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The Persistence of Human Norovirus Surrogate in Leafy Greens Production System

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The Persistence of Human Norovirus Surrogate in Leafy Greens Production System

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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Abstract

Since mostly consumed raw, foodborne pathogen contamination of leafy greens has led to a large number of foodborne disease outbreaks and illnesses each year in the United States. Human noroviruses (hNoV) are the most common viral pathogen transmitted by leafy greens. In this dissertation, the persistence of the hNoV surrogate Tulane virus (TV) on pre-harvest lettuce and microgreens was investigated. Lettuces are the most studied leafy green model, while previous hNoV research has mainly focused on the post-harvest stage of production. Here, pre-harvest hydroponically grown lettuce were used to determine TV persistence on leafy greens. After inoculation on leaves at 40 days age, TV reached over 4 log PFU/leaf reductions over the subsequent 4 days of observation. On day 45, TV was still detected on leaves, indicating that the pre-harvest viral contamination may last to post-harvest stages including consumption.

Meanwhile, microgreens are a group of novel salad greens whose color, texture, flavor and nutritional values have attracted more consumers in recent years. Currently, the understanding of viral risks in microgreen cultivation systems is limited. This dissertation used sunflower (SF) and pea shoots (PS) as model microgreens to study the virus transmission from two types of soil-free cultivation matrix (SFCM)—biostrate and peat. Without the presence of plants, TV survived over 10 days in SFCM with only 2.08 and 1.76 log PFU/tray reduction in biostrate and peat, respectively. However, when TV were inoculated in SFCM on day 0 before sowing seeds, no virus was detected in harvested microgreen edible tissues on day 10, regardless of the plant variety and SFCM type. Notably, there were significantly lower virus concentrations in the planted SFCM compared to the unplanted control areas. Later, the virus transfer from SFCM to microgreen was further investigated when inoculated with TV at day 7 of plant age. On day 10, there were minor reductions in virus concentrations in SFCM, but in microgreen edible tissue,

TV was still not detected. In addition, another study was carried out to characterize virus persistence on microgreen leaves surfaces. A significantly higher virus persistence was observed on PS than SF. From plant age of 7 to 10 days TV reduced on average over 4.5 log PFU/plant (n=2) on SF, while the reduction was only 2.52 on average (n=2) for PS, indicating a plant variety-dependent virus persistence on microgreens. The findings in this dissertation provides insights on virus transmission during pre-harvest production stage of two types of leafy greens—head lettuce and microgreens. This information will help to develop more effective virus prevention and control strategies within leafy green production systems.

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Dedication

This dissertation is dedicated to my parents for their understanding, supports, and unconditional love.

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List of Published Papers

Chapter 1. Deng, W. & Gibson, K. E. (2017). Interaction of microorganisms within leafy green phyllospheres: Where do human noroviruses fit in?. *International Journal of Food Microbiology*, 258, 28-37.

Introduction: Leafy Green Production System

I. Conventional and modern leafy green production

Fresh produce occupies a higher proportion of the human daily diet with the increased awareness of natural foods and the concept of a balanced diet (Randhawa et al., 2015). Traditionally, leafy greens are produced in soil under open air with irrigation and application of fertilizers, pesticides, and herbicides (Lages Barbosa et al., 2015). In modern agriculture, controlled environment agriculture (CEA) such as greenhouses and hydroponics has been implemented to improve the yield and quality of fresh produce (Ferguson et al., 2014). In addition, the choice of growing media and production system types for fresh produce has become more diversified, and it can be classified into soilless medium, hydroponic or aquaponic, and aeroponic (Aatif Hussain et al., 2014; Ako and Baker, 2009; Lakhiar et al., 2018; Touliatos et al., 2016).

The soilless cultivation media usually include a mix of different components such as vermiculite, coconut coir, peat moss, sphagnum moss, sand, or perlite at a designated ratio for different leafy green types. In hydroponic, aquaponic, and aeroponic systems, the roots of the plant are exposed continuously or periodically to a nutrient solution in a closed space (Lakhiar et al., 2018; Savvas and Gruda, 2018). Compared to the conventional production mode, there are several advantages of soilless media production, including more water efficient, no pesticide use, less impact to the environment, lower land space occupation, and lower risk of fecal and spoilage microorganisms (Lages Barbosa et al., 2015; Sirsat and Neal, 2013). These new sustainable cultivation techniques are suggested to be the possible solutions to future food security caused by the rapidly increasing human population (AlShrouf, 2017).

II. Indoor microgreen production

Microgreens, also called “vegetable confetti”, are a special group of leafy green. They are immature vegetables that are usually harvested when the seed leaves fully develop and first pair of true leaves emerge, which takes 1 to 3 weeks after seed germination (Pinto et al., 2015).

Various vegetables and herbs can be grown as microgreens including red beet, cilantro, radish, sunflower, mustard, and pea shoots (Kyriacou et al., 2016) (Table 1). In the 1980s, microgreens were first used by chefs in San Francisco, California (Renna et al., 2017). Now microgreens, as a new culinary trend, are mostly consumed in restaurants for enhancing the texture and flavor of foods and embellishment purposes (Kyriacou et al., 2016). Moreover, as a rich source of antioxidants and minerals (e.g. iron and zinc), microgreens can provide a higher dietary intake of macroelements, microelements, and bioactive compounds vegetables, while containing less anti-nutrients compared to mature plants (Lenzi et al., 2019; Weber, 2017).

The small scale of microgreen production and its relatively simple production setup requirements offer the potential of diversifying the food system especially in urban areas (Weber, 2017). Most commercial microgreen production systems are indoor operations which belong to the CEA production category. A recent survey of 176 microgreens operations in the US revealed that the most used a production setup is comprised of trays on stacked artificially lit shelves in indoor residential places (26.7%), followed by container farms inside a climate-controlled greenhouse (8.5%), and trays on shelves in indoor commercial spaces (6.8%) (Misra and Gibson, 2021). Interestingly, 75% of operations in the survey were opened after 2010, which indicates the growing demand of the microgreen market in recent years.

III. Microbial food safety risks during leafy green production

The foodborne pathogens that are frequently associated with leafy greens include Shiga toxin-producing *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, norovirus, Hepatitis A, and *Cyclospora* (Carstens et al., 2019). Microbial contamination during leafy green production may be caused by various components in the growing system, such as the irrigation water, environment, soil amendment and fertilizer, and harvesting process (FAO, 2008). The interconnection of farmers, livestock, plant and environment contribute to the complexity of the possible contamination routes (U.S. Food and Drug Administration, 2021). For instance, the contamination routes of human norovirus in fresh produce production can be a result of (1) heavy rain and flooding causing sewage water to spread to irrigation water sources or to the plant growing field, (2) cross-contamination from worker or equipment during production and harvest, and (3) improper use of organic waste in field as fertilizer (EFSA, 2014; Sofy et al., 2018; Terio et al., 2020). According to an investigation on the irrigation water from five farms located in Finland, the Czech Republic, Serbia and Poland, human enteric viruses Hepatitis E viruses (1/20) and human norovirus (hNoV) genogroup 2 (GII) (4/28) were detected in leafy green irrigation water (well water) from three countries. Also, 3.6% of hNoV GII (2/56) were detected in berry fruit irrigation water (river and well water) from all four countries (Kokkinos et al., 2017).

In addition, the contamination routes and microbial hazards related to different leafy green production methods share some similarities but also vary due to their different characteristics. For example, a study comparing aquaponic and hydroponic systems revealed that the introduction of Shiga toxin-producing *E. coli* was mainly through fish feces in the former system; however, for the hydroponic system (i.e., without fish presence), the introduction of *E.*

coli was possibly through accidental cross contamination or biofilm in equipment (Yi-Ju et al., 2020). Thus, for each production system, the microbial risks need to be evaluated specifically.

IV. Norovirus and leafy greens

Noroviruses (NoV) are non-enveloped single-strand RNA viruses that cause acute gastroenteritis. As a member of family *Caliciviridae*, NoV are classified into 10 genogroups (GI-GX) so far, among which genogroups I, II, VIII and IX are infectious to humans (Parra, 2019). The GI, GII, GVIII and GIX are known to consist of 9, 27, 1 and 1 genotypes, respectively (Chhabra et al., 2019). On average, hNoV leads to 570-800 deaths, 56,000-71,000 hospitalizations, 400,000 emergency department visits, 1.7-1.9 million outpatient visits, and 19-21 million illnesses each year in the US (Hall et al., 2013).

Between 2004 to 2012, hNoV was found responsible for 59% and 53% of fresh produce caused foodborne outbreaks in the United States and European Union, respectively (Callejón et al., 2015). Fresh produce is an important food source that can be contaminated with hNoV. Among single commodity caused hNoV outbreaks (n=364) from 2001 to 2008, leafy greens constituted 33%, fruits and nuts were 16%, and mollusks caused 13% outbreaks (Hall et al., 2012). Hall et al. (2014) reported that leafy vegetables constituted 30% of the hNoV outbreaks caused by single commodity (n=67) in the US, 2009-2012.

In this dissertation, viral risks were evaluated in hydroponic and indoor farming systems for the production of lettuce and microgreens, respectively. The hydroponic lettuce was used as a model for studying the virus persistence on pre-harvest leaf surface (Chapter 2). On the other hand, microgreens are a new class of leafy greens. In contrast to the numerous foodborne outbreaks associated with contaminated lettuces, so far there have been no documented

outbreaks linked to microgreens and only several recalls have been reported due to potential *Salmonella* and *Listeria* contamination of commercially available microgreens (Turner et al., 2020). Nevertheless, hNoV is one of the top causes in leafy green outbreaks, while current knowledge about hNoV risk in microgreen production is very limited (Herman et al., 2015). Therefore, several potential viral risks during indoor microgreens production were investigated in this dissertation. The virus survival in different types of microgreens growing media and the subsequent transfer to plant edible tissue was tested (Chapter 3). Also, the impact of virus contamination occurrence when close-to-harvest stage of microgreens was examined. Last, the impact of contamination route (irrigation water or direct contact with leaves) was investigated in order to understand virus transfer and persistence on the edible microgreen tissue (Chapter 4).

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VI. Tables

Table 1. Summary of common microgreens by families and species

Microgreen	Families	Species
Vegetable	<i>Brassicaceae</i>	Cauliflower, broccoli, cabbage, kale, radish, rappini, watercress, mizuna, arugula, rocket
	<i>Asteraceae</i>	Lettuce, endive, chicory, radicchio, sunflower
	<i>Apiaceae</i>	Dill, carrot, fennel, celery, cilantro, cumin
	<i>Amarillydaceae</i>	Garlic, onion, leek
	<i>Amaranthaceae</i>	Amaranth, quinoa, swiss chard, beet, spinach, quinoa
	<i>Cucurbitaceae</i>	Melon, cucumber, squash
Herb	<i>Gramineae</i>	Oat, wheat, corn, barley, rice
	<i>Leguminosae</i> (<i>Fabaceae</i>)	Chickpea, alfalfa, bean, green bean, fenugreek, fava bean, lentil, pea, clover
	<i>Lamiaceae</i>	Basil
	<i>Liliaceae</i>	Chives

Chapter 1: Interaction of microorganisms within leafy green phyllospheres: Where do human noroviruses fit in?

I. Abstract

Human noroviruses (hNoV) are one of the major causes of foodborne disease outbreaks linked to leafy greens. However, the interactions—including attachment and persistence—of hNoV with leafy greens are not well characterized. In the present review, three mechanisms are hypothesized for the interaction of hNoV with leafy green phyllospheres: 1) specific binding to histo-blood group antigen (HBGA)-like carbohydrates exposed on leaf surfaces and present on bacterial microbiota; 2) non-specific binding through electrostatic forces; and 3) internalization of hNoV through contaminated water (e.g. hydroponic feed water). To add more complexity, there is a rich diversity of microbial communities (i.e., bacteria, fungi, protozoa) residing in leafy green phyllospheres, and the attachment and persistence of hNoV could be largely impacted by these microorganisms through direct and indirect interactions. For instance, enzymes produced by bacteria and fungi could potentially compromise the structure of HBGA-like carbohydrate binding sites on leaves, leading to a reduction in hNoV binding. On the other hand, some bacteria also possess HBGA-like binding sites on their cell surface, which may provide extra binding locations for hNoV. There are also numerous metabolic compounds that can be produced by leafy greens and its microbial inhabitants and be subsequently distributed within leafy green phyllospheres. These compounds could theoretically play roles in enhancement or reduction in the attachment of hNoV. Overall, increasing the understanding of the various types of hNoV attachment and interactions with leafy green phyllospheres will be crucial for elucidating hNoV transmission via leafy greens as well as for the development of effective control measures.

II. Introduction

i. Public health burden of human noroviruses

Human noroviruses (hNoV) are non-enveloped, single-strand RNA viruses that are a causative agent of acute gastroenteritis. Norovirus genus belongs to the family *Caliciviridae*. The genus is classified into at least 6 genogroups and further divided into at least 38 genotypes (Vinjé, 2015). Human noroviruses from genogroups I, II, and IV are infectious to humans (de Graaf et al., 2016; Verhoef et al., 2015). This group of viruses is transmitted through multiple routes: food, water (drinking and recreational contact), environmental surfaces, and person-to-person, among which person-to-person transmission is predominant. Specifically, 24% of hNoV outbreaks in the United States (U.S.) are foodborne, and in the European Union (EU) the percentage is estimated to be lower at 10% (Belliot et al., 2014). Globally, 14% of hNoV caused diarrheal diseases are due to food contaminations (Lopman et al., 2015). Among all of the hNoV genogroups and genotypes, GII.4 are more related to person-to-person transmission while non-GII.4 genotypes are frequently related to foodborne transmission (de Graaf et al., 2016).

Infections caused by hNoV are usually self-limiting among healthy populations, while more severe in elderly, young children, and immunocompromised populations. As reported by the U.S. Centers for Disease Control and Prevention (CDC), hNoV causes 56,000-71,000 hospitalization and 570-800 deaths annually, which are 15-20% and 2-10% of the total food-caused hospitalizations and deaths, respectively (CDC, 2016). The total cost of hNoV illness in the U.S. every year is \$2 billion with 5,000 quality-adjusted life-years for illnesses and deaths (Belliot et al., 2014). Worldwide, hNoV results in a median number of 699 million illnesses (95% uncertainty interval [UI]: 489–1,086 million) and 219,000 deaths (95% UI: 171,000–277,000)

annually (Bartsch et al., 2016). These illnesses and deaths result in \$4.2 billion in costs directly to health care and \$56.2 billion related to loss in productivity.

