fixative solution (3% glutaradlehyde and 0.1M PBS) then

stored at 4°C until processed. Samples were washed three times for 15 min in 0.1M PBS. The samples were post-fixed in 2% OsO_4 (Sigma Aldrich) in PBS for 1 h at 4°C in the dark, and rinsed as above. The pistils were centrifuged for 1 min at 2,000 *x g*, the supernatant removed, and 0.5 ml of a molton 2% agarose solution [prepared in 0.1M Na_3PO_4 (Sigma Aldrich), pH 7.4] was added to cover the pistils. The samples were placed briefly in cold dH_2O and dehydrated with a graded series of cold ethanol washes of 30%, 50%, 70%, 95%, and three times with100%, respectively. Samples were infiltrated in Spurr's resin (Sigma Adrich) with vacuum, and then embedded in BEEM capsules (Ted Pella Inc, Redding, CA) at 70°C overnight. Cured blocks were hand-trimmed with a single-edge razor blade and thin-sectioned at 80nm using a Leica EM UCT6rt Ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) and a diamond knife (DDK, Inc., Wilmington, DE). Grids were double-stained with 4% aqueous uranyl acetate for 1 h in the dark at RT, followed by 4 min in Reynold's lead citrate (electron opaque stain). Grids were viewed using a JEOL JEM-1200EX transmission electron microscope (JEOL USA, Peabody, MA) at 80kV. Images were digitally acquired using a Gatan Model 795 ES 1000W Erlangshen CCD camera (Gatan Inc, Pleasanton, CA), and minimally processed in Photoshop CS6 (Adobe, San Jose, CA). All TEM imaging was performed at the Center for Electron Microscopy (NCSU, Raleigh, NC).

Glycosyl compositional and linkage analysis of pistils

To remove low molecular weight residues that would saturate analytical detectors, pistils were dialyzed prior to analysis. Fifty mg of Camarosa pistils were dialyzed in CE

dialysis tubing (molecular weight cut off 3.5-5 kD) (Spectrumlabs, Rancho Dominguez, CA) for 48 h against 2 L of deionized water at 4°C, with a buffer exchange after 24 h. The sample was collected and sent to the Complex Carbohydrate Research Center (Athens, GA) for analysis. Briefly, glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis as described previously (Santander et al., 2013). For glycosyl linkage analysis, the samples were permethylated, depolymerized, reduced, and acetylated; and the resultant partially methylated alditol acetates (PMAAs) were analyzed by gas chromatography-mass spectrometry (GC-MS) as described by Heiss et al. (2009).

Extraction of strawberry cell wall materials

Extraction of strawberry cell wall material (CWM) was performed at 4°C according to the method of Hoffman et al. (2005). Briefly, ripe Camarosa strawberries were surface sterilized for 10 min in 0.1% sodium hypochlorite, rinsed with sterile water, and dried (Koch and Nevins, 1989). Using a culinary mandolin, 70 g of 1.5 mm strawberry surface slices were collected and CWM was extracted by grinding the slices with mortar and pestle in liquid nitrogen to a fine powder. The powder was then homogenized in 80% ethanol using a Branson Digital Sonifer 450 (Branson, Danbury, CT) followed by centrifugation for 20 min at 2,500 *x g*. The subsequent pellet was washed in series (two washes with 80% ethanol, followed by one with 100% ethanol) and stirred for 2 h at 4°C in 60 ml methanol-cholorform [1:1 (vol/vol)]. The pellet was then collected on Whatman glass microfiber filters, grade

GF/A (Sigma Aldrich) and washed with ice-cold methanol-choloroform and lastly, with ice-cold acetone. The pellet was vacuum dried overnight at RT and provided a yield or approximately 2 g. The resulting CWM was tightly sealed and stored at 4°C under dark conditions until use in assays.

Detection of HuNoV VLP binding to CWM by ELISA

An ELISA similar to that used by Esseili et al. (2012), with minor modifications, was used to evaluate the binding of HuNoV VLPs to strawberry CWM. Specifically, strawberry CWM was resuspended in PBS at a concentration of 5 mg/ml, homogenized twice for 10 sec, and centrifuged twice at 500 *x g* for 2 min. One hundred microliters of the resulting supernatant was used to coat individual wells of a MaxiSorp 96-well plate (Thermo-Fisher Scientific, Rockford, IL). The plates were incubated at 4°C for 18 h. Following three washes with PBS supplemented with 0.05% Tween (PBS-T), wells were blocked with 200 µl of SuperBlock (Thermo-Fisher) for 15 min. The plate was then washed three times again with PBS-T, and once with PBS. One hundred microliters of HuNoV GII.4 (GRV) VLPs (3 µg/ml) was added to each well and incubated at RT for 1 h. After four more washes, 100 µl of primary antibody NS14 (courtesy of Dr. Robert Atmar, Baylor College of Medicine, Houston, TX) and the secondary antibody [goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (KPL, Gaithersburg, MD)], diluted in PBS supplemented with 0.1% skim milk (3 µg/ml and 1:3000, respectively), were added to each well sequentially. Both antibodies were incubated for 1 h at 37°C, and plates were washed thrice with PBS-T and once more with PBS. One hundred microliters of the two-component substrate

tetramethylbenzidine (TMB) (KPL) was added to each well and incubated at RT for 15 min. The reaction was stopped using 100 µl of the TMB stop solution (KPL), and the plate read at OD_{405} using an Infinite M200 Pro instrument (Tecan, Mannedorf, Switzerland). The positive controls were PBS-coated wells with VLPs added. Negative controls included (i) wells coated with CWM but having no VLPs added; and (ii) SuperBlock-coated wells with VLPs added.

Inhibition treatments

The potential role of complex carbohydrates, proteins, and simple sugars on HuNoV VLP binding to strawberry CWM was done in conjunction with the ELISA described above. The general approach was to pretreat CWM prior to its coating on ELISA plates. Specifically, carbohydrates of CWM were oxidized using freshly made sodium periodate ($NaIO_4$) at 10 and 100 mM in PBS for 1 h at RT under dark conditions, prior to coating the ELISA plates. To determine if there was potential protein involvement, CWM suspensions were boiled for 5 min at 100°C before coating the ELISA plates.