Nevertheless, the disease burden due to hNoV is nearly always underestimated due to the underreporting of outbreaks. For instance, during an epidemiological investigation in the U.S., Hall et al. (2013) observed a 25-fold difference between the highest and lowest states reporting hNoV outbreaks on a population-based rate. These discrepancies are partly due to incidence variations among states, but more likely, this is an indication of outbreak reporting and investigation resources at the state level. Also, hNoV outbreaks on a global scale are underestimated as the epidemiological investigations are normally performed within each individual country with varying resources (de Graaf et al., 2016).

ii. hNoV and leafy greens

A majority of confirmed hNoV outbreaks in Belgium from 2002 to 2007 were caused by food handlers (42.5%) followed by contaminated water (27.5%), bivalve shellfish (17.5%), and fresh produce (12.5%) (Baert et al., 2009). While the reporting on hNoV outbreaks in water and shellfish has been intensive, reporting of outbreaks associated with fresh produce is less frequent (Baert et al., 2011). According to the outbreak summary for leafy greens and fresh fruits in the U.S. and EU, hNoV is the primary causative agent followed by *Salmonella* (Raquel M Callejón et al., 2015). In the U.S., hNoV outbreaks are more often related to consumption of salad, and in the EU, reported outbreaks are mainly due to contaminated berries (Raquel M Callejón et al., 2015).

Between 1973-2012 in the U.S., there were a total of 606 outbreaks associated with leafy greens, leading to 20,003 illness, 1,030 hospitalization and 19 deaths. Among all outbreaks, most

of them were caused by hNoV (55%), followed by Shiga toxin-producing *Escherichia coli* (18%), and *Salmonella* (11%) (Herman et al., 2015a). Leafy green contamination with hNoV is more often related to GI hNoVs with positive GI results found in 100% (2/2), 72.5% (133/181), and 66.7% (2/3) of tested samples collected from food companies or supermarkets in Belgium, Canada, and France (Baert et al., 2011). This is potentially due to the fact that GI genotypes are more often associated with contaminated environmental sources such as water and have been shown to persist longer in the environment when compared to GII hNoVs (Bitler et al., 2013; Escudero et al., 2012; Matthews et al., 2012). In addition, the risk of hNoV contamination of leafy greens is conceivably greater due to the globalization of the food supply chain, especially when products are imported from countries with poor sanitation practices (Callejón et al., 2015; Nyachuba, 2010). With respect to fresh vegetables including leafy greens, 25% of those on the U.S. market are imported each year (Johnson, 2015). Meanwhile, this globalization provides increased opportunities for viral strains to come together and possibly increase the chance for viral recombination—one of the primary ways for viruses to evolve—leading to more challenges related to prevention and control through vaccine and anti-viral compound development (de Graaf et al., 2016).

III. Overview: Interactions of microorganisms with leafy greens

In recent decades, outbreaks related to consumption of leafy greens are becoming more frequent and recognized. Unexpected pathogens have been associated with fresh produce including *E. coli* O157:H7 in baby spinach, *Yersinia pseudotuberculosis* in lettuce, and *Listeria monocytogenes* in bagged salads, etc. (Lynch et al., 2009). To control and reduce these undesired pathogens as well

as to hypothesize the less well-understood interactions of human enteric viruses with leafy greens, it is crucial to understand how microorganisms colonize and interact with leafy greens.

i. Bacteria

a *General bacterial habitants on leafy greens*

Fresh produce harbors a diverse population of residential bacterial communities, which are determined by many factors. The microbial diversity is large across different fruits and vegetables, but often the same type of fresh produce (i.e., lettuce, spinach, tomato etc.) share more common microbial compositions compared to the other types (i.e., apple, peach, mushroom etc.), (Leff and Fierer, 2013). Bacteria are able to colonize most organs of plants including leaves, stems, and roots (Bais et al., 2006). While some bacteria can be found throughout a plant, there are also unique taxa that are only found in certain parts of the plant. Fresh produce leaves are colonized by numerous bacterial cells with an average of 10^6 - 10^7 cfu/cm² (Lindow and Brandl, 2003). As reported previously, the majority of bacteria genera found on lettuce leaf surfaces include *Pseudomonas*, *Pantoea*, *Arthrobacter*, *Flavobacterium*, *Acinetobacter*, and *Bacillus* (Table 1). In rhizoplane, bacteria composition varies between different soil types (Maloney et al., 1997). Cardinale et al. (2015) reported the most abundant bacteria families on lettuce roots are *Pseudomonadaceae*, *Xanthomonadaceae*, *Cellvibrio*, *Flavobacterium*, and *Sphingomonadaceae*. The plant age can also impact bacterial compositions since the variety of bacteria decreases during the maturation of lettuce leaves. Similarly, the concentration and diversity of the bacteria decrease during spinach leaf maturation (Dees et al., 2015). In addition to the above mentioned, climate and storage conditions, the bacteria location, and even the

microbial detection methods (i.e., culture-based, molecular) used in a given lab can all contribute to the reported microbial composition diversity in fresh produce (Rastogi et al., 2012, 2010).

Aside from just the bacterial composition of leafy greens, there is great interest in the plant-microbe interaction known as symbiosis, which can be categorized as pathogenic, mutualistic, or parasitic (Newton et al., 2010). Mutualism is beneficial for both plant and microbes. The most well-known mutualistic interaction is between the nitrogen fixation bacteria *Rhizobium* and legumes (Oldroyd, 2013). Parasitic and pathogenic interactions are both harmful to hosts with the former leading to collateral damage while the latter one causes trophic loss and even necrosis (Newton et al., 2010). Pathogenic interactions have been the most intensively studied compared to other types of interactions. For example, the phytopathogen *Pseudomonas syringae* is able to cause disease in a wide range of plants. This is achieved through the Type III secretion system (T3SS), which secretes host-specific effectors into plants (Feng et al., 2016).

In turn, plants have immune systems for defense during interactions with bacterial pathogens. The first line of defense relies on the binding between pathogenic-associated molecular patterns (PAMPs) from bacteria and pattern recognition receptors (PPRs) from plant (Feng et al., 2016). This further activates PAMPs triggered immunity (PTI) in plants to respond to invaders. Since PTI is non-specific and can be triggered by any bacteria, its effects are very limited. When a pathogen successfully outcompetes PTI, the second line of the plant immune system starts to react. Effectors secreted by pathogens will be specifically recognized and therefore activate the effector-triggered immunity (ETI) of the plant. Generally, if ETI is able to block all the pathogen effectors, then the plant is not impacted. Otherwise, the plant can develop diseases (Jones and Dangl, 2006; Xin and He, 2013).

The composition of the leaf surface provides the basic nutrient uptake for microorganism colonization. As reported by Hunter et al. (2010), the soluble carbohydrates, calcium, and phenolics on lettuce leaf surfaces have significant influence on bacterial community structure. Meanwhile, the morphology of the leaf is another factor effecting colonization. Specifically, the size of the lettuce head can determine air flow as well as water and soluble nutrient deposit (Hunter et al., 2010). Interestingly, bacterial colonization on leaves can sometimes alter the surface to make a better habitat. To better colonize and survive on leaves under harsh conditions, microbial aggregates can be formed with mixed bacterial species and even fungi. The matrix of aggregation is called extracellular polymeric substances (EPS) produced by bacteria. The EPS on leaf surfaces shield bacteria and protect them from some outer stresses (Lindow and Brandl, 2003).

Given the complexity of bacterial composition on leafy greens, it is not surprising that interactions have been reported among bacterial communities. The presence of *Xanthomonas campestris* pv. *vitiensis* (Xcv) on lettuce leaves was found to be positively related to genus *Alkanindiges*, while negatively related to *Bacillus*, *Erwinia*, and *Pantoea*. Several hypotheses have been raised to explain these relationships while the mechanisms behind it remain unclear (Rastogi et al., 2012). First, it might be due to the antagonistic relationship between Xcv with *Bacillus*, *Erwinia* and *Pantoea*. Second, the establishment of Xcv on lettuce leaves may have a specific impact on the phyllosphere community including *Bacillus*, *Erwinia* and *Pantoea*. In addition, the relationship might be due to other less well-defined factors such as the plant genotype.

b *Human bacterial pathogens on leafy greens*

Human pathogenic and opportunistic bacteria colonization on plants can be an important part of their life cycle as an alternative host for these human pathogens. A study revealed that *Salmonella* Typhi introduced by contaminated water to lettuce can survive from the seeding stage to maturation (Brandl et al., 2013). Human bacterial pathogens can attach to leafy greens through polysaccharides, bacterial lectins, and structural adhesins such as fimbriae, pili, and flagella (Gorski et al., 2003; Hassan and Frank, 2004; Tan et al., 2016). After attachment, bacteria are able to internalize in the plant through natural openings and damages, root uptake, or migrations through the vascular system (Quilliam et al., 2012). Unlike plant pathogens which trigger all available plant immune responses, human pathogens only induce very basal defenses of the plant. For instance, *Salmonella* and *E. coli* O157:H7, although recognized by lettuce immune cells, only trigger a weak defense response (Brandl et al., 2013).

Numerous studies have focused on leafy green colonization with foodborne pathogens including *Salmonella*, *E. coli*, and *L. monocytogenes* (Klerks et al., 2007; Quilliam et al., 2012; Solomon et al., 2002). The colonization of bacterial pathogens on plants can be significantly affected by plant genotypes. *Salmonella enterica* colonization of the phyllosphere of four types of tomatoes showed a 100-fold difference depending on the type of tomato (Barak et al., 2011). (Quilliam et al., 2012) also observed that the metabolic activities of *E. coli* O157:H7 on lettuce phyllosphere vary depending on the cultivar.

It has also been shown that plant pathogens that cause leaf maceration favor the growth of human pathogens such as *Salmonella* and *E. coli* O157:H7, whose cell density can be 10-fold higher on damaged plants compared to healthier plants. More specifically, the leaf maceration causes the leakage of nutrients such as sugars which can then be utilized by *Salmonella* and *E.*

E. coli O157:H7 (Brandl et al., 2013). Conversely, competition also happens between *E. coli* O157:H7 and indigenous spinach-biofilm bacteria since they utilize the same type of carbon and nitrogen sources (Carter et al., 2012). Bacterial pathogens can also interact with inhabitants on leafy greens. Studies have shown that vacuoles released by certain protozoa on lettuce and spinach can support the growth and survival of foodborne pathogens including *E. coli*, *L. monocytogenes*, and *Salmonella enterica* subspecies *enterica* (Gourabathini et al., 2008).

ii. Protozoa

The presence of free-living protozoa (FLP) is common on leafy greens such as lettuce and spinach (Gourabathini et al., 2008; Vaerewijck et al., 2011). Protozoa are ubiquitous in the environment, and they can be introduced to leafy greens through irrigation water or soils (Hsueh and Gibson, 2015). The estimated number of FLP on butterhead lettuce leaves ranges from 9.3×10^2 MPN/g to 2.4×10^5 MPN/g leaf and is dominated by *Spumella*(-like) flagellates and *Cercozoa* (Vaerewijck et al., 2011). Protozoa can also favor the growth and survival of certain bacterial pathogens. For instance, *E. coli* and *Salmonella enterica* Typhimurium were able to survive the digestion of *Tetrahymena* sp. and then egested via fecal pellets, whereas *Helicobacter pylori* was digested (Rehfuss et al., 2011; Smith et al., 2012). Furthermore, *S. Typhimurium* that passed through the FLP was reported to have an elevated gene expression for acid tolerance, compared to *S. Typhimurium* that had not passed through *Tetrahymena* (Rehfuss et al., 2011).

iii. Fungi

Fungal communities on leafy greens are less densely populated compared to their bacterial counterparts, though studies in this area are also not as prevalent (Vorholt, 2012). Fungi can interact with the plant by delivering small RNAs (sRNAs) into cells to defect plant immunity. Fungal pathogen *Botrytis cinerea* (Bc) can silence tomato and *Arabidopsis* immunity genes by secreting small RNAs (Bc-sRNAs) (Weiberg et al., 2013). In turn, endogenous sRNAs in many plants (e.g., wheat, rice, eggplant, cotton) were found to play important roles in immune response against fungal pathogens (Li et al., 2014; Llave et al., 2002; Weiberg et al., 2014; Zhang et al., 2016). For instance, cotton plants can excrete microRNAs to silent virulence genes in the fungal pathogen *Verticillium dahlia* (Zhang et al., 2016). These cross-kingdom interactions were further utilized as genetic tools by researchers to construct a transgenic plant to biologically control natural enemies such as demonstrated by Koch et al. (2013) using *Arabidopsis* and barley plants to inhibit *Fusarium* colonization and infection.

Bacteria have also been utilized as another tool for controlling plant fungal pathogens. The pathogenic fungus *Rhizoctonia solani* can cause crop losses; however, Chowdhury et al. (2013) discovered a strategy to attenuate the adverse impact by introducing *Bacillus amyloliquefaciens* FZB42 to the lettuce rhizosphere. As a result, *B. amyloliquefaciens* FZB42 successfully reduced the bottom rot of lettuce caused by pathogen *R. solani*. Also, *B. amyloliquefaciens* FZB42 were observed to lower the impact of *Rhizocotonia* on microbiome on lettuce phyllosphere (Erlacher et al., 2014). In addition, studies on plant fungi have also focused on the utilization of the arbuscular mycorrhizal fungi (AMF) to enhance the accumulation of mineral compound (e.g., Cu, Fe) and antioxidants such as anthocyanins, carotenoids, and phenolic compounds in lettuce leaves (Baslam et al., 2011; Li et al., 2012). Conversely, when AMF was present, the time of

persistence was extended for foodborne pathogens *Salmonella* and *E. coli* O157: H7 which were internalized in leek roots (Gurtler et al., 2013).

iv. Viruses

While viruses pathogenic to leafy greens as well as human enteric viruses that may contaminate leafy greens have both been intensively studied and reviewed in the literature, there are fewer studies characterizing the virome of leafy greens. A recent study by Aw et al. (2016) reported on the diversity of viruses present in field grown and retail lettuces using metagenomics. The authors found that plant pathogenic viruses dominated the romaine and iceberg lettuces, with a relative abundance of 66.7 and 64.4% respectively. Other viruses were found that infected a wide range of hosts including bacteria, invertebrates, amoeba, fungi, and algae. The bacteriophages (phage) present on tested lettuces were associated with 63 different bacterial hosts including homologs of *Salmonella* and *E. coli* phages. Rotaviruses and picobirnaviruses—common human and animal viruses—were identified on tested samples, while more well-known foodborne viruses such as hNoV, hepatitis A and E were absent, possibly due to the high detection limit or a seasonal effect. Human enteric viruses can be introduced during the production of leafy greens through contamination with human waste. Mattison et al. (2010) reported the detection of hNoV and rotaviruses in packaged leafy greens collected from retail stores in Canada. Among 275 samples, 6% were hNoV positive and 0.4% were positive for rotavirus.

Independent of the immune response to bacterial pathogens, plants have a different response mechanism against pathogenic viruses called RNA silencing (Voinnet, 2005). The viral genome replication can happen in the nucleus or the cytoplasm of the host cell. Under both environments, viral gene replications are recognized by DCL (dicer-like) which further triggers the production of viral siRNA (small interfering RNA). The viral siRNA interacts with viral DNA or RNA,

resulting in silencing of viral gene expression (Voinnet, 2005). However, viruses are not always pathogenic and can also be mutualistic with the plant. As reported by Roossinck (2015), viruses can ameliorate the adverse effects of abiotic stresses on plants including drought, heat, and cold. For example, Xu et al. (2008) inoculated four viruses—brome mosaic virus, cucumber mosaic virus, tobacco mosaic virus, and tobacco rattle virus—onto plants then cultivated them under water withholding conditions. Surprisingly, all four viruses postponed the appearance of drought symptoms.

IV. Human noroviruses (or hNoV surrogates) and leafy green interactions

i. Brief overview of hNoV structure and function

While the structure and function of hNoV has been covered thoroughly in the literature (Hardy, 2005; Karst et al., 2014; Tan and Jiang, 2010, 2014), a brief overview is provided here. The virion of hNoV is icosahedra and is composed of 90 dimers of a major capsid protein (VP1) and one or two copies of a minor structural protein (VP2) (Hardy, 2005). The protruding (P) domain on VP1 plays the main role in binding to carbohydrate receptors such as histo-blood group antigens (HBGAs)—the presumptive hNoV receptor on target host cell (Tan and Jiang, 2010, 2014). Specifically, the P domain is located on the outermost portion of the virus particle forming arches extending from the shell and contains two subdomains—P1 and P2—with the latter responsible for the strain diversity, HBGA binding, and antigenicity (Shanker et al., 2016; Tan and Jiang, 2007). Additional ligands have also been identified including glycosphingolipids with negatively charged sialylated structures; however, the recognition of these alternative ligands varies by hNoV genotype (Rydell et al., 2009; Han et al., 2014). It can be hypothesized that the binding specificity (i.e. the composition of the central binding pocket and variable

surrounding region) of a given hNoV genotype would likely impact interactions and binding of hNoV with leafy greens via carbohydrate moieties present on the phylloplane as well as hNoV persistence as covered in Chapter 1 Section III-iii.

ii. Introduction to hNoV surrogates

Although the hNoV research community has unveiled numerous aspects of hNoV pathogenesis in the past decade, an *in vitro* culturing method for hNoV remained unavailable until recently. Ettayebi et al. (2016) reported on the successful cultivation of hNoV in human intestinal enteroids. However, until the cultivation method is widely available and part of routine hNoV research, cultivable surrogate viruses will continue to be used to understand and predict the physicochemical properties, interactions, infectivity, and pathogenesis of hNoV. The most common hNoV surrogates include other caliciviruses such as feline calicivirus strain F9 (FCV), murine norovirus type 1 (MNV), and Tulane virus (TuV) (Cromeans et al., 2014; Li et al., 2012). Additional less commonly used surrogates include porcine enteric calicivirus, or sapovirus (PSaV; Cowden strain), Aichi virus A (AiV), and Hepatitis A virus (HAV) with AiV and HAV human pathogens in their own right (Bozkurt et al., 2014; Cromeans et al., 2014; D'Souza et al., 2016). With respect to structural similarities of surrogates with hNoV, FCV is in the same family but differs from hNoV in some biochemical properties and is a feline respiratory virus as opposed to enteric. Meanwhile, MNV is more similar to hNoV in that it is a member of the *Norovirus* genus, but its symptoms of infection present differently in mice, and it recognizes sialic acid as their functional receptor as opposed to HBGAs (Karst et al., 2014). On the other hand, TuV does recognize HBGA receptors in rhesus macaques similar to hNoV recognition of HBGAs in humans for the majority of genotypes (Farkas et al., 2010). Similar to hNoV structure,

surrogate virus properties are likely to impact the type of interactions observed in studies with leafy greens as outlined below in Sections III-iii and III-iv of Chapter 1.

iii. Attachment of hNoV (or surrogates) to leafy greens

Although hNoV is not a plant pathogen, it can contaminate the phylloplane of leafy greens. As shown with other microorganisms, the leaf surface structure and morphology of leafy greens can impact the distribution and persistence of viruses. Using immunofluorescence analysis, hNoV virus-like particles (VLPs) preferably distributed around cut edges, stomata, and minor veins of lettuce leaf surface (Esseili et al., 2012a). Hirneisen and Kniel (2013) found that the rougher and more irregular spinach leaf surfaces allowed hNoV to persist longer under thermal conditions. However, it is unclear how the viruses actually interact with the leaf surfaces. It is hypothesized that the attachment of hNoV to leafy green surfaces can be achieved in various ways, including specific binding, non-specific binding, and internalization as outlined in Table 3.

a *Specific binding*

Upon entry into the human body, hNoV needs to attach to the host cell in order to cause infection. As mentioned previously, the attachment of the majority of hNoV genotypes relies on the specific recognition of HBGAs present on the membranes of cells that line the body's mucosal layers (Huang et al., 2005). The binding specificities among hNoV genotypes rely on the recognition of different carbohydrate moieties on HBGAs (Hirneisen and Kniel, 2013). In addition to HBGAs, hNoV has been found to bind with other receptors depending on the genotype (Chapter I, Section III-i). Tian et al. (2005) demonstrated that recombinant norovirus-like particles (rNVLP) bound to porcine gastric mucin (PGM) through the recognition of sugar moieties. Han et al. (2014) revealed that GI.3 and GII.4 VLPs were able to bind to the

oligosaccharide of ganglioside. In addition, Rydell et al. (2008) found α -2,3-sialylated carbohydrates as another binding site for select hNoV GII strains. Based on hNoV attachment to host cells, researchers became interested in the attachment of hNoV to food matrices—an important vehicle of transmission as detailed in Chapter 1 Section I-ii. To investigate the interaction of hNoV with common food commodities implicated in hNoV outbreaks, numerous studies have been conducted with lettuces (Hirneisen and Kniel, 2013).

Most studies on hNoV binding to lettuce focus on GII.4 strains, which are known to bind to the widest variety of HBGAs (Huang et al., 2005). Esseili et al. (2012a) reported on the specific binding of GII.4 hNoV VLPs to lettuce cell wall materials (CWM), especially various carbohydrate moieties presence on the cell wall. Binding of hNoV VLPs to young leaf (2-6 cm) and old leaf (20-25 cm) CWM were quantified and compared with an enzyme-linked immunosorbent assay (ELISA) method. It was revealed that binding of hNoV VLPs to old lettuce leaf CWM was significantly higher than that for young leaves. This can likely be attributed to the different sugar concentrations and composition between old and young leaves (Esseili et al., 2012a). Later, Gao et al. (2016) further revealed that GII.4 hNoV VLPs specifically recognize and bind to α -1,2-fucose moiety of HBGA-like carbohydrates on lettuce leaves. The authors also identified the presence of HBGA-like carbohydrates in the hemicellulose fraction of the cell wall. Cellulose R-10 digestion pre-treatment can increase binding efficiency of hNoV VLPs since the HBGA-like binding sites are not directly exposed but rather under the surface of leaves (Gao et al., 2016).

In addition to leafy greens, the binding of GII.4 hNoV VLPs to other types of fresh produce was also tested (Gao et al., 2016). After digestion by the cell wall degrading enzyme R-10, GII.4 hNoV VLPs were able to bind to celery veins while not to basil leaves. This indicates that hNoV

specific binding can occur in a variety of fresh produce (Gao et al., 2016). However, in a study by Gandhi et al. (2010), the authors did not find any HBGA-like carbohydrates in romaine lettuce. The authors stated that hNoV GI.1 VLPs bind instead to unknown proteinaceous compounds found on lettuce surfaces. In contrast, (Esseili et al., 2012a) indicated that hNoV GII.4 VLPs bind weakly or non-specifically to cell wall proteins of lettuce leaves. The distinction could be an indication of the differences in binding specificity between hNoV GI and GII.

Previous studies have also investigated the localization of hNoV surrogates including MNV and TuV. Similar to distribution patterns of hNoV VLPs, hNoV surrogates TuV and MNV were also found to aggregate around lettuce stomata (DiCaprio et al., 2015b; Esseili et al., 2016). DiCaprio et al. (2015b) also observed a variation in affinities between TuV and MNV during attachment to romaine lettuce leaves possibly due to differences in receptor binding. As stated previously, only TuV mimic the majority of hNoV that specifically recognize HBGAs while MNV bind to sialic acids on glycoproteins (Esseili et al., 2016; Taube et al., 2009). Therefore, whether or not these distribution patterns of hNoV surrogates are related to specific binding or presence of viral cellular receptor analogs over the other on leafy greens is difficult to determine.

b Non-specific binding

Vega et al. (2008) conducted studies on the attachment of viruses to butterhead lettuce and subsequently revealed the major role of electrostatic forces in this interaction. The authors tested four viruses: echovirus 11, FCV, MS2, and ϕ X174. Then NaCl was used to reduce or eliminate the electrostatic forces. The inhibitory effect of 1M NaCl varied depending on type of viruses and pH conditions. At pH 7 and 8, 1 M NaCl blocked all viral attachment except ϕ X174. The

authors hypothesized that the strong absorption at pH 5 was due to Van der Waals forces (Vega et al., 2008). Wang et al. (2012) also reported that at pH 5 (capsid isoelectric point for PSaV), the binding of PSaV to lettuce leaves was most significant, and its infectivity remained after 1 week at 4°C.

c Internalization

Viral internalization can occur during both soil production and hydroponic production of fresh produce. DiCaprio et al. (2012a) cultured romaine lettuce in hydroponic feed water with around 10^6 RNA copies/mL of hNoV or 10^6 PFU/mL of TuV and MNV (strain type 1). High levels of viral-genome RNA were detected for hNoV (10^5 to 10^6 RNA copies/g) at day 1 while it took 3-7 days for TuV and MNV to reach a level of 10^5 to 10^6 PFU/g. After reaching some maximum level, the hNoV and surrogate concentrations remained stable for 14 days in lettuce tissue (DiCaprio et al., 2012a). Similar studies were carried out in kale and mustard microgreens, using both plaque assay and real-time reverse transcription PCR (RT-qPCR), (Wang and Kniel, 2015). The plaque assay results indicated that MNV remained stable (2.5-1.5 log PFU/sample) during the first 12 hours and then decreased from day 8 to 12. However, RT-qPCR results indicated relatively higher levels of MNV (4.5-5.5 copies/sample) which also maintained stability (Wang and Kniel, 2015). Besides root uptake, the internalization could also happen through cut lettuce leaves or the stomata of lettuce. Wei et al. (2010) compared internalization of intact lettuce to those with cuts on leaf and stem; although more MNV was observed under confocal microscopy, the difference was not statistically different. Nevertheless, once internalized, viruses cannot be easily removed compared to those existing on the surface of fresh produce.

In addition, DiCaprio et al. (2015a) studied the effect of biotic stress (infection with lettuce mosaic virus) and abiotic stresses (drought and flood) on the internalization and dissemination of hNoV surrogates in soil grown romaine lettuce. The results indicate that only abiotic stress alters rates of internalization for TuV and MNV. More specifically, drought stress significantly decreased the rate of internalization and dissemination for MNV and TuV but not flooding stress. The authors explained that drought stress may have led to more hNoV surrogates binding to the soil matrix due to an increased presence of cations, thus rendering the virus unavailable for passive uptake through the roots. Another possibility introduced by the authors was virus inactivation due to osmotic stress in an increase in reactive oxygen species in the plant.

iv. The effect of leafy green (surface) metabolites on hNoV or its surrogates

The lettuce leaf contains a vast number of metabolites that are water-soluble (carbohydrates, polyols, organic acids, and amino acids) or soluble in organic solvents (e.g. sterols, fatty acids, diacylglycerophospholipids, etc.), (Sobolev et al., 2005). As lettuce grows and matures, the energetic compounds in leaves decrease. Pereira et al. (2014) observed metabolite changes in lettuce leaves under four main categories during leaf maturation. Most amino acids (6/7), organic acids (2/2), carbohydrates (2/3), and other compounds (7/8) showed a decrease in concentration with leaf maturation. These details may be of important as those metabolites on lettuce surfaces could play roles during hNoV binding and survival.

a Metabolic compounds that impact hNoV

The metabolomes of leafy green surfaces can negatively impact attachment or survival of viruses. According to a study by Lamhoujeb et al. (2008), HAV exposure to potentially toxic compounds (e.g. phenolics, ethanol, and acetaldehyde) on lettuce surfaces accelerated virus inactivation. Additional studies have focused on the inactivation of hNoV and its surrogates

when exposed to natural plant compounds (Li et al., 2012; Su and D'Souza, 2013, 2011). Several phytochemicals extracted from fruits have been shown to have anti-hNoV activity, such as flavonoids from grape seeds, polyphenols and anthocyanidin in the berry secondary metabolite catechin, and polymeric tannins from persimmons (Ryu et al., 2015). Polyphenol and flavonoids both exist in lettuce, though the concentrations vary between different lettuce species. Llorach et al. (2008) characterized the concentration of polyphenols and Vitamin A in five types of lettuce including iceberg, romaine, continental, red oak leaf, and lollo rosso. The highest level of phenolic compounds and Vitamin A was detected in red-leaf and continental varieties, respectively. Interestingly, Lee and Ko (2016) observed that Vitamin A was able to inhibit MNV replication during *in vitro* and *in vivo* experiments.

There are also compounds that may enhance the binding of hNoV. Binding of hNoVs specifically to lettuce leaf surfaces relies on the carbohydrates exposed on the cell walls (Esseili et al., 2012a). For surrogates, it is known that MNV attachment requires sialic acid, glycolipids, and glycoproteins (Ryu et al., 2015). Aside from this, very little information is known with regards to the potentially beneficial impact of metabolic compounds on hNoV.