For greater specificity in carbohydrate inhibition assays, commercially available lectins MAA, BS-1, UEA-1, LcH, DBA, SNA, LEA (Sigma Aldrich), MNA-G, MNA-M, MOA EYLabs, San Mateo, CA) that bind specific sugar moieties (summarized in Table 1) were incubated with CWM after the blocking step and subsequent washes (washed thrice with PBS-T and once more with PBS). Lectins were diluted in TBS buffer (50 mM Tris-HCl, 150 mM NaCl 2.5 mM $MnCl_2$ and 2.5 mM $CaCl_2$) to a concentration of 10 and 100 µg/ml and 100 µl of this suspension was added to each well for 1 h at 37°C. Negative control

wells (no lectin) were incubated with TBS buffer under the same conditions. Other negative controls included (i) boiled PBS or CWM with no VLPs added; and (ii) CWM with no VLPs but with sodium periodate or lectin added, to control for nonspecific binding. No increased nonspecific binding of the primary or the secondary antibodies used in the ELISA was observed (data not shown). The data were expressed as inhibitory ratios, i.e. the ratio of the absorbance reading of the positive control [VLPs bound to CWM under the corresponding treatment buffer (i.e. PBS, TBS, boiled PBS)] to that of the given experimental treatment. Values were considered significant if the positive/negative ratio was greater than 2.0 (Tian et al., 2006; Almond and Jaykus, 2016). All wells were done in triplicate and all experimental treatments were performed in triplicate independent runs.

Statistical analysis

Data were expressed as mean ± standard deviation of three independent experimental replicates. The data were analyzed by unpaired T test and one-way Analysis of Variance (ANOVA) with the Tukey's multiple comparison test using GraphPad Prism ver. 5.0 d (San Diego, CA). Values of $p \leq 0.05$ were considered statistically significant.

Results

HuNoV binding of strawberry pistils across a range of pH values

To determine the impact of environmental pH on HuNoV binding to strawberry pistils, suspension assays, followed by RT-qPCR, were done at pH 3.5, 5.5, and 7.0 using HuNoV GI.6 and GII.4 (New Orleans). Both strains bound to the pistils with consistently

high efficiency (93.5-96.9% for GI.6 and 95.5-98.2% for GII.4), regardless of pH (Figure 1A). Overall, there were no statistically significant differences ($p \leq 0.05$) in binding efficiency when comparing both HuNoV strains at a given pH or a given HuNoV strain across the pH range tested.

Zeta potential of HuNoV and Camarosa pistils across a range of pH values

The overall electrical surface charge (zeta potential) of HuNoV GII.4 (Houston) VLPs and Camarosa pistils was tested across a broad pH range to infer if non-specific electrostatic forces were likely to influence binding (Figure 1B). The zeta potential of the VLPs was positive at pH 2.1 (electrical charge of 14.2 mV) and they steadily became more electronegative as the pH increased. At approximately pH 5.2, the net charge of the VLPs changed from electropositive to negative, and reached -12.3 mV by pH 7.3. Beyond this pH, VLP aggregation interfered and further zeta potential readings were not possible. The Camarosa pistils remained electronegative across the entire pH range tested (pH 3.5-9.2), with a zeta potential values ranging from -26.8 to -43.3 mV.

TEM images of HuNoV VLPs binding to the surface of pistils

Transmission electron microscopy was performed on cross sections of Camarosa pistils. The negative control (Figure 2D) showed the pistils having significant surface area and composed of several layers of different densities. Within the interior of the hollow tube of the pistil styles, asymmetrical cellular debris lined the walls (Figure 2D). When the pistils

were exposed to GII.4 (Houston) VLPs, they aggregated on the pistil surfaces, specifically along contours and irregularly shaped areas (Figure 2A-C).

Glycosyl composition and linkages of Camarosa pistils

The strawberry Camarosa pistils were compositionally analyzed to determine the specific glycosyl residues that may be contributing to HuNoV binding. The most predominant sugars present were glucose (39.1%) and arabinose (36.0%) (Figure 3 and Appendix A). The pistils were also composed of a moderate amount of galactose (13.0%), rhamnose (5.3%), and xylose (3.7%), and small amounts of galaturonic acid (1.8%), fucose (0.7%), and mannose (0.4%). Notable glycosyl residues not detected included ribose, glucuronic acid, N-acteyl galactosamine, N-acetyl glucosamine, N-acetyl mannosamine, and sialic acid.

Inhibition of NoV VLP binding to CWM by chemical/physical treatments

The impacts of chemical oxidation and physical denaturation on HuNoV VLP binding to strawberry CWM were also determined (Figure 4A). Sodium periodate was used at 10 mM and 100 mM concentrations to oxidize the carbohydrates of the CWM prior to ELISA. The results showed significant reduction in overall VLP binding upon chemical oxidation, with inhibitory ratios of 1.9 and 2.5 for 10 mM and 100 mM periodate treatments, respectively. To assess whether proteins could be contributing to HuNoV binding, CWM was boiled prior to ELISA. This treatment resulted in a significant ($p \leq 0.05$) reduction in HuNoV VLP binding, with an inhibitory ratio of 3.0.

Inhibition of HuNoV VLP binding to CWM by lectins

Competitive ELISA was performed to determine if select lectins (Table 1) were able to effectively inhibit HuNoV VLP binding to strawberry CWM. All of the lectins having HBGA-specific moieties inhibited VLP binding, except UEA-1 [binding specificity to HBGA O (H) type 2/Le^y] (Figure 4B). All lectins except UEA-1 also showed a statistically significant concentration effect ($p \leq 0.05$), with the higher concentration (100 mM) providing greater inhibition than the lower concentration (10 mM). Lectin BS-1 (specificity to HBGA B>>A) was the most effective inhibitor of HuNoV VLP binding to strawberry CWM, with inhibitory ratios of 1.8 and 3.3 for 10 mM and 100 mM lectin concentrations, respectively. Less pronounced inhibition occurred at 10 mM for lectins DBA (specificity to HBGA A1>>A2), MNA-M (specificity to HBGA A), MNA-G (specificity to HBGA A), and MOA (specificity to HBGA B>>A), but at 100 mM, inhibitory ratios were 2.5 for MOA; 2.2 for DBA; 1.9 for MNA-M; and 1.6 for MNA-G.

Similar competitive ELISA experiments were also performed using lectins without HBGA-specific activity (Table 1). Lectin SNA [specificity to αNeuNAc(2→6)gal] was the most effective inhibitor of HuNoV VLP binding to strawberry CWM at both 10 mM and 100 mM concentrations, with inhibitory ratios of 2.0 and 4.1, respectively (Figure 4C). Lower inhibitory ratios occurred with at mM for lectins LEA (specificity to GlcNAc) and MAA (specificity to sialic acid glycoconjugates), but at 100 mM, inhibitory ratios were 2.0 for LEA and 1.7 for MAA. Lectin LcH (specificity to α-D-Man/α-D-Glc) did not effectively inhibit HuNoV binding to strawberry CWM at either concentration.