V. Potential interactions between hNoV and microorganisms that colonize leafy greens

i. Bacteria identified on leafy green surfaces

As mentioned in Chapter 1 Section II-i, the bacterial community has the largest population among all microbial groups inhabiting leafy green surfaces (Leff and Fierer, 2013). The major groups of bacteria found on lettuce were very similar according to several reports summarized in Table 2. These bacteria can interact with plants, within the bacterial community on the plants, with fungi and protozoa that inhabit the plants, and possibly with viral inhabitants as well. There

are very few reports about the potential interactions between hNoV and bacteria on leafy greens. However, it has been reported that bacteria can interact and associate with hNoV in other environments or *in vitro* (Almand et al., 2017; Li et al., 2015; Miura et al., 2013).

ii. Bacteria influence hNoV binding

After hNoV was found to specifically bind to HBGAs expressed on intestinal epithelium, it was further discovered that some bacteria also express HBGA-like binding sites. Miura et al. (2013) reported an enteric bacteria *Enterobacter* sp. SENG-6 bears HBGA-like binding sites on their EPS. Li et al. (2015) also discovered that hNoV VLPs from GI.1 and GII.4 bound to HBGA expressing *E. coli* LMG8223 and *E. coli* LFMFP861 though the HBGA epitopes may not be the same as those present on human red cells. The authors also demonstrated that pre-incubation with HBGA expressing *E. coli* protected the antigen integrity and mucin-binding ability of both hNoV GI.1 and GII.4 VLPs under heat treatment at 90°C for 2 min (Li et al., 2015). In addition, it was reported that the amount of epitopes for GII.4 were significantly increased for HBGA expressing *E. coli* when detected after heating, revealing that heat treatment potentially helped to unmask epitopes (Li et al., 2015). Another study evaluated the binding of hNoV GII.4, GI.6, surrogate TuV and Turnip Crinkle Virus (TCV) to several representative gut microbiota (*Klebsiella* spp., *Citrobacter* spp., *Bacillus* spp., *Enterococcus faecium*, and *Hafnia alvei*) and reference strains (*Staphylococcus aureus* and *E. cloacae*). While hNoV GI.6 and GII.4 bound to all tested bacteria with more than 90% binding efficiency, TuV only bound selectively to five bacteria and no bindings occurred for TCV (Almand et al., 2017). Although it is not clear the mechanisms of binding of hNoV, the authors hypothesized that the HBGA-like antigens on bacteria played the role since TuV—genetically close in relation to hNoV—also bound to

bacteria, though more selectively, while TCV—not related to hNoV—did not. Of additional importance, some of these bacteria (i.e. *Bacillus* spp., *Enterobacter* spp.) are also relevant to and present in the leafy green phyllosphere (Jackson et al., 2015, 2013).

Conversely, some studies have focused on the antiviral effect of bacteria against hNoV surrogates. Shearer et al. (2014) tested antiviral effects of the metabolic products from a range of bacteria (*Enterococcus faecalis*, *Pseudomonas fluorescens*, *E. coli*, *S. epidermidis*, *B. subtilis*, *B. coagulans*, *Clostridium sporogenes*) as well as a commercial probiotic mixture (*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Lactobacillus salivarius*, and *Streptococcus thermophiles*) against MNV and TuV; however, no inhibitory effect was found during virus infectivity assays.

iii. Impact of fungi and protozoa on the interaction of lettuce and hNoV

Some fungi can favor hNoV binding to leafy greens indirectly. For instance, a fungal habitant *Trichoderma viride* on lettuce leaves is able to produce a multi-enzymatic system called R-10, which has cellulase, pectinase, and hemicellulase activities. As reported previously by Gao et al. (2016), R-10 can digest lettuce cell wall structure and exposing HBGA-like carbohydrates, leading to significantly increased binding of hNoV GII.4 VLPs to lettuce leaves. In addition, some leafy greens can be contaminated by the mold *Aspergillus flavus* leading to subsequent decay. During the decay process, more HBGA-like binding sites on lettuce leaves can be exposed. In contrast, bacteria and fungi on plant surfaces could secrete enzymes that depolymerize the main structural polysaccharide or decipher the carbohydrate structure on plant cell wall (Gao et al., 2016).

In regards to protozoa, the FLP *Acanthamoeba* sp. can be found in similar environments (i.e. water and fresh produce) as hNoV. The study by (Hsueh and Gibson, 2015) revealed that hNoV surrogate MNV could attach to *A. castellanii* and *A. polyphaga* and be internalized into the trophozoites and survive a complete life cycle (i.e. encystment through excystment), while another surrogate, FCV, could not. However, although the authors speculated about the exact interaction, neither the binding type nor specific binding site and the internalization mechanisms for MNV were confirmed.

VI. Future research directions

Current research on hNoV and leafy green interactions has mainly focused on the various mechanisms of attachment of hNoV to lettuce. However, the interaction between hNoV and leafy greens is such a complex process that many other factors could be involved aside from simple attachment. Due to the difficulty of culturing hNoV *in vitro*, research must involve surrogates to gain a better understanding of hNoVs. Therefore, to what extent the particular surrogate mimics hNoV will affect the research outcomes. A previous study compared the performance of hNoV and surrogate MNV during the attachment to both inert and food surfaces (lettuce leaves, strawberry and raspberry). It was found that hNoV and MNV attachments were comparable only on inert and lettuce surfaces, but not strawberries and raspberries (Deboosere et al., 2012). However, lettuce and inert surfaces clearly have different properties with lettuce surfaces presenting a much more complex environment—a living environment. As indicated, leafy green surfaces are habitats for a vast number of diverse microbes. The viral binding and dwelling on leafy greens is not only dependent on an exposed binding site but could also be affected by the microbial community that inhabit the surface. More thorough understanding is

needed on the relationship between the bacterial population and viral binding properties. It is known that bacteria can modify leafy green surfaces through aggregation and production of EPS to aid in survival on the leafy green phyllospheres during osmotic stress, oxidative stress, etc. (Lindow and Brandl, 2003). However, it is unclear so far if the EPS also protects viral inhabitants and potentially provide binding sites for hNoV (Miura et al., 2013). Additionally, some bacteria and fungi are able to produce carbohydrate degrading enzymes which unveil the binding sites for hNoV (Gao et al., 2016). Thus, characterization of the enzymes produced on leafy green surfaces would also be worthwhile.

The metabolic compounds on leafy green surfaces might also play an important role in hNoV binding and persistence. One study reported that it took only 4 days for infectious MNV to be reduced by 1-log on lettuce surfaces whereas it took 29 days in water, 15 days on stainless steel, and 12 days in soil to get the same reductions (Fallahi and Mattison, 2011). This possibly indicates that some anti-viral metabolites, or other compounds, exist on lettuce leaves. In addition, it was reported that the latex sap of lettuce leaves was able to damage the capsid of PSaV while not destroying the RNA. Latex sap, located in the continuous tube of lettuce leaves, is formed by leaf secretions and secondary metabolites (Esseili et al., 2012c). Although minimal, these studies reveal some possible interactions between hNoV and leafy greens metabolites. Therefore, future studies are needed to discover these functional compounds with potential anti-hNoV effects.

VII. Conclusions

In summary, the interaction of hNoV and leafy greens is an under developed area of research that warrants future investigations based on the evidence presented in this review. Given the status of

hNoV and leafy greens as an important pathogen-commodity pair responsible for numerous outbreaks each year, further elucidating the interactions between hNoV and leafy greens will move forward attempts to design effective control and prevention strategies, understand viral infectivity, and ameliorate detection methods.

The surfaces of leafy greens are colonized by bacteria, fungi, protozoa, and other microbial inhabitants, that utilize the nutrients from plants for growth and survival. Plants have their physical barriers and immune systems, while bacteria use different strategies to replicate. The microbial inhabitants interacting with leafy greens have been described from the standpoint of both human and plant pathogens. Overall, human pathogens only trigger a low level of plant immune response. Meanwhile, their replications are very limited as they are unable to infect plant cells. Therefore, interactions between human pathogen and leafy greens are relatively simple.

In this review, the interactions of hNoV with leafy greens were categorized into three types: specific binding, non-specific binding (i.e. electrostatic force), and internalization. Specific binding is stronger than non-specific interactions, and their specificity will vary depending on the hNoV genotype. The well-characterized GII.4 hNoV recognizes and binds to HBGA-like antigens on the lettuce surface. However due to the strict specificity, the specific binding can be interrupted once the binding sites are damaged through some force or compound such as carbohydrate enzymes. In addition to providing binding sites, leafy green surfaces also contain a variety of metabolic compounds such as polyphenols and flavonoids. Some anti-viral compounds might damage or even inactivate hNoV. So far there are few studies characterizing the impact of lettuce metabolites on hNoV survival.

Though the effect of most microbial communities on hNoV remains unclear, some bacteria with HBGA-like antigens could provide extra binding sites for hNoV and thus allow for some form of protection. Also, certain enzymes (e.g. R-10) produced by bacteria and fungi help to expose more binding sites on leafy green surface, which indirectly assist hNoV interactions with the leafy green surface. However, some enzymes could also damage the binding sites by decomposing carbohydrates. Overall, the vast diversity of bacterial and fungal communities on leafy green surfaces could be either beneficial or challenging to hNoV persistence and survival.

VIII. References

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IX. Tables

Table 2. The bacterial community identified on leafy greens.

Leafy Green Type	Location	Microorganism	Method	Proportion	Reference
Romaine lettuce	Leaf surface	<i>Pseudomonas</i> (17%), <i>Bacillus</i> (7%), <i>Massilia</i> (5%), <i>Xanthomonas</i> (4%), <i>Arthrobacter</i> (1%), <i>Pantoea</i> (6%)	Washing of leaf samples (n = 106)	Percentage of microorganisms in all tested samples.	(Rastogi et al., 2012)
Romaine lettuce, baby spinach, green leaf lettuce, iceberg lettuce, red leaf lettuce	Leaf surface	<i>Pseudomonas</i> , <i>Pantoea</i> , <i>Chryseobacterium</i> , and <i>Flavobacterium</i>	Sterile or unsterile samples were placed in bottle and shake at 200 rpm; culture isolate	Exist in more than 20% of samples	(Jackson et al., 2013)
45 Lettuce (<i>Lactuca sativa</i>)	Leaf surface	<i>Xanthomonas</i> sp., <i>Pantoea</i> sp. (Enterobacteriaceae), <i>Pectobacterium</i> sp., <i>Leuconostoc</i> sp., <i>Janthinobacterium</i> sp.	Swabbing conventional and organic lettuce	Large proportions (>5% of bacterial community on lettuce).	(Leff and Fierer, 2013)
Spinach	Leaf surface	<i>Pantoea</i> sp. (Enterobacteriaceae), <i>Klebsiella/Raoultella</i> sp.	Sterile water rinse of conventional and organic spinach	Large proportions (>5% of bacterial community on spinach).	(Leff and Fierer, 2013)
Lettuce	Leaf tissue	<i>Pseudomonas</i> (53%) <i>Acinetobacter</i> (10%) <i>Alkanindiges</i> (5%), <i>Pantoea</i> (4%)	5 g lettuce leaf tissue were broken down and re-suspended in 10 mL of 0.85% NaCl.	Percentage of microorganisms in all tested samples.	(Erlacher et al., 2014)
Lettuce (<i>L. sativa</i>)	Leaf surface	<i>Pseudomonas</i> (30%), <i>Arthrobacter</i> (12%), <i>Pantoea</i> (10%), <i>Acinetobacter</i> (8%)	Shaking at 100 rpm in 0.15M NaCl, 0.1% Tween 20 solution at room temperature for 15 min.	Percentage of microorganisms in all tested samples.	(Dees et al., 2015)

Table 3. Currently known interactions of hNoV or its surrogates with leafy greens as well as colonizing microorganisms on leafy green surface.

Interaction type	hNoV or surrogates	Interact with	Details	Reference
Non-specific binding	echovirus 11, FCV, MS2, and ϕ X174	Butterhead lettuce	The nonspecific binding is mainly by electrostatic forces	(Vega et al., 2008)
Specific binding	hNoV GI.1 VLPs	Lettuce surface	hNoV GI.1 VLPs bind to unknown proteinaceous compounds on lettuce surface	(Gandhi et al., 2010)
Internalization	hNoV GII.4, TuV, MNV	Romaine lettuce	During hydroponic cultivation, the romaine lettuce took 1-7 days to reach the similar levels of virus concentrations as that in feed water.	(DiCaprio et al., 2012a)
Indirectly help binding	MNV	Protozoa on leafy green	<i>Acanthamoeba</i> sp. can be found on fresh produce. MNV was reported to be internalized into the trophozoites of the protozoa.	(T.-Y. Hsueh and Gibson, 2015)
Internalization	MNV	Kale and mustard greens	After 2 hours inoculation, MNV was detectable in edible tissue and root of both fresh produces, which are cultivated on hydroponic pad.	(Wang and Kniel, 2015)
Specific binding	hNoV GII.4 VLPs	Lettuce cell wall	GII.4 hNoV VLPs specifically bind to HBGA-like carbohydrates on lettuce leaves	(Gao et al., 2016)
Indirectly increase viral binding	hNoV	Fungi on leafy green	The fungi <i>Trichoderma viride</i> on lettuce leaves produce enzymes that digest cell wall structure; the mold <i>Aspergillus flavus</i> cause decay on lettuce. These help to expose HBGA-like carbohydrates on lettuce.	(Gao et al., 2016)

FCV = feline calicivirus; hNoV = human norovirus; MNV = murine norovirus; TuV = Tulane virus; VLP = virus like particle

Chapter 2: Virus Persistence on Pre-harvest Hydroponic Lettuce Leaf Surface

I. Abstract

Human norovirus (hNoV) is one of the major causes of outbreaks linked to leafy greens. This study aimed to investigate the persistence of Tulane virus (TV), a hNoV surrogate, on pre-harvest hydroponically grown lettuce leaf. TV were characterized for virus survival on adaxial surface of 40 days age oakleaf lettuce grown hydroponically. On day 40, TV were inoculated on one random leaf for each of five lettuce heads. On post-inoculation day (PID) 0, 1, 2, 3, and 4, TV were recovered from leaves and quantified immediately by viral plaque assay. Tulane virus were found to survive throughout all four days. Virus reduction on PID 2 was highest (average 2.19 log PFU/leaf) and on PID 3 and 4 virus concentration only decreased by 0.14 and 0.6 log PFU/leaf, respectively. This study showed that virus contamination that happens close to harvest day might sustain infectious virus through post-harvest or even consumption. The understanding of virus persistence on pre-harvest leafy greens will help to characterize the virus transmission route as well as to develop specific control strategies.

II. Introduction

Human noroviruses are a group of enteric viruses that lead to epidemic and sporadic gastroenteritis worldwide (Ramani et al., 2016). The most common food vehicles for hNoV transmission include leafy greens, berries, and seafoods (Bozkurt et al., 2021). In recent decades, the consumption of fresh produce has increased remarkably in order to obtain a healthier and balanced diet (Chatziprodromidou et al., 2018; Machado-Moreira et al., 2019). Since often consumed raw or with minimal processing, there is an increased risk of foodborne illnesses in consumers. In industrialized countries, the top three foodborne pathogens leading to fresh produce-related outbreaks include hNoV (42.4%), *Salmonella enterica* (19.9%) and

Staphylococcus aureus (7.9%) in 2010 to 2015 (Li et al., 2018). An investigation by Herman et al. (2015) found the food-etiology pair of lettuce and hNoV accounted for 25% of leafy green caused outbreaks (n=97) in the US between 1973-2012, only behind the lettuce and Shiga toxin-producing *Escherichia coli* (30%) food-etiology pair. Contamination of lettuce due to hNoV has been increasingly reported in recent years (Müller et al., 2016). Moreover, due to globalization of the food system and different hNoV transmission modes, many international outbreaks are difficult to investigate (Verhoef et al., 2011). For instance, in April of 2016, there were 23 separate point-source gastroenteritis outbreaks reported in Denmark within one week. In total, 1,497 persons were exposed when dining in café, company, high school, nursing home, restaurant, or catering located in different cities. Later a national investigation found that the source of the outbreak was hNoV genogroup I contaminated green coral lettuce imported from France (Müller et al., 2016).

Because of the significant economic and health burdens caused by hNoV, it is crucial to understand virus attachment and persistence in the leafy green production system. On farm, enteric viruses can be transmitted to leafy greens through irrigation water, virus-shedding farm workers, packaging, and food handlers (Stals et al., 2015). Human norovirus and its surrogates previously have been characterized for internalization into leafy greens from hydroponic nutrient solution while virus persistence on hydroponic lettuce remains unclear (DiCaprio et al., 2012). This study focused on the understanding of virus persistence on lettuce leaves that are close to harvest date using the TV surrogate.

III. Material and method

i. Mammalian cell growth and virus production

Monkey kidney cells LLC-MK2 (ATCC CCL-7; American Type Culture Collection, Manassas, VA) were cultured in M199 medium (Corning, VA, USA) containing 10% Fetal Bovine Serum (FBS, Cytiva, MA, USA), 1% Penicillin-Streptomycin (100 U/mL, 100 µg/mL; Cytiva) and 1% Amphotericin B (Corning) supplementation. The incubation of cells was at 37°C, 5% CO₂ and cells were split when they reached 100% confluency. Tulane virus was kindly provided by Dr. Jason Jiang from Cincinnati Children's Hospital Medical Center (Cincinnati, OH). Virus propagation and titration were carried out as described previously with minor modifications (Arthur and Gibson, 2015). Briefly, viruses were produced by infecting MK2 cells in T175 flasks at multiplicity of infection (MOI) of 0.1 and rocking at 37°C, 5% CO₂ for 1h. Afterwards, maintenance medium (2% FBS supplemented Opti-MEM) (Gibco Life Technology, Scotland, UK) was added to the flask and further incubated at the same condition without rocking for 48h. Tulane virus was harvested by three freeze-thaw cycles and centrifugation at 3000×g, 4°C for 15 min. The virus supernatant went through 0.45µm cellulose acetate membrane filter (Corning) to remove any remaining cell debris. Harvested viruses were aliquoted and stored at -80°C until use.

For virus quantification, MK2 cells were seeded in 6 well plates at a concentration of 8×10^5 cells/well and incubated overnight. Five hundred microliters of serial diluted virus in maintenance medium were added to each well followed by 1h rocking at 37°C and 5% CO₂. After aspirating viruses, 2mL of 1:1 ratio mixture of 3% low melting agarose and maintenance medium were added in each well to cover the cell monolayer. The plates were further incubated

at 37°C without rocking for 120h. At the end of incubation, the virus plaque forming units (PFU) were visualized by staining with 0.01% neutral red for 1h at 37°C without rocking.

ii. Optimization of viral recovery method

Green oakleaf lettuces purchased from local supermarket were used. One milliliter of TV stock at known concentration (4.53 log PFU/mL) was inoculated to one young leaf by pipetting tiny droplets (approximately 50µL) (Figure 3). Two leaves were inoculated and allowed to air-dry (approximately 2h) in the biosafety cabinet. Following air drying, the two leaves inoculated with TV were added to 10mL of elution buffer (1x MEM supplemented with 2% FBS and 1% Penicillin-Streptomycin). The samples were then recovered by (1) shaking by hand and vortex at maximum speed for 1 min, or (2) placed in beaker and rocked at 200 rpm 4°C for 15min. The eluent was passed through 0.45µm filter, serially diluted and quantified in plaque assay. The recovered virus concentration showed minor difference from the virus stock, indicating that either method was reliable (Figure 5). In the end, the vortex method was chosen for the formal experiment.

iii. Cultivation of hydroponic lettuces

Hydroponic nutrient solution was prepared following the instruction of Hydro-Gro Leafy Green (CropKing, OH, USA) supplemented with Calcium Nitrate (Hi-Yield, TX, USA). The oakleaf lettuce (*Lactuca sativa* var. *crispa*) seeds (Seed Needs LLC, MI, USA) were planted in rockwool cubes (Cropking) that were pre-soaked in nutrient solution for 5 minutes. The rockwool cubes (Grodan, Roermond, NL) were placed on Petri dishes under 6400K growing light (Agrobrite, Hydrofarm, PA, USA) with a photoperiod of 16h at room temperature (21 ± 1 °C) and relative humidity 45-55%. The rockwool cubes were kept moist via daily watering. Once seeds

germinated and the roots developed to about 1 inch long (around 5 to 7 days), the rockwool cubes were moved to a styrofoam raft floating on 20L nutrient solution in a 27L size plastic container (Sterilite, MA, USA) (Figure 1). The outer surface of the container was covered by aluminum foil to prevent light from entering. An air-pump (ActiveAQUA, Hydrofarm) was immersed in the nutrient solution for generation of oxygen. The nutrient solution was monitored and maintained within a pH range of 5.8 to 6.0 and an electrical conductivity (EC) of 1ms/cm. The lettuces were cultivated to 40 days of age for experiment.

iv. Inoculation of virus on lettuce and sampling

At the age of 40 days, five lettuce heads were inoculated with TV. For inoculation, one young leaf from each lettuce head was randomly chosen and labeled with a sticker on the leaf tip. Five hundred microliters of deionized water (DI water) containing approximately 10^6 PFU of TV were inoculated on the adaxial surface of each labeled leaf by evenly pipetting small droplets. The leaves were allowed to air dry for 1 to 2h. On post-inoculation day (PID) 0, 1, 2, 3 and 4 (i.e. plant age 40, 41, 42, 43, and 44 days), one random lettuce head was harvested and the labeled leaf was detached to recover the surface viruses (Figure 2). The leaf was placed in a 50mL centrifuge tube containing 10mL elution buffer (1× MEM supplemented with 2% FBS and 1% Penicillin-Streptomycin). The tubes were vigorously shaken by hand followed by 1 min vortexing to recover the surface viruses. Afterwards, the eluate was filtered through 0.45µm cellulose acetate membrane to remove bacteria and leaf tissue debris. The samples from PID 3 and 4 were then concentrated by ultrafiltration using a molecular weight cut-off (MWCO) 100 kDa centrifugal filter unit (Amicon-15, Millipore Sigma, Germany) spinning at 5000×g for 7 min. The regular or concentrated virus samples were then quantified by plaque assay.

v. Total aerobic bacteria, mold and yeast count on lettuce leaves

To determine the indigenous microorganisms on hydroponic lettuce, lettuce leaves of similar size (around 3g) were sampled and placed in stomacher bags containing 10mL of PBS. The surface microbes were recovered by hand massaging without breaking the leaf for 1 min. The surface and internalized microorganisms were recovered by smashing the stomacher bag with hammer followed with stomaching at 230 rpm for 1 min. The samples were spread plated on Tryptic soy agar (TSA) plates and incubated at 35°C for 5 days and counted.

vi. Data analysis

The virus recovered and PID were analyzed by one-way ANOVA in RStudio (version 1.4.1106, implementing R version 4.0.4) (<https://www.rstudio.com>). Afterwards, post-hoc analysis Tukey's HSD test was used for paired comparisons. The significance level of 0.05 was used. The average count of two replications for recovered TV against PID were plotted as dot plot.

IV. Results

i. TV persistence on lettuce leaf surface

TV persisted from PID 1 to 4 (Figure 4). Starting from 5.69 log PFU/leaf on PID 0, the virus decreased by over 4 log PFU/mL during the monitored time. The main reduction happened on PID 1 and 2 which reached on average 1.4 and 2.19 log PFU/leaf reduction, respectively. While the reduction on PID 3 and 4 was minor with only 0.15 and 0.6 log PFU/leaf, respectively. Overall, the major virus titer drop was observed on PID 2 with a recovered TV of only 2 log PFU/leaf. The recovered TV on PID 3 and 4 showed a large variation.

ii. Comparison of two recovery methods

The vortex and shaking incubator showed a recovery rate of 71% and 53% respectively. Their difference from TV stock were both less than 0.5 log PFU/mL, so either of them are suitable for virus recovery. In this study, the vortex method was chosen.

iii. Microorganism count on hydroponically grown lettuce

There were no colony forming units (CFU) on plates after 24h incubation, while after 120h the count from leaf surface and smashed leaf were 75 and 25 CFU/leaf respectively. Bacteria, yeast and mold colonies were observed.

V. Discussion

Human norovirus contamination during pre-harvest stage of leafy green production can occur through contaminated seeds, growing media, irrigation water, and production equipment, as well as the cross-contaminations from sewage and farmer (CDC, 2021; Iwu and Okoh, 2019; Riggio et al., 2019). It is concerning if virus contamination on lettuce leaves before harvest will persist to post-harvest stage. As loose-leaf type lettuce, oakleaf lettuce are usually mature and harvested between 45 to 55 days (Loresco et al., 2018). The present study used hydroponically cultured lettuce as a model to investigate virus persistence at the pre-harvest stage (40 to 45 days) under indoor farming conditions. This study showed that the contamination of TV on lettuce leaf surface was able to persist over 4 days. On the last PID (45 days age), there was still on average 1.34 log PFU/leaf virus remaining, which poses a risk to post-harvest and consumption stage.

Previously several studies have been carried out related to virus persistence on lettuce leaf surfaces with a large portion of them focused on post-harvest stage (Allwood et al., 2004; Esseili et al., 2016, 2015; Fallahi and Mattison, 2011). Esseili et al. (2016) studied the survival of

hNoV and hNoV surrogates murine norovirus (MNV), sapovirus (SaV), and TV on abiotic stressed (physical damage, heat or flood) lettuce and spinach leaves at pre-harvest stage. The authors did not observe any significant difference with infectious virus titer on stressed leaves for all tested surrogates until PID 7, and after that the virus titers became undetectable (Esseili et al., 2016). Unlike the infectious viruses for surrogates, the RNA titer for hNoV and surrogates were detected throughout PID 14. It was shown that hNoV was significantly enhanced on the physically damaged lettuce leaves on PID 14. Meanwhile, the RNA titers of MNV and TV were significantly enhanced by three stresses in different extent (Esseili et al., 2016). Nevertheless, the RNA titer is not equal to the detection of infectious viral particles. Thus, the real number of infectious viruses on PID 14 is unknown.

In addition to lettuce, virus persistence on pre-harvest produce leaves was also carried out on 4-week-old basil (Li and Uyttendaele, 2018). In their study, TV and MNV titer were undetectable on PID 3, which was over a 5.5 and 3.3 log PFU/leaf reduction for MNV and TV, respectively (Li and Uyttendaele, 2018). These results are comparable to the present study which observed 3.7 log PFU/leaf reduction of TV on PID 3. While in another study carried out on spinach, Hirneisen and Kniel (2013) reported insignificant different decimal reductions of 2.25 and 2.61 days for TV on smooth and semi-savory spinach adaxial leaves. Overall, the plant type, experiment setup, and plant growth conditions all lead to difficulties for cross-study comparisons.

In this study, two recovery methods—shaking incubator and vortex—were compared (Figure 5). The difference in recovery efficiency was negligible, though the vortex seemed like a more intensive force to the leaf than shaking. Moreover, the vortex method is easier for handling different sizes of leaves by rolling it up to fit the centrifuge tube. For the shaking method, leaves

with smaller size can better fit the beaker bottom. The bacterial counts of lettuce leaves in this study were less than 100 CFU per leaf (~3g) regardless of the leaf surface or whole leaf sample. According to a study on field grown lettuce, the surface bacterial community ranged between 10^5 to 10^6 CFU per gram tissue, and the actual culturable population was estimated at 1-log lower than the number (Rastogi et al., 2012). The huge difference in their counts into the present study indicates that the indoor hydroponically grown lettuces have reduced microbial populations. Moreover, it was reported that the microbial diversity on lab grown lettuce was significantly lower than field grown (Williams and Marco, 2014).

There are some limitations in this study. First, the experiment was carried out with surrogate TV. The cultivation system of hNoV is a long-standing barrier to studying the virus in past decades. Due to the unavailability of an economical and easily manipulated cell culture system, most studies on hNoV are carried out using virus surrogate models (Estes et al., 2019). The virus surrogates are genetically, morphologically, or biochemically similar to hNoV (Feng et al., 2011). The most commonly used hNoV surrogates include TV, MNV, feline calicivirus (FCV) and MS2 bacteriophage (Kamarasu et al., 2018). Despite the similarities shared by surrogates and hNoV, the extrapolation of experimental results to hNoV should be done with caution. Previously, virus persistence on semi-savory spinach whole plant (foliar surface and stem) was carried out for hNoV genogroup II and surrogate MNV and TV (Hirneisen and Kniel, 2013). There were significant differences in survival observed between hNoV and its surrogates, though the surrogates were analyzed for infectious virus by plaque assay, and hNoV RNA were quantified by PCR.

Second, in this study, the lettuce was grown at an ambient indoor temperature which is different from the greenhouse or field conditions such as day and night temperature change,

outdoor humidity fluctuation, UV exposure, etc. In reality, the lower temperature at night may favor virus persistence while the exposure to UV radiation may give the opposite effect. Also, as mentioned previously, the large populations of bacteria on lettuces grown in field or high tunnels might also contribute to a different virus survival pattern (Esseili et al., 2016; Liu et al., 2020; Williams and Marco, 2014). Moreover, the starting concentration of TV in the study was high (average 5.69 log PFU/leaf) while in reality the virus concentration in a farm environment would likely be much lower (Miranda and Schaffner, 2018).