Discussion

In Chapter 3, effective binding of HuNoV to strawberries was demonstrated, and preliminary evidence suggested that the berry pistils had increased affinity for the virus. Strawberry pistils, the remnant female organs that include the stigma (where pollen grains germinate) and the style (the pollen tube that delivers pollen to the ovary), are attached to the outer portion of each of the ~200 achenes on the surface of a mature strawberry receptacle (Poling, 2012). The specific mechanisms that can theoretically contribute to HuNoV binding to the pistils include non-specific interactions (e.g., electrostatic and/or hydrophobic) and more specific interactions (e.g., binding to HBGA-like and/or non-HBGA-like ligands). The purpose of this study was to further elucidate the mechanism(s) governing the binding of HuNoV to strawberry tissue, with a focus on the pistil structures.

Transmission electron microscopy confirmed that HuNoV VLPs bind to pistils, apparently aggregating around the contours of the pistil surface. The importance of electrostatic and hydrophobic interactions on this binding was indirectly investigated by Tian et al. (2011). These investigators were characterizing the efficacy of electrolyzed water and hydrophobic rinses to promote removal of HuNoV from Romaine lettuce and raspberries, finding minimal to no differences between these products when compared to a simple tap water rinse. In a more focused study, Da Silva et al. (2011) evaluated the influence of environmental pH on HuNoV GI.1 VLP attachment to silica surfaces. Binding efficiency significantly decreased as the experimental pH increased above the isoelectric point (pI) of HuNoV (above pH 5.5); however, the binding efficiency was extremely erratic at pH below the pI, making it difficult to make overarching conclusions about electrostatic mediation of

binding. In the study described here, pistil suspension assays were performed on solutions pH-adjusted to 3.5, 5.5, and 7.0. This pH range was selected so as to observe effects on HuNoV-pistil binding under environmental conditions well below the pI of the virus (pH 3.5); at/near HuNoV pI (5.5); and well above HuNoV pI (pH 7.5). The lower and upper pH values could be said to correspond to strawberry juice and intact strawberry surfaces, respectively. Clearly, changing pH within this range had virtually no effect on the binding efficiency of HuNoV to strawberry pistils.

To further investigate a possible relationship between the overall electrical charge of pistils and HuNoV binding, the zeta potential of VLPs and pistils was determined across a broad pH range. The zeta potential is a quantitative measurement of the magnitude of the electrostatic (charged) repulsion/attraction between particles (Nik, 1997). It provides an estimate of the overall electrical surface charge of an entity and hence allows inferences to be made regarding the degree of positivity/negativity of particles, which can theoretically mediate their interactions with one another. While the HuNoV VLPs drifted from an electropositive zeta potential at pH 2-5, and an electronegative zeta potential above pH 5, the strawberry pistils were highly electronegative (-26.8 to -43.3) across the entire pH range tested (Figure 2B). The environmental surface of the berry is estimated to be between a pH range of 6.0 to 6.8 (Demchek et al., 2010). Because both the HuNoV VLPs and the pistils are electronegative at that pH, these data suggest that strawberry pistils and HuNoV are able to overcome natural electrostatic surface repulsions and still bind to one another. The implication is that there are more specific mechanisms mediating virus-berry binding behavior.

One logical candidate for more specific binding mechanisms between HuNoV and fresh produce is histo-blood group antigen (HBGA)-like moieties. Human HBGAs are distinct terminal polysaccharides of glycoproteins or glycolipids found in various cell types and tissues, including the epithelial cells lining the gastrointestinal mucosa (Ravn and Dabelsteen, 2000). These include H type, A and B blood groups, and Lewis carbohydrates. The capsid of HuNoV strains show HBGA binding pockets in the P2 domain (Tan et al., 2009) and HBGA binding occurs across genotypes, with various strains showing different HBGA binding patterns (Harrington et al., 2004). Molluscan shellfish were shown to effectively bind HuNoV GI.I through HBGA A-like moieties found in the oyster gastrointestinal lining (Tian et al., 2006). This specific binding was shown to allow the virus to concentrate inside the gastrointestinal tract and resist removal through depuration (Tian et al., 2007). Recently, H-type HBGA-like moieties were discovered in the cell wall material (CWM) of Romaine lettuce (Gao et al., 2016), although no such work has been done with berries.

In the previous study, we were unable to find appreciable concentrations of HBGA-like moieties in berry extracts. While similar studies were undertaken to look for these molecules in the pistils, the intact pistils and homogenates could not be prepared in a manner amenable to the ELISA method used here. Due to lack of migration of pistil components through SDS-PAGE gels, detection of HBGA-like moieties via Western blots as performed by Almond et al. (2016) were also unsuccessful. Nonetheless, identification of the biochemical composition of strawberry pistils is an important step in supporting the hypothesis that binding to HuNoV is structurally and compositionally specific.

Unfortunately, little is known about the composition and chemical structure of achenes and pistils, making inference of putative binding ligands impossible. We therefore performed compositional analysis of the strawberry pistils to determine the glycosyl composition and linkages present in the pistils. While globally we were unable to ascertain many details regarding the specific linkages and carbohydrate bonds present, we were able to detect the presence of important pistil carbohydrate components such as galactose (13%) and fucose (0.7%). It has previously been shown that there are four core carbohydrates associated with HBGAs (i.e. fucose, galactose, galactosamine, and glucosamine), and a combination of two of the four are required for HBGA activity (Springer, 1970).

Glycans (including HBGAs) consist of a chain of monosaccharides connected primarily by O-glycosidic linkages. It is possible that HuNoV binding is mediated by one of any number of monosaccharides, disaccharides, or oligosaccharides that are similar but not identical to HBGAs. This may explain the difficulties in identifying HBGA-like moieties in berries in previous studies. Lectins are carbohydrate-binding proteins having high specificity for sugar molecules; they can be disabled upon exposure to their target sugar. One could make the argument that HuNoV capsid proteins act as putative lectins, binding to specific carbohydrate moieties found on produce items (Van Damme et al., 1998). Because lectins bind so tenaciously to their specific target, they have utility in blocking sugar-specific binding. Esseili et al. (2012) demonstrated that the binding of HuNoV VLPs to Romaine lettuce leaves was significantly inhibited by pre-treating CWM with lectins that bind the carbohydrate moieties α-D-Gal, α-D-Man/ α-D-Glc, and α-L-Fuc. Interestingly, the lectin binding patterns changed throughout plant maturation and post-harvest, suggesting that

different surface sugars may be present at different phases of plant development and that HuNoV binding may involve several different carbohydrate molecules.