The present study showed that virus contamination at late growth stage persisted though reduced over time on the mature plant. In future research, more virus persistence study on pre-harvest fresh produce should be carried out. Currently, most related studies were for post-harvest stage virus survival and sanitizing as it is closer to the consumption part of ‘farm to fork’ supply chain. However, the prevention or reduction of virus contamination during the production period will also alleviate the burden of post-harvest stage cleaning and disinfection procedures.

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VII. Figures

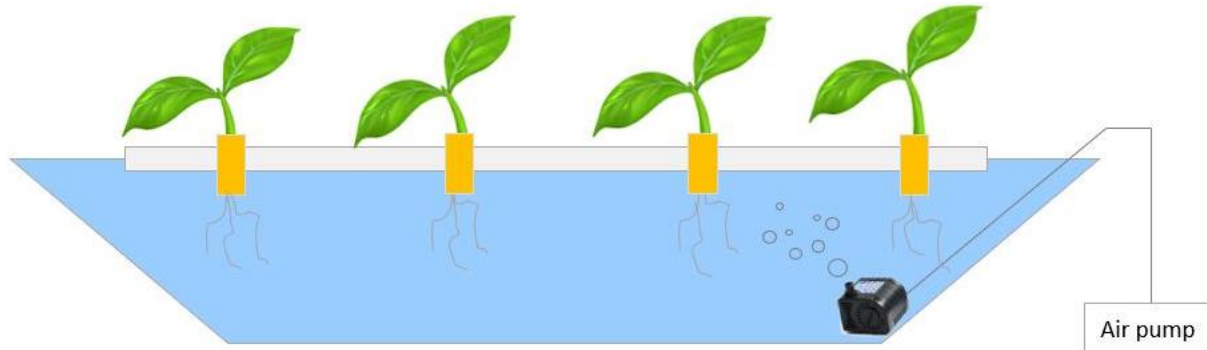


Figure 1. Lab-scale oakleaf lettuce hydroponic growing system. A container filled with 20L of nutrient solution was covered by a styrofoam raft with six holes where each hole held a lettuce head. An air pump was located in the bottom of the container to supply air. The system was maintained at pH 5.8 to 6 and EC 1ms/cm.

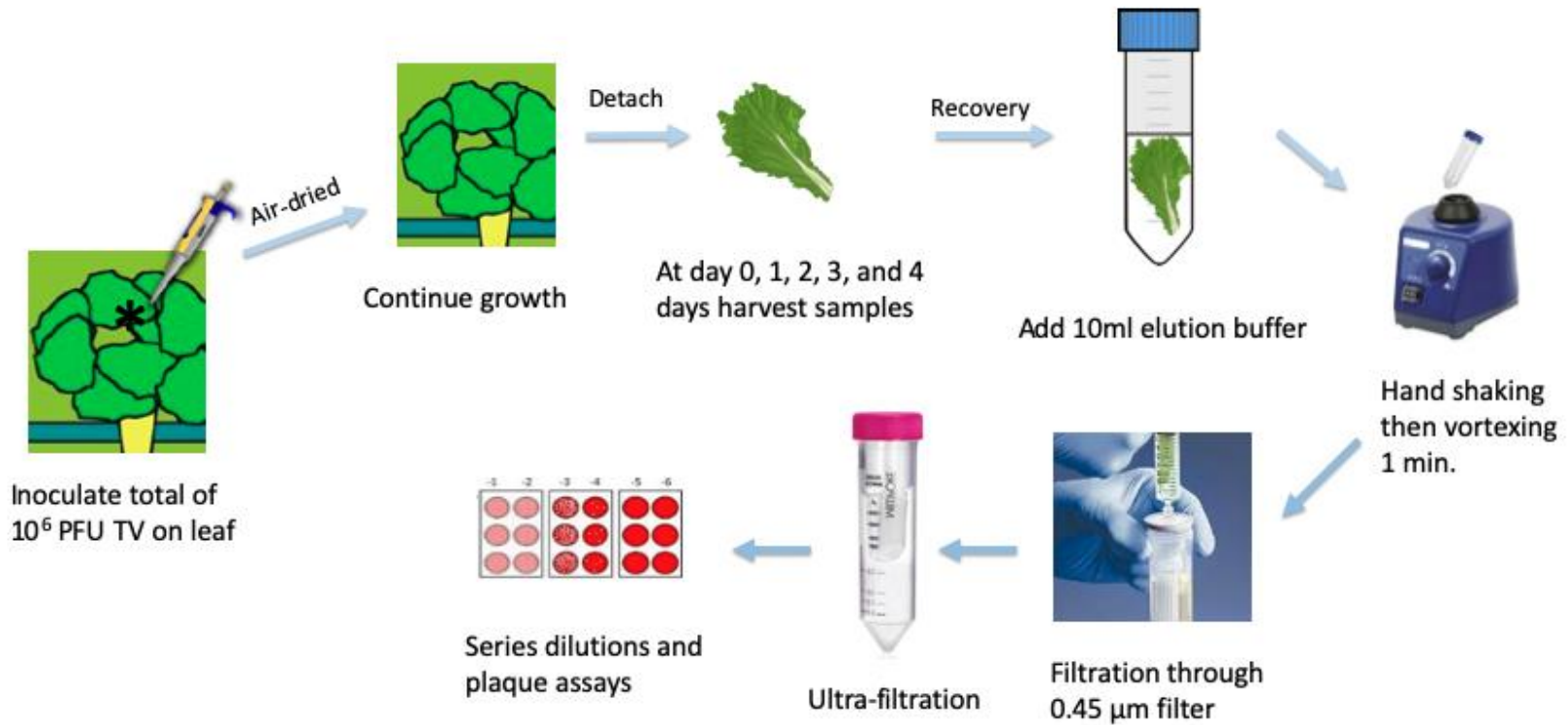


Figure 2. Tulane virus inoculation and recovery on oakleaf lettuce leaves at age of 40 days. The TV were inoculated on one leaf per lettuce head. For each PID, one lettuce head was harvested, and the leaf was recovered for TV following above procedures.

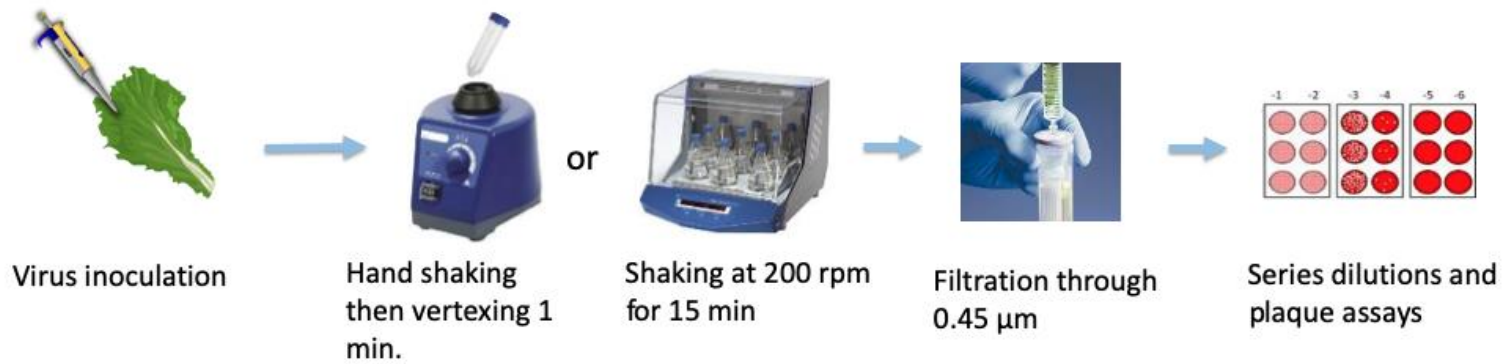


Figure 3. Flow diagram for virus recovery method comparison. The oakleaf lettuces inoculated with TV were either vortexed or subject to shaking to recover viruses from leaves surfaces. Both samples followed the same subsequent steps for virus quantification.

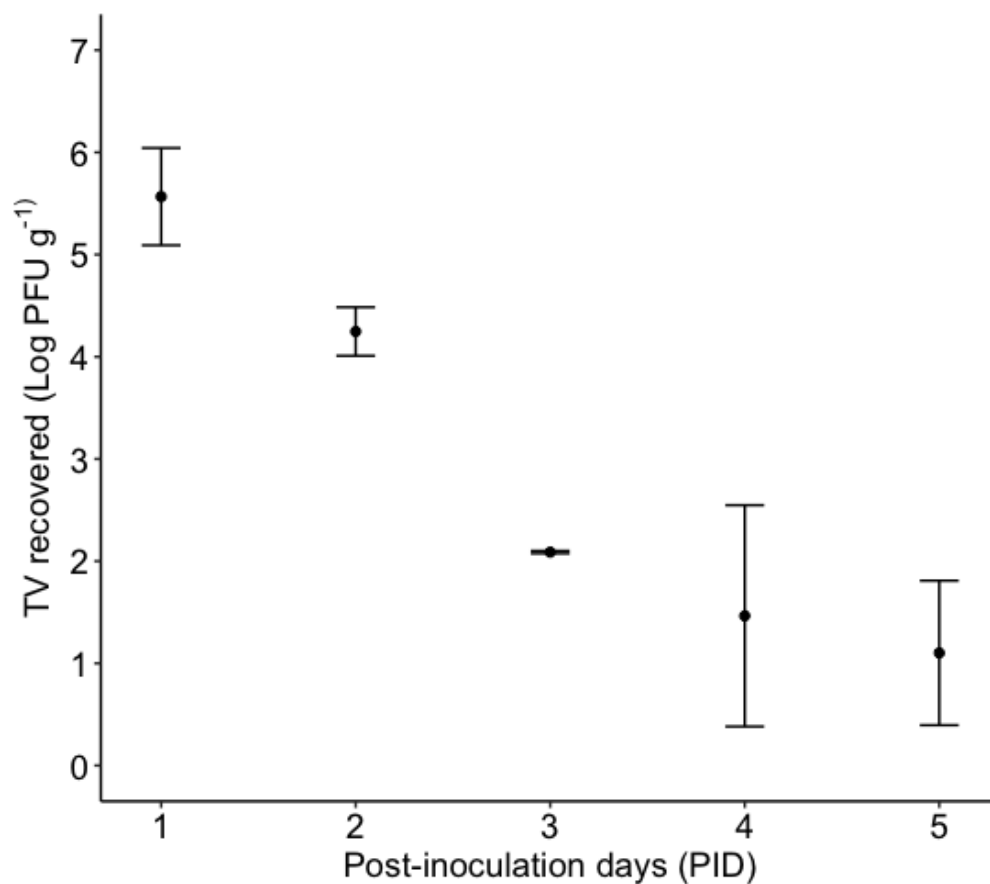


Figure 4. Tulane virus persistence on oakleaf lettuces in continuous five days from plant age of 40 days. The inoculated TV was approximately 6 log PFU/leaf. The dots in graph represent the average of two biological replications. Error bars were plotted on each mean value. The detection limit is 0.3 log PFU/leaf and throughout the experiment every sample was above the detection limit.

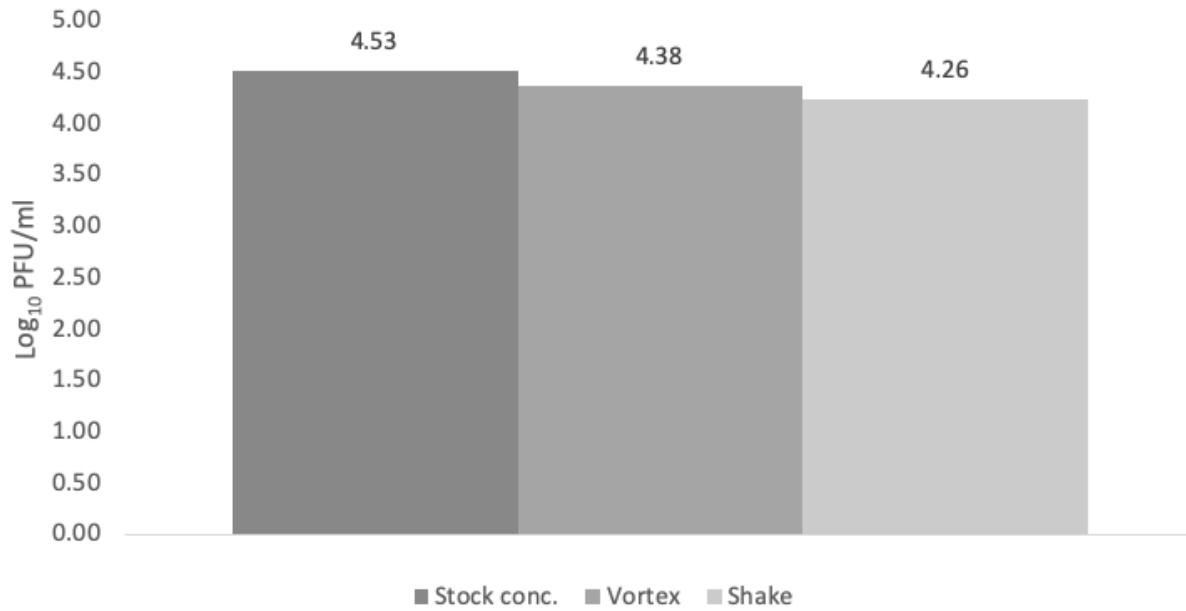


Figure 5. Tulane virus recovered concentrations from vortexing and shaking method. Recovered virus concentrations were converted into log PFU/mL to compare with the original stock concentration.

Chapter 3: Virus Persistence in Plant Growing Medium and Virus Internalization from Medium into Microgreen Plant Tissue

I. Abstract

As a novel salad green, the microgreens market has expanded in recent years due to an increase in popularity amongst consumers. Meanwhile, the lack of standard risk management practices for commercial microgreen cultivation has prompted safety concerns. So far, several studies have evaluated the risks of pathogenic bacteria in microgreens growing systems including *Listeria monocytogenes* and *Salmonella* spp., but there have been few investigations on human pathogenic viruses such as human noroviruses (hNoV). In this study, a hNoV surrogate Tulane virus (TV) was first tested for persistence in two types of soil-free cultivation matrix (SFCM)—biostrate and peat—without plants. On day 0, approximately 7.6 log PFU of TV was mixed with irrigation water and inoculated on biostrate and peat in growing trays. The trays were maintained under a 16-h photoperiod with a growing light and watered daily to mimic the microgreen growing condition. At post-inoculation day (PID) 0, 1, 3, 5, and 10, TV was recovered from SFCM samples and quantified. It was observed that the reduction of TV was on average 2.08 and 1.76 log PFU for biostrate and peat, respectively. No significant difference in persistence of TV was shown between peat and biostrate ($p > 0.05$). For both SFCM, the reduction pattern for TV was gradual over time. Subsequently, the transfer of TV from inoculated SFCM to mature microgreen edible tissue was determined. After inoculation of SFCM with 7.6 log PFU of TV, sunflower (SF) or pea shoot (PS) seeds were planted on half of the area of each SFCM, while the other half was left unplanted and served as a control. On day 10, the mature microgreens were harvested, and SFCM samples were collected from planted and unplanted areas of each tray. No TV was recovered from the edible tissue of either type of microgreen. However, TV was still

present in the SFCM on day 10. Interestingly, the level of TV was significantly lower in the root-containing planted area compared with the unplanted area for both biostrate and peat ($p < 0.05$). The difference between unplanted and planted was on average 1.15 and 0.49 log PFU/g for biostrate and peat, respectively. In this study, it was found that TV was able to survive in SFCM during the complete microgreen cultivation period and possibly beyond. Although the direct transfer to edible tissue was not observed, there is still a risk of cross contamination from SFCM to microgreens during commercial production.

II. Introduction

Microgreens are a novel category of plants produced with vegetable, herb, or cereal seeds. These were initially used in 1996 in San Francisco, California to embellish the cuisine in restaurants (Turner et al., 2020). Microgreens are harvested within 1 to 3 weeks after seed germination, usually when cotyledon have fully developed or the first pair of true leaves has appeared (Teng et al., 2021). The introduction of a diverse variety of microgreens provides more alternatives for healthy diet given their rich contents of phytonutrient and bioactive compounds (Galieni et al., 2020). Compared to seeds and mature plants, microgreens are reported to contain lower antinutrients and are more abundant in polyphenols, minerals (e.g. Ca, K), carotenoids, and vitamins (Paradiso et al., 2018; Renna et al., 2020; Xiao et al., 2012).

Depending on the farm size, microgreens are grown in soil, hydroponics or soil-free alternative substrates under open air, greenhouse, or indoor settings (Kyriacou et al., 2016; Misra and Gibson, 2021). The soil-alternatives include substrates made of natural fibers (agave fiber, coconut fiber, peat moss) or synthetic substitutes (capillary mat and cellulose sponge), or mixes

of peat, bark, perlite, and vermiculite (Kyriacou et al., 2016; Teng et al., 2021). Unlike other fresh produce, research on microbial risks during microgreen production is limited.

Human norovirus is one of the major food pathogens contributed to foodborne outbreaks in fresh produce (CDC, 2021). One of the most prevalent causes of viral contamination in fresh produce production is sewage contaminated irrigation water and growth substrate (Alegbeleye et al., 2018). According to an investigation, one liter of community sewage water contains as many as 5000 enterovirus particles, 7000 cells each of *Salmonella* spp. and *Shigella* spp., and 100 *Vibrio cholerae* cells (Iwu and Okoh, 2019). In addition to water contamination, the transmission of hNoV at the farm level can also occur through farmer workers' hands and contaminated harvesting equipment (Bouwknegt et al., 2015).

So far, microgreen production safety research has been mainly focused on bacterial hazards (Misra and Gibson, 2020; Reed et al., 2018; Wright and Holden, 2018; Xiao et al., 2015). Human enteric virus risks during microgreen production has only been studied within a hydroponic system; however, virus survival in hydroponics is likely not representative of virus survival in solid growth media (Wang and Kniel, 2016). Gioia et al. (2017) previously characterized the microbial population in microgreen growth substrates including a peat-based mix and synthetic mat. The authors found that peat contains significantly higher aerobic bacteria, yeast, mold, and Enterobacteriaceae than the other three types of fiber-based media evaluated in the study. Currently, studies characterizing virus persistence in different types of growing media is lacking. Therefore, the two aims of this study include comparing two types of soil-free cultivation matrix (SFCM)—biostrate and peat—for virus persistence without planting. The biostrate felt mat is designed for microgreen and salad green cultivation while peat is one of the most commonly used cultivation matrices for microgreens (Misra and Gibson, 2021).

Furthermore, virus uptake from contaminated SFCM into microgreen tissue was studied. Here, sunflower and pea shoot were chosen since they are within the top three produced microgreen varieties in the US and have not been characterized in previous studies (Misra and Gibson, 2021). In addition, due to the limitations of the hNoV *in vitro* cultivation system, the surrogate Tulane virus (TV) was used for studying virus persistence and transmission (Bhar and Jones, 2019).

III. Material and method

i. Mammalian cell cultivation, virus production and quantification.

a *Cell cultivation*

The LLC-MK2 cells (ATCC CCL-7; American Type Culture Collection, Manassas, VA) were grown in M199 medium (Cytiva, MA, USA) and supplemented with 10% Fetal Bovine Serum (FBS, Cytiva), 1% Penicillin-Streptomycin (100 U/mL, 100 µg/mL; Cytiva), and 1% amphotericin B (250 µg/mL; Corning, VA, USA) at 37°C, 5% CO₂ condition. Tulane virus was kindly provided by Dr. Jason Jiang from Cincinnati Children's Hospital Medical Center (Cincinnati, OH).

b *Virus production and quantification*

Virus production and plaque assay followed the method described previously (Arthur and Gibson, 2015). Briefly, MK2 cells were infected with TV at a multiplicity of infection (MOI) 0.1. The flask with inoculated MK2 cells was rocked under 37°C, 5% CO₂ for 1h followed by adding 20mL of maintenance medium (2% FBS supplemented Opti-MEM) (Gibco Life Technology, Scotland, UK). The infected cells were incubated for an additional 48h at 37°C, 5% CO₂ without rocking. At the end of incubation, the flask was tap vigorously to detach all cells.

Viruses were harvested by three times freeze-thaw (-80°C and 37°C) to release the viruses from the cells. The lysed cells were pelleted by centrifugation at 3000×g, 4°C for 15 min. The virus supernatant was then filtered through a 0.45µm pore bottle top vacuum filter (Corning).

The day before the plaque assay, MK2 cells were seeded in 6 well plates at a concentration of 8×10^5 cell/well. After overnight incubation, 500µL of TV or sample were added per well with technical duplicates. The plates were rocked for 1h at 37°C and 5% CO₂. Samples were then removed, and cell monolayers were covered with 2mL overlay containing 1.5% low melting agarose and maintenance medium. The plates were further incubated for 5 days at 37°C without rocking. On day 5, the cells were stained with 2mL of 0.01% neutral red diluted in phosphate buffered saline (PBS) (1×, pH 7.4) followed by 1h incubation at 37°C without rocking for visualization of plaque forming units (PFU).

ii. Virus inoculation on SFCM without plants

Soil-free cultivation matrix were prepared before virus inoculation. BioStrate® Felt 185gsm growing mat (biostrate) (Grow-Tech, ME, USA) was cut into 10-inch by 10-inch square pieces (equivalent to approximately 11 g) that fit the bottom of a growing tray (True Leaf Market, UT, USA). Three hundred grams of Canadian *sphagnum* peat and vermiculite mix (peat) (Jiffy-Mix®, Jiffy Growing Solutions, NL) were weighed and added to the growing tray. Tulane virus in total of 4×10^7 PFU was mixed into 200mL and 500mL of sterile deionized (DI) water for inoculation of biostrate mat and peat, respectively. The virus contaminated water was evenly distributed in the biostrate tray by tilting the tray in different directions. The peat and water were mixed uniformly by hands wearing sterile gloves. To mimic the plant growing condition, the trays containing SFCM were placed under growing lights with a 16h photoperiod at room temperature

(21°C) and relative humidity (RH) 50-60%. Also, the biostrate and peat trays received 100mL and 150mL of watering daily, respectively, from day 1 to 10.

On day 0, the biostrate and peat samples were taken immediately after virus inoculation. Biostrate samples of 2×2cm size (approximately 0.1g) were cut off from a random location in the tray by sterile scissors and tweezers. The sample was transferred to a 50mL centrifuge tube containing 5mL of phosphate saline buffer (PBS) (1×, pH 7.4). Approximately 0.5g peat samples were taken by a sterile metal spoon and stored in 50mL centrifuge tube containing 10mL of PBS. Post-inoculation day (PID) 1, 3, 5 and 10 samples were taken following the same procedure as day 0.

Tulane virus was recovered from biostrate samples by vortexing (VWR Analog Vortex Mixer, PA, USA) at maximum speed (3200 rpm) for 1 min. The eluent was then passed through a 0.22µm PVDF filter (Foxy Life Science, NH, USA) syringe filter in order to remove potential bacteria present. Peat samples were vortexed at intermediate speed for 30 seconds then centrifuged (Allegra X-30R Centrifuge, Beckman Coulter, CA, USA) at 800 rpm for 5 min to spin down peat. The supernatant was collected and passed through filter paper (VWR Grade 417, Avantor) and a 1µm nylon filter (Whatman, UK) to further remove the low weight and fine soil particles. Lastly, the eluent was passed through a 0.22µm PVDF filter to remove any bacteria present. Most peat was pelleted after the centrifugation while some lighter particles were floating on top of the supernatant. Thus, the filter paper was used afterwards to separate those light particles. These peat particles left on filter paper were scraped off and transferred back to the original tube containing the centrifuged peat pellet.

The biostrate and peat eluents were serially diluted and titered for TV by plaque assay as described in Chapter 3 Section III-i. The tubes containing biostrate and peat sample were dried

without lid covering in 80°C oven for 48h. Sample dry weights (gram) were recorded. The PFU/g in biostrate and peat was calculated based on the sample dry weight. Furthermore, the total virus per tray was calculated by multiplying the PFU/g with total weight of biostrate or peat in trays. All samples were tested with biological and technical duplicates.

iii. Microgreen cultivation on TV contaminated SFCM

a *Day 0 TV inoculation on SFCM, SFCM sampling, and microgreen sowing*

Sunflower (*Helianthus annuus*) (Tiensvold Farms, NE, USA) and pea shoots (*Pisum sativum*) (Tiensvold Farms) were separately grown on two types of soil-free cultivation matrix (SFCM), biostrate and peat. The preparation of SFCM before planting followed the same procedure as described in Chapter 3 Section III- ii. Two hundred milliliters and 500mL of sterile DI water containing approximately 4×10^7 PFU TV were added to biostrate and peat trays, respectively, to hydrate the cultivation matrices. One sample each of biostrate and peat was taken from each tray before planting and denoted as day 0 sample. The sampling and recovery method of day 0 sample followed the same steps in Chapter 3 Section III- ii.

Organic black oil sunflower (SF) seeds and field pea shoot (PS) seeds were soaked in sterile DI water for 6h before sowing. At the end of soaking, the seeds were drained in sterilized sieves. Approximately 25g of SF or 40g of PS seeds were evenly planted on half of the area of tray while the other half was left unplanted as control (Figure 6). After sowing seeds, the trays were covered with black lids and incubated in the dark for 48h to favor seed germination. During the covered period, the water loss in the trays was minor so trays were only misted 1 to 2 times daily to keep moist. When lids were removed, the growing trays were set on shelves installed with three compact fluorescent lamps (GrowBright 4-foot T5 6400K, HTG supply, PA, USA). The photoperiod was set at 16h. The SFCM were visual checked daily to determine the watering

volume. The biostrate and peat trays were irrigated overhead with approximately 100mL and 150mL, respectively, of water daily. During the sprouting stage the trays were also misted several times per day to help maintain the moisture of roots. The indoor temperature and relative humidity (RH) were maintained within a range of 21 to 23°C and 50 to 60%. Both SF and PS were harvested on day 10.

b Day 10 harvesting of microgreen and SFCM sampling

At day 10, microgreens and SFCM were both sampled, and microgreens were analyzed to determine TV transfer from SFCM to microgreen edible parts. The SF and PS plants were held by sterile tweezers at the top of the stem and were cut at the bottom of the stem (1cm above SFCM) using sterile scissors. For each tray, approximately 5 to 10 plants were sampled, weighed, and stored in stomacher bags. Each sample was weighed and then 5mL of PBS were added to each bag. Microgreen samples were smashed by gently hitting a hammer on the bag to release the virus in plant tissue. The samples were further blended in a stomacher machine (Stomacher 400 Circulator; Seward, UK) for 2 min at 230 rpm. The eluent was transferred to a 15mL tube by serological pipette. To remove the plant debris, the samples were centrifuged at $3000\times g$ for 5 min. The supernatant was transferred to a clean tube and subsequently passed through 1 μ m and 0.22 μ m pore size filters. The plant eluent samples were serially diluted and plated on 6-well plates for plaque assay as described in Chapter 3 Section III-i with biological and technical duplicates.

The SFCM were sampled from the planted and unplanted areas of each tray. In the planted area, the microgreen roots and SFCM were mixed. There were no extra steps to separate roots from the SFCM. The elution of virus from peat samples was similar to day 0 samples. Briefly, 10mL of PBS were added to the 50mL tube containing peat samples and vortexed gently

for 30s. Afterwards, the samples were centrifuged at 800 rpm for 5 min to pellet the heavier components in peat. The supernatant was then passed through a filter paper to remove the light particles. The resulting sample was then passed through 1 μ m and 0.22 μ m pore size filters to remove tiny particles and background microorganisms. The samples were plated to quantify virus as described in Chapter 3 Section III-i.

For biostrate samples, the recovery was slightly different from the day 0 sample recovery. The day 10 samples were collected in 50mL tubes and immersed in 5mL PBS. After vortexing for 1 min, the eluent was centrifuged at 3000 $\times g$ for 5 min to pellet the any bacteria present. The supernatant was then passed through 1 μ m nylon and 0.22 μ m PVDF filters to remove remaining bacteria. Also, 1mL of Penicillin-Streptomycin solution (100 U/mL, 100 μ g/mL; Cytiva) was added to each sample to inactivate bacteria in case any remained following filtration. Samples were serially diluted and plated in duplicate for detection of TV by plaque assay. All peat and biostrate samples were dried in 80°C oven for 48h and weighed for the calculation of per gram concentration of viruses recovered.

iv. Statistical analysis

Virus counts were log transformed for statistical analysis. The virus survival comparison in biostrate and peat were analyzed using one-way ANOVA in RStudio (version 1.4.1106, implementing R version 4.0.4) (<https://www.rstudio.com>). The Tukey's HSD test was applied in the post-hoc analysis to compare the means among different PID. The result in log PFU/tray was reported in dot plot. The viral transfer from SFCM to microgreens experiment was a nested design, and it was analyzed using mixed effect model. The virus counts in SFCM (log PFU/g) were reported as boxplot. A significant difference level of 0.05 was used for all above analysis.