Similar to Esseili et al. (2012), an ELISA preceded by inhibition treatments was used to determine if proteins, carbohydrates, and select lectins were able to effectively inhibit HuNoV VLP binding to strawberry CWM. Boiling the CWM (total protein denaturation) caused a significant reduction in HuNoV VLP binding, with an inhibitory ratio of 3.0. This was not previous observed with the boiling of Romaine lettuce CWM (Esseili et al., 2012). Chemical oxidation of carbohydrates in CWM also caused increased VLP binding inhibition. These data suggest that VLP binding to strawberry CWM is mediated by carbohydrates, and perhaps proteins, although additional studies would be required to prove the latter.

These studies were followed by lectin inhibition assays that allowed for the identification of specific sugar moieties that, by inference, may be involved in HuNoV VLP binding. Choice of lectins to include in these studies was informed by the compositional and linkage analysis described above. Several lectins effectively inhibited HuNoV binding to strawberry CWM, notably lectin SNA, which binds αNeuNAc(2→6)gal (No HBGA specificity); lectin BS-1, which binds α-D-Gal >> GalNAc (HBGA specificity B>>A); lectin MOA, which binds Galα1, 3Gal, 3Galβ1 (HBGA specificity B>>A); and lectin DBA which binds GalNAc (HBGA specificity A1>>A2). These lectins all produced inhibition ratios greater than 2.0 (Figure 4A and 4B). The majority of lectins that significantly inhibited HuNoV binding have sugar specificity with various conformations of galactose. Lectins with binding specificity to HBGA moieties A and B were found to be most effective in blocking HuNoV binding to strawberry CWM. Of all the fruit-based lectins assayed, only lectin SNA

(originating from elderberry) effectively inhibited HuNoV binding, although this lectin has no HBGA specificity. Taken together, these data suggest the HuNoV VLPs bind to strawberry CWM through interactions with carbohydrate moieties, with most (but not all) of these showing HBGA specificity. It is entirely possible that structural carbohydrate combinations uncommon to human HBGAs may be predominant structures of strawberry pistils that serve as binding pockets to lectins and HuNoV.

This study involved development and application of some novel methods that have not previously been applied to berry tissues. Further, this work was subject to the complexities of carbohydrate analysis, which is much more difficult that analysis of proteins or nucleic acids. Data interpretation is, therefore, impacted by methodological limitations. For instance, despite rigorous dialysis to prepare the pistils for compositional analysis, residual low molecular weight carbohydrates remained in the extract. These may have originated from the berry receptacle, not the pistils themselves and this could have skewed compositional percentages estimated in the linkage analysis. Because the competitive ELISA methods are an indirect measure of VLP binding, HuNoV binding to specific carbohydrate moieties can only be inferred. In addition, there are many, many lectins, so the data presented here are only representative and there may be other carbohydrate moieties to which HuNoV VLPs bind, and/or those moieties may be strain-specific. Processing of berry tissue for analysis may release chemicals and byproducts not otherwise available to the viruses in nature, which could also impact interpretations. However, the methods described here are the most advanced to date for characterizing HuNoV binding to fresh produce, and the

preponderance of data suggests that specific carbohydrate interactions are important for this phenomenon.

Currently, there are no effective methods available to remove or inactivate HuNoV from the surface of fresh berries. Consistently documented in the literature is the vulnerability of fresh-cut produce items and the exposure of plant carbohydrate moieties that serve as virus attachment sites (Wei et al., 2010; Esseili et al., 2012; Gao et al., 2016 Baugher and Jaykus, 2016). Our approach was to systematically identify specific and non-specific interacting forces that contributed to HuNoV attachment to strawberries, with a focus on the pistil structure. This study provides a deeper understanding of the HuNoV binding mechanisms to this fruit. This information and future study can aid in the development of effective removal strategies such as targeted washes, as well as provide a strong basis for resolving the binding dynamics of HuNoV to other foods and/or surfaces.

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Table 4.1. List of plant origins, common names, sugar specificity, and HBGA specificity of lectins used in competitive inhibition ELISA.

Lectin Code	Plant Origin	Common Name	Sugar specificity[a]	HBGA specifity[a]
MAA	*Maackia amurensis*	Amur maackia	Sialic acid glycoconjugate	None
BS-1	*Bandieraea simplicifolia*	Griffonia simplicifolia	α-D-Gal >> GalNAc	B >> A
UEA-1	*Ulex europaeus*	Common gorse	α-L-Fuc	O (H) type 2/Le^y
LcH	*Lens culinaris*	Lentil	α-D-Man/α-D-Glc	None
DBA	*Dolichos biflorus*	Horsegram	GalNAc	A1 >> A2
SNA	*Samucus nigra*	Elderberry	αNeuNAc(2→6)gal	None
LEA	*Lycopersicon esculentum*	Tomato	GlcNAc	None
MNA-G	*Morniga G*	Black mullberry	Galactose	A
MNA-M	*Morniga M*	Black mullberry	Mannose	A
MOA	*Marasmium oreades*	Mushroom	Galα1, 3Gal, 3Galβ1	B >> A

[a] Adapted from *Handbook of Plant Lectins* (Van Damme et al., 1998) and the work (Zakhour et al., 2009)

Figures

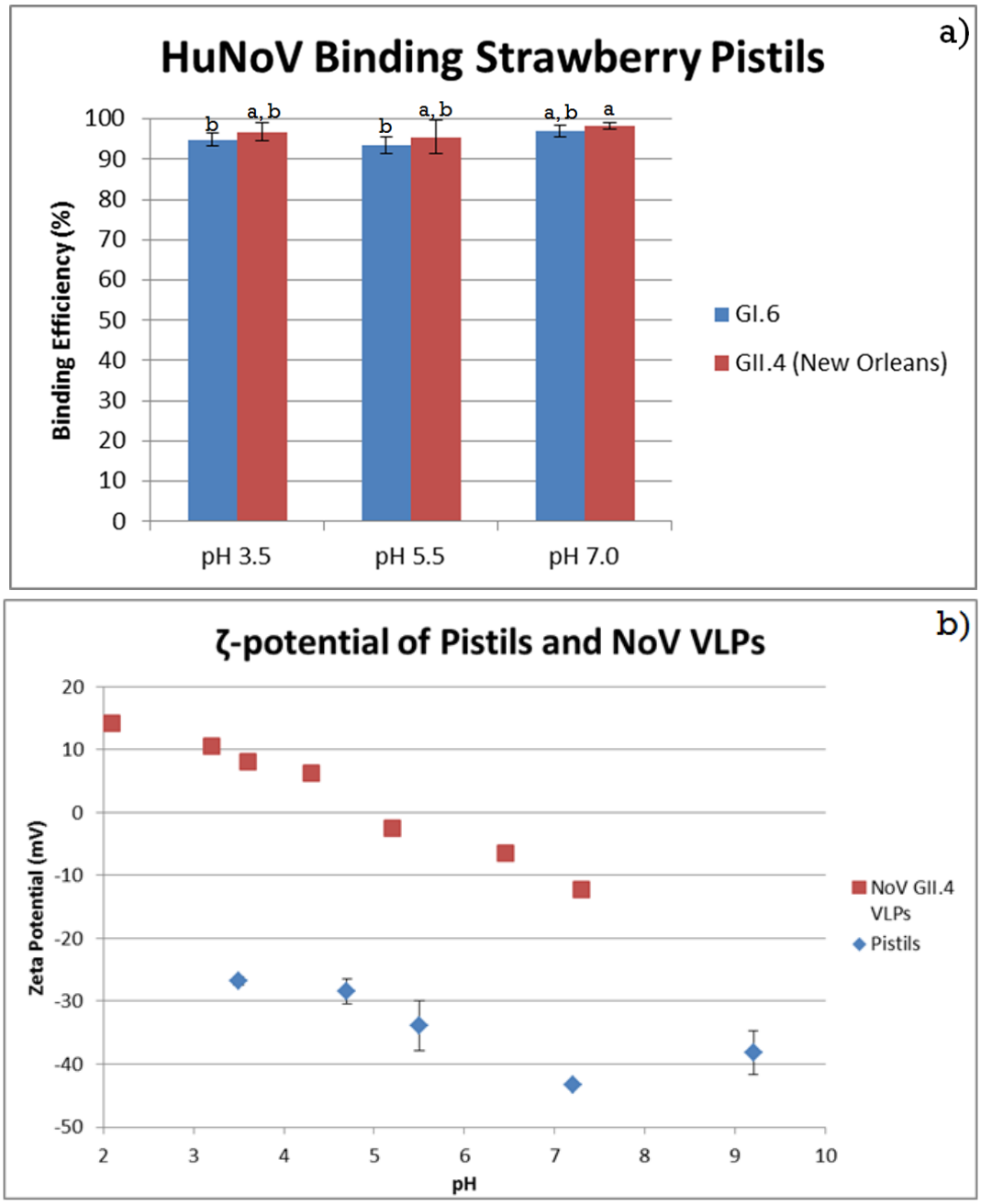

Figure 4.1. Evidence that electrostatic interactions have minimal impact of HuNoV GI.6 and GII.4 (New Orleans) binding to Camarosa strawberry pistils. Panel A shows results of suspension-RT-qPCR assays done to evaluate virus-berry binding across pH range of 3.5-7.0. Panel B is the zeta potential of HuNoV GII.4 (Houston) VLPs, and that of Camarosa strawberry pistils across a broad pH range (2.1-9.3). Letters in Panel A denote statistically significant differences ($p \leq 0.05$) when comparing samples means of both HuNoV strains across the pH range.

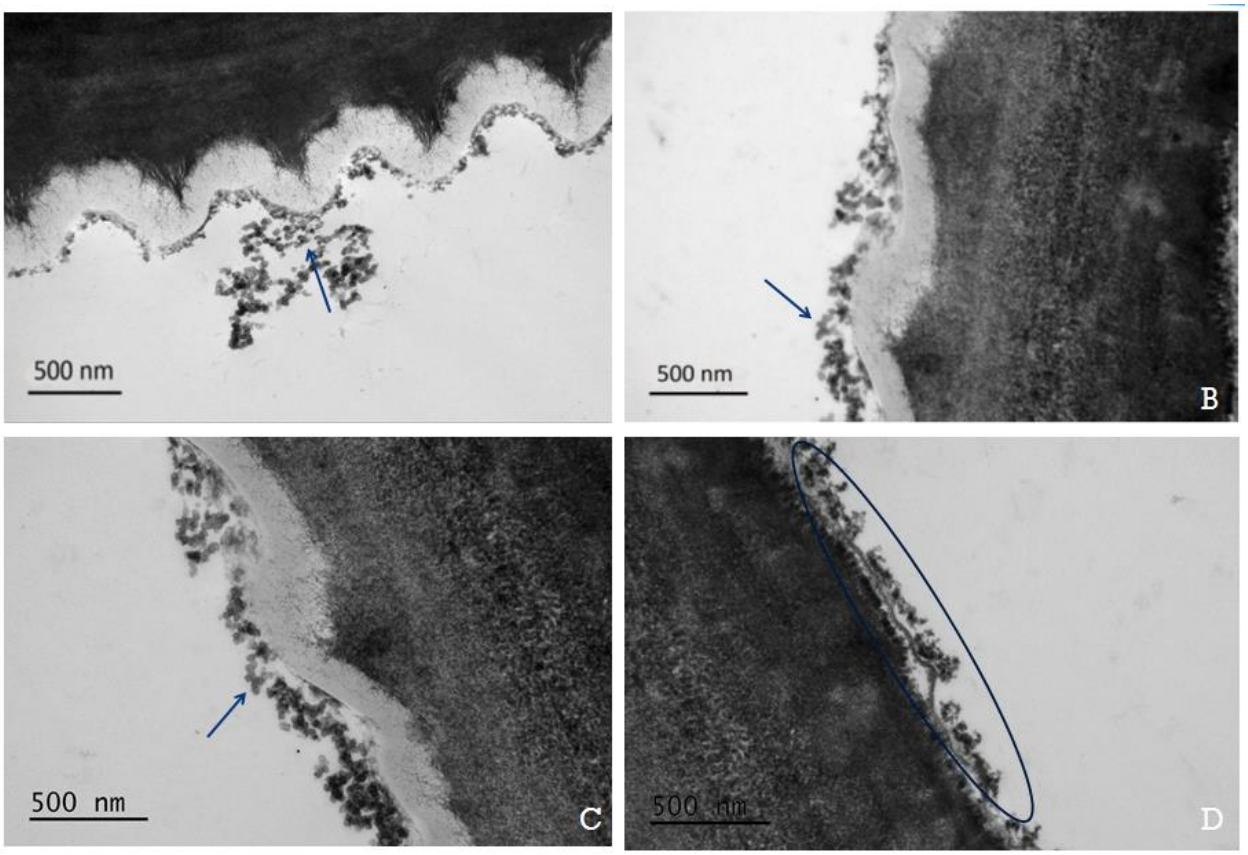

Figure 4.2. TEM images of GII.4 (Houston) VLPs bound to Camarosa pistils. Panels A-C show VLP aggregation on the pistil surface, arrows pointing to individual aggregates. D) Control showing internal pistil debris circled.

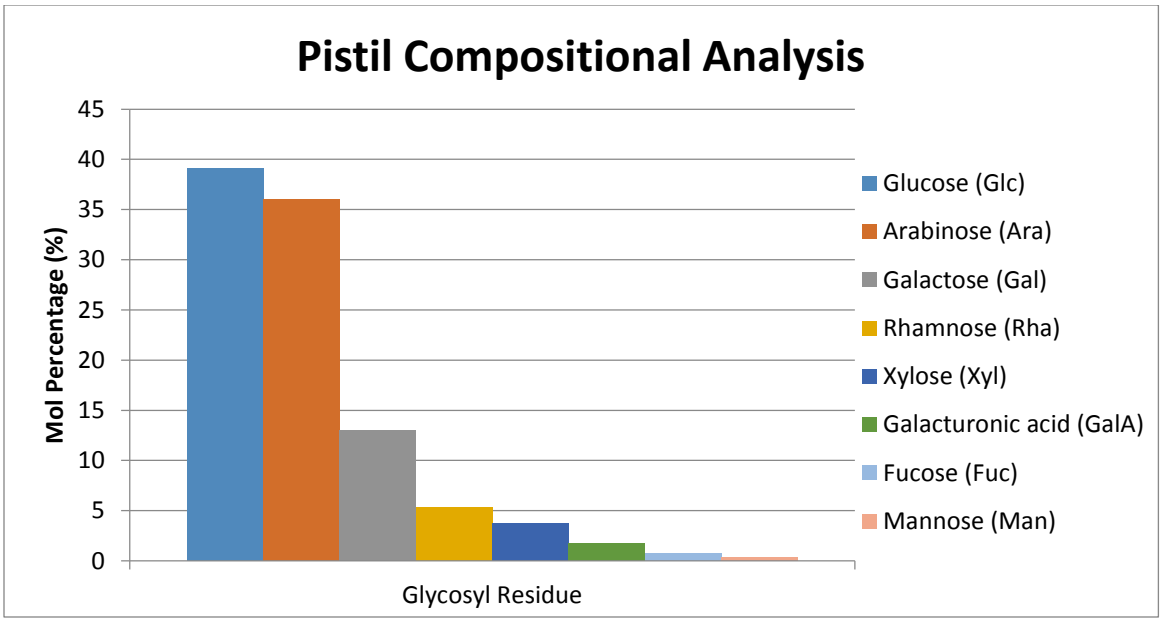

Figure 4.3. Molecular glycosyl residue composition (by percentage) of Camarosa pistils. Glycosyl residues not detected included ribose (Rib), glucuronic acid (GlcA), N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), and N-acetyl mannosamine (ManNAc).

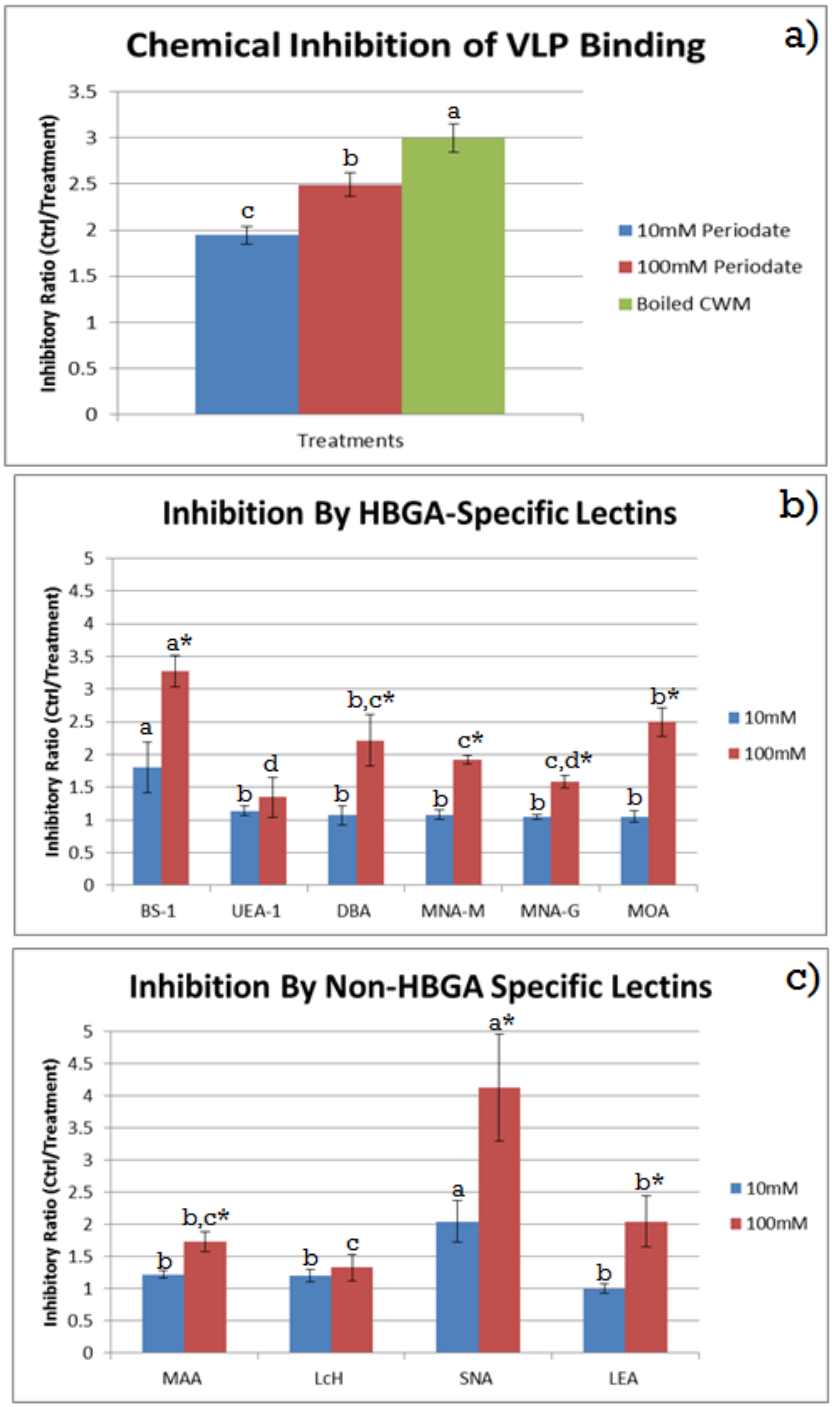

Figure 4.4 Competitive ELISA inhibition assays for CWM. Pre-treated by chemical and physical disruption (Panel A); HBGA-specific lectins (Panel B); and non-HBGA specific lectins (Panel C). Letters in Panels B and C denote statistically significant differences ($p \leq 0.05$) between lectins of the same concentration. * denotes statistically significant differences ($p \leq 0.05$) between the same lectin at different concentrations.

CONCLUSIONS

Throughout the world, human norovirus (HuNoV) is the leading causative agent of foodborne illness, causing millions of infections and costing billions of dollars. Fresh produce, specifically fresh berries, have been recognized as a significant contributor to the overall burden of foodborne disease. In the US, nearly half of the fresh fruit is imported, often from developing countries with limited measures to monitor safe irrigation/processing waters and personal hygiene. Despite the likelihood of produce contaminations, little is known about the intimate interactions of how and why HuNoV attaches to surface of strawberries.

The majority of HuNoV-produce binding studies have been performed on Romaine lettuce, a produce item with very little in common to that of strawberries. Most of the previously reported produce studies began with a very specific hypothesis that was thoroughly examined, and the subsequent results were reported. These studies attempted to build directly off the research findings from other food commodities such as Molluscan shellfish that determined HBGA-like moieties (similar to the putative HuNoV receptors found in humans) in the gastrointestinal tract of oysters effectively bound and bioaccumulated HuNoV. Unfortunately, the presence of HBGA-like moieties were not detected in the initial produce items tested (i.e. Butterhead and Romaine lettuce, spinach, and cilantro).

Other produce studies looked to determine the mechanisms of action for HuNoV attachment to produce (specifically Romaine lettuce and raspberries) by disrupting the HuNoV-produce bonds. Electrolyzed waters, hydrophobic rinses and the addition of salts

were added to wash waters to determine what non-specific interactions governed the binding interactions. When none of these treatments were determined to be effective, researchers concluded that unknown specific interactions governed the HuNoV-produce interactions. With virtually no studies performed on fresh strawberries, this reiterated the huge knowledge gap that little was known about how HuNoV attached to fresh strawberries.

The work presented in this dissertation clearly shows that HuNoV readily binds to the surface of fresh strawberries. We aimed to take a systematic approach to determine the physiochemical forces and mechanisms of action that govern human norovirus binding interactions with fresh strawberries. Specifically, we wanted to investigate if the HuNoV-strawberry binding interactions were mediated via the natural-berry microflora, non-specific electrostatic interactions, and/or specific berry ligands.

To investigate if the biofilm (EPS) producing natural berry microflora mediated HuNoV-strawberry binding interactions, a microbial survey was performed, bacterial isolated/sequenced, and biofilm production of selected isolates performed. With only a few microbial surveys of strawberry juice previously performed, this work is the first microbial survey of the microflora of the strawberry surface. Although biofilm production varied greatly, strains of *Pantoea agglomerans*, *Pseudomonas moraviensis*, *Klebsiella oxytoca,* and *Enterobacteriaceae spp*. were the strongest biofilm producers (A_{405} > 2.2). These biofilm producers were tested for their ability to bind HuNoV GI.6 and GII.4 (New Orleans) and only *P. moraviensis* was shown to have a moderate binding efficiency (>90%) for GI.6. All the strains were tested for the presence of HBGA-like moieties A, B, H, Lewis B, and Lewis Y, with only *P. moraviensis* showing detectable levels of HBGA A, B, H(O). It was

concluded that the native berry microflora has low potential to facilitate HuNoV binding to strawberries, with minimal evidence to suggest the native berry microflora as a significant vehicle for HuNoV transmission.

We then characterized HuNoV GI.6 and GII.4 (New Orleans) attachment to fresh strawberries by quantifying physical attachment to the berry surface under various environmental pH conditions (pH 3.5, 5.5, and 7.0) by developing a suspension assay using whole berry surface slices. These experiments provided data showing that regardless of HuNoV strain and pH, whole strawberry slices had a binding efficiency of ~90%. This gave supporting evidence that HuNoV-strawberry binding was governed by specific interactions that overcame environmental conditions.

ELISAs were performed on homogenates of five strawberry and three raspberry varietals for the presence of HBGA-like moieties A, B, H, Lewis B, and Lewis Y, with only minimal levels of Lewis A detected. This suggested that if specific interactions governed HuNoV-binding, it was not specifically HBGA-like moieties; however, the prepared samples were homogenized whole berries that may have diluted low levels of HBGA-like moieties below detectable levels. The HBGA ELISAs have a high degree of variability for berry samples and due to poor reagent availability and poor assay sensitivity, the results were unreliable. This highlights the need for standardized methods for the detection of HBGA-like moieties in produce samples.

An important question to be answered when investigating HuNoV-strawberry binding interactions is, at what developmental stage are the berries susceptible to HuNoV attachment. By collecting Camarosa strawberries throughout the developmental stages (strawberry

flowering, green berries, and ripe berries), it was determined that HuNoV have consistently high affinity for binding strawberries. Because HuNoVs are known to be environmentally persistent and strawberries typical grow from flowers to ripe within 30 days, an important future study will be to determine if HuNoV can survive on strawberries throughout berry development. It is critical to determine if HuNoV contaminated irrigation waters on flowering strawberry plants pose a significant risk to subsequent consumers.

The use of confocal microscopy was able to visualize Alexa-fluor labeled VLPs binding to the achenes and pistils on the surface of strawberries. The confocal images showed compelling evidence that the VLPs aggregated at sites of fracture and injury on the surface of pistils. This is an important finding that supports previous fresh produce work that showed cut and injured Romaine lettuce leaves were sites of HuNoV aggregation. The data support the hypothesis that injured produce and damaged produce is highly susceptible to HuNoV attachment (i.e. produce quality equates to overall produce safety).

Quantitative data showed that throughout strawberry development pistils bound HuNoV with high affinity (>94%), and the removal of the pistil/achene from the surface significantly reduced the overall binding efficiency. The identification of specific strawberry structures with high HuNoV binding efficiency, specifically strawberry pistils, provided us with a model system for further investigation into the mechanisms of action of HuNoV-strawberry binding. The pistils were shown to consistently bind to both GI.6 and GII.4 (New Orleans) across a broad pH range (pH 3.5, 5.5, and 7.0). Analysis of the zeta potential of VLPs and pistils showed that while VLPs transitioned from electropositive at low pH to electronegative at pH 5.2 and higher, the pistils were negatively charged across the entire pH

range tested. This provided strong supporting evidence that the binding interactions between HuNoV and pistils are not governed by non-specific charge interactions and that specific HuNoV-pistil binding interactions were required to overcome the electronegative, repulsive forces at environmental pH higher than 5.2. The TEM images provided visual confirmation that HuNoV was physically attaching to surface of the pistils.

Previous investigations have assessed the potential HuNoV binding implications of carbohydrate moieties with minimal insight into of the composition of the produce item being investigated. We employed a targeted approach by performing a compositional and linkage analysis of the strawberry pistils to determine potentially relevant carbohydrate binding moieties. Although the linkage analysis of the pistils was more global in nature and failed to provide some of the specificity that we initially desired, we were able to employ these data in our lectin selection process. Although important HBGA component residues n-acteyl glucosamine (GlcNAc) and n-acetyl galactosamine (GalNAc) were not present in the sample, galactose and fucose were found in the pistil composition. We chose a panel of relevant HBGA and non-HBGA lectins to assess their potential role in inhibiting HuNoV binding. Because we used cell wall material (CWM) and not pistils for the lectin binding assays, we also selected several lectins that had specificity to GlcNAc and GalNAc to determine if those residues were present in other components of the CWM than the pistils. The data showed lectins LEA and DBA specific to GlcNAc and GalNAc, respectively, did significantly inhibit HuNoV binding to the CWM.

Based on the results of the lectin inhibition assays, HuNoV interacts specifically to multiple carbohydrate moieties found on the surface of strawberries. Of the interacting

strawberry carbohydrate moieties, some residues have HBGA specificity; while others have no direct HBGA binding specificity associated with them. Carbohydrate exposure to sodium periodate caused oxidation of the carbohydrates and significantly inhibited HuNoV from binding to CWM. Boiling of the CWM resulted in a large inhibition of HuNoV binding to CWM, suggesting that the protein portion of the CWM significantly influences HuNoV binding. However, the assumption that boiling only denatures the protein portion of CWM without disrupting specific carbohydrate bonds remains unclear. Non-specific interactions were shown to have minimal effects on the HuNoV-strawberry binding interactions throughout all experimentation.

There are several future directions that we could readily take this research. The data indicates that HuNoV binds to strawberries with high affinity throughout strawberry development; therefore, HuNoV applied directly on strawberry plants need to be performed to determine how long intact and infectious HuNoV persists as on strawberry plants. This could have significant implications for better early monitoring of irrigation systems and fertilizers. The potential protein contribution of CWM does need to be determined in future work. We have indirectly inferred that a number of strawberry carbohydrates are critical to HuNoV-strawberry binding interactions. Enzymatic cleavage assays designed to remove these candidate carbohydrate residues and assess if the same HuNoV binding inhibition occurs, would provide confirmation that these residues are directly involved in HuNoV binding. Lastly, we hypothesize that performing the confocal microscopy assays with punctured or injured berries would fully visually elucidate the hypothesis that injured or damaged produce strawberries are highly susceptible to HuNoV. This would support the

statement that produce quality should be the same conversation as produce safety. We contend that this research will aid in the development of intelligently designed produce rinses and novel HuNoV removal strategies, as well as potential advancements in good agricultural practices (GAPs) that may reduce the burden of HuNoV disease associated with strawberries.

APPENDICES

Appendix A. Glycosyl linkage analysis of Camarosa pistils.

Residue	**Peak Area**	**Area %**
Terminal Rhamnopyranosyl residue (t-Rha)	545967	1.2
Terminal Arabinofuranosyl residue (t-Araf)	2200812	4.9
Terminal Fucopyranosyl residue (t-Fuc)	222296	0.5
Terminal Xylopyranosyl residue (t-Xyl)	840578	1.9
2 linked Rhamnopyranosyl residue (2-Rha)	390663	0.9
Terminal Manopyranosyl residue (t-Man)	454955	1
Terminal Glucopyranosyl residue (t-Glc)	9315388	20.5
3 linked Arabinofuranosyl residue (3-Araf)	259694	0.6
Terminal Galactopyranosyl residue (t-Gal)	1138979	2.5
4 linked Arabinopyranosyl residue or 5 linked Arabinofuranosyl residue (4-Arap or 5-Araf)	609965	1.3
2 linked Xylopyranosyl residue (2-Xyl)	536071	1.2
4 linked Xylopyranosyl residue (4-Xyl)	536071	1.2
3 linked Glucopyranosyl residue (3-Glc)	6939916	15.3
2 linked Mannopyranosyl residue (2-Man)	122519	0.3
2 linked Glucopyranosyl residue (2-Glc)	1055881	2.3
3 linked Galactopyranosyl residue (3-Gal)	479159	1.1
4 linked Mannopyranosyl residue (4-Man)	964004	2.1
2 linked Galactopyranosyl residue (2-Gal)	121218	0.3
6 linked Glucopyranosyl residue (6-Glc)	1589574	3.5
4 linked Galactopyranosyl residue (4-Gal)	1751663	3.9
4 linked Glucopyranosyl residue (4-Glc)	8421813	18.6
6 linked Galactopyranosyl residue (6-Gal)	983954	2.2
3,4 linked Glucopyranosyl residue (3,4-Glc)	782011	1.7
2,3 linked Glucopyranosyl residue (2,3-Glc)	509504	1.1
2,4 linked Glucopyranosyl residue (2,4-Glc)	585875	1.3
4,6 linked Glucopyranosyl residue (4,6-Glc)	1523594	3.4
3,6 linked Galactopyranosyl residue (3,6-Glc)	2450623	5.4
Total	45332747	100.0