IV. Results

i. TV persistence on SFCM

To compare TV persistence in different kinds of SFCM for microgreen growth, peat and biostrate were studied in the absence of plants (Figure 7). Virus count from each sample was calculated for log PFU/g and then multiplied by the SFCM weight to get a total virus number in the whole tray in order to compare the biostrate and peat. For biostrate, the virus titer on PID 10 was significantly lower than other tested days (i.e., PID 1 to 5) ($p < 0.05$), while no significant differences were observed for the virus counts among PID 0, 1, 3, and 5 (Table 4). For peat, no significant differences in virus titers were detected among all PID samples (Table 5). Throughout the tested time, the total reduction of TV was higher for biostrate than peat (average 2.08 vs. 1.76 log PFU/tray), but the effect of SFCM was not significant ($p = 0.72$).

ii. Virus transfer from day 0 inoculated SFCM to microgreen edible plants

To determine the TV transfer to microgreens, the above ground edible portions, SFCM planted (containing roots), and SFCM unplanted area were all tested on PID 10. No virus was detected at the limit of detection range of 0.32 to 0.74 log PFU/g (this range was based on the microgreen sample weight) in the microgreen edible tissue of SF or PS grown in both types of SFCM. This may indicate that no virus transferred from SFCM to edible tissue during the growing period.

Besides microgreens, the virus concentrations in SFCM were also monitored on PID 0 and 10 (Figure 8). In order to better interpret the data, the TV concentrations in SFCM was not calculated back to PFU/tray. The recovered TV concentration on PID 0 were on average 6.41 and 4.67 log PFU/g for biostrate and peat trays, respectively. Compared to PID 0, the virus titer from all samples on PID 10 decreased in the range of 1.27 to 3.21 log PFU/g, and no sample was below detection limit. When looking at biostrate or peat individually, the virus titers in planted

and unplanted area were compared within each SFCM type. Without considering the microgreen variety, in biostrate the unplanted area contained 1.15 log PFU/g higher of TV than the planted area ($p=0.035$). The unplanted areas were on average 1.07 and 1.46 log PFU/g higher than planted area for SF and PS biostrate trays, respectively. Similar patterns were also observed in peat, where the unplanted area of combined microgreen types was on average 0.49 log PFU/g higher than planted area ($p=0.0081$). For SF and PS peat tray, the unplanted areas were 0.67 and 0.34 log PFU/g than planted areas, respectively.

V. Discussion

Microgreens are perishable leafy greens that are usually consumed with minimal or no processing (Mir et al., 2017; Riggio et al., 2019). Thus, it is critical to understand the foodborne pathogen risks during microgreen production. In recent years, several studies were carried out to investigate the fate of bacterial foodborne pathogens in different microgreen growing conditions including in hydroponics and SFCM (Wright and Holden, 2018; Xiao et al., 2015). However, research on the risks related to contamination of microgreens with human enteric viruses is limited. Only one published study on murine norovirus (MNV) in hydroponic systems has been carried out (Wang and Kniel, 2016). To our knowledge, this is the first study that characterizes virus persistence in microgreen SFCM. Also, the subsequent virus transfer from SFCM to edible tissues of microgreens was first described here.

Without plants presence, TV reductions in biostrate and peat were similar with minimal reduction over the 10-day experimental trials. In a previous study on virus persistence in a hydroponic system for the production of microgreens, Wang and Kniel (2016) reported on the survival of murine norovirus (MNV) in the circulating nutrient solution of hydroponic system

over five days without the presence of microgreen. No significant differences in MNV were observed over the 5-day post inoculation period in the (Wang and Kniel, 2016). Both the present study and the one by Wang and Kniel (2016) indicate that viruses (i.e., TV and MNV) are relatively stable under common microgreen production conditions. This may indicate that virus-specific risk management practices should be development to prevent and control virus contamination within microgreen growing environments, specifically as it relates to soil-free media and nutrient solutions.

When microgreens were cultivated in TV contaminated SFCM, we were not able to detect virus transfer to edible tissue of either microgreen type even though SFCM on PID 10 still contained virus. The virus uptake from media can be plant type or cultivation matrix dependent. A study by Yang et al. (2018) compared TV internalization from hydroponics and soil into pre-harvest green onions, lettuce, and radish. While TV were recovered from all three studied plants grown in hydroponic system, only lettuce cultivated in soil was TV positive. The TV was not detected in any part (i.e., root, shoot, or leaf) of the plant for radish and onions (Yang et al., 2018). Similarly, another study also reported the absence of infectious TV and RNA internalization into bell peppers grown in contaminated soil (DiCaprio et al., 2015). As discussed in the above two studies, a possible reason for the absence of virus internalization into certain plant types was due to the presence of the antiviral compounds in plants. Sunflower seeds were previously found to contain antiviral peptides, and its crude extract effectively reduced the herpes simplex virus (HSV-1) (Oliveira et al., 2009; Rauf et al., 2020). However, the antiviral activity that was observed for HSV-1 can be different for hNoV. Specifically, HSV-1 is an enveloped double-strand DNA virus while hNoV is a non-enveloped single-strand RNA virus

(CDC, 2021; Gavanji et al., 2015). Nevertheless, the specific reasons for the lack of detection of TV in edible microgreen tissue need further exploration.

Interestingly, the planted and unplanted areas within each tray showed significantly different virus titers, regardless of the SFCM types. The planted and unplanted areas were treated the same (i.e., irrigation, photoperiod, temperature, and humidity) while their major differences were the planted coverage and the presence of roots. The reason for this difference requires further investigation to uncover, but there are two possible explanations for it. The possibility is, that even though the virus concentrations in plant and unplanted areas were at the same level, the plant roots have their own microbial communities and produce secondary metabolites that could be antiviral. With respect to the potential rhizosphere bacterial community in the planted areas (Ofek et al., 2011; Reed et al., 2018), some viruses in planted areas might associate with the bacteria through unknown mechanisms. Previous research has reported the binding activities of TV with certain bacteria including binding with *Escherichia coli* O86:H2 through the exposed histo-blood group antigens (HBGAs) on the bacterial surface (Li et al., 2017). It has been reported that TV can selectively bind to some types of HBGAs and sialic acids (Tan et al., 2015; Zhang et al., 2015). Moreover, hNoV has been reported to bind with several bacteria derived from leafy greens; thus, TV may bind to certain plant indigenous bacteria through similar binding mechanisms. There are also some virus and bacteria bindings by unknown mechanisms. Almand et al. (2017) observed the binding of hNoV genogroup I. 6 (GI. 6), GII. 4 to eight selected human gut microbiota while TV bound to four of them (*Lactobacillus plantarum*, *L. gasseri*, *Klebsiella* spp., and *Enterococcus faecium*). Based on the previous finding, if virus binding activity occurred in the present study, the recovery procedures (i.e., centrifugation and filtration steps) might have removed the viruses associated with bacteria or other larger particles,

and only the remaining unbound viruses were detected. The unplanted area might harbor much lower bacterial population, so the viruses and bacterial binding effect was limited.

Second, virus concentration in planted area was possibly reduced by certain secondary metabolites secreted by rhizosphere bacteria. The *Pseudomonas fluorescens* strain Gpf01 isolated from ginseng rhizosphere was found to produce antiviral compounds against the cucumber mosaic virus (Cho et al., 2009). Similarly, cotton rhizospheric *Bacillus amyloliquefaciens* (VB7) contains 10 antimicrobial peptide genes encoding iturin, bacilysin, bacillomycin and other antimicrobial compounds (Vinodkumar et al., 2018). The antimicrobials together with VB7 secreted fatty acids synergistically complemented the antiviral effect against tobacco streak virus.

In this study, the virus titration of TV in microgreen root was performed on the mixture of SFCM and roots since the biostrate mat fiber and roots were combined tightly and difficult to distinguish. To maintain consistency, the roots in peat were also not separated. Because of this limitation, the TV titer in root was not determined alone. Another limitation of this study was that the microgreen growth rate on peat and biostrate differed. A visually lower canopy height and yield were observed for biostrate than peat. The substrate effect on microgreen production has been reported in several studies (Bulgari et al., 2021; Kyriacou et al., 2020; Wieth et al., 2020). A study compared the growth of three types of microgreens on six substrates (agave fibers, capillary mat, coconut fibers, peat moss and cellulose sponge), revealing that all microgreens varieties achieved their tallest canopy on peat, and the shortest on capillary mat (Kyriacou et al., 2020). Third, the growth condition of microgreen had some fluctuations due to difficulties in controlling the indoor air conditioning system as previously described (Deng et al., 2021). On PID 10, a big variation within biostrate samples was observed, and the possible reason

could be related to the room temperature and relative humidity (RH) fluctuations in the plant cultivation room. Even though a humidifier was placed next to the microgreen trays, its buffering capacity was limited if there was a drastic change of RH in the plant room. Comparably, the peat was less affected by the changes in environmental conditions. Last, this study was carried out using the hNoV surrogate TV which shares many biochemical and genetical similarities with hNoV (Tian et al., 2013). However, the results from this study should be extrapolated with caution in regard to how hNoV might behave under similar conditions.

There are also some questions to be solved in future research. First of all, as discussed earlier, the bacteria in microgreen root areas have potential interactions with viruses which may lead to lower TV titer in planted area. Additional research should aim to characterize the microbial communities and investigate whether virus-bacteria binding or bacteria produced antiviral compounds played a role on TV titer. Second, more studies should focus on the mechanism of virus internalization or transfer from different SFCM to leafy greens. So far, several fresh produce are reported to have lower or to be absent of virus internalization within certain growing systems (DiCaprio et al., 2015; Yang et al., 2018). If a certain cultivation system were proved to reduce the risk of virus internalization, it should be considered a risk management practice to enhance the safety of fresh produce.

Overall, this study revealed that TV was able to persist for a fairly long time in biostrate and peat. Although virus transfer from SFCM to the edible tissue of microgreens was not observed, the virus in SFCM could potentially lead to cross-contamination. For instance, during production, any contact between the edible tissue of mature microgreens with SFCM may lead to virus transmission. Also, in the real world, some farms reuse the microgreen trays during a continuous production cycle. In this case, the cleaning and sanitizing procedures should be

validated for sufficient inactivation of pathogens including viruses (Turner et al., 2020). To summarize, the findings in this study provide valuable information on viral transmission routes during microgreen production.

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VII. Figures

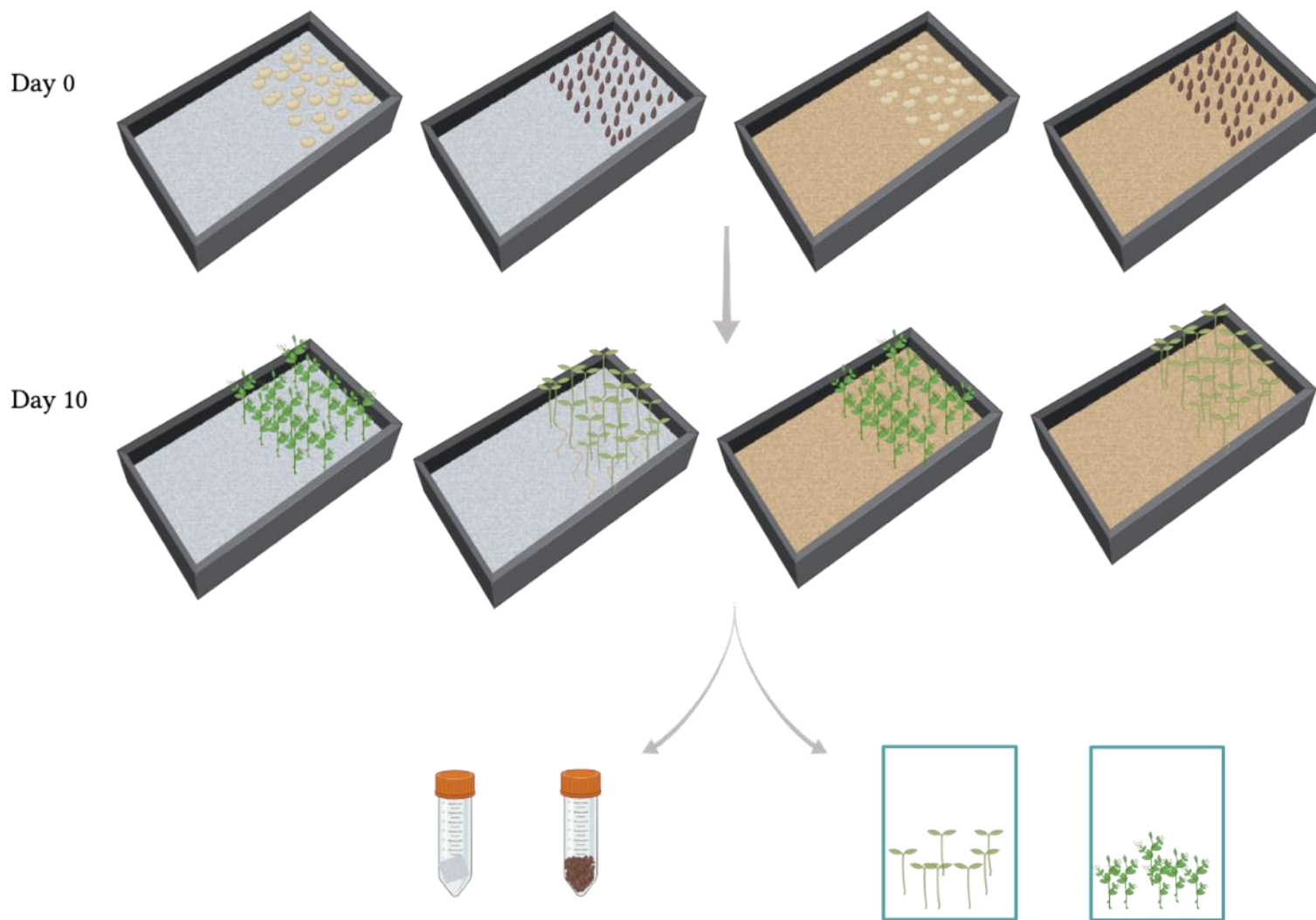


Figure 6. Experiment layout for microgreens planting on day 0 and sample collection on day 10. The microgreen planted and unplanted areas in each tray are shown above. On day 0, SFCM samples from each tray were collected. On day 10, microgreens edible tissues were harvested in stomacher bags, and SFCM from planted and unplanted areas were sampled respectively in tubes.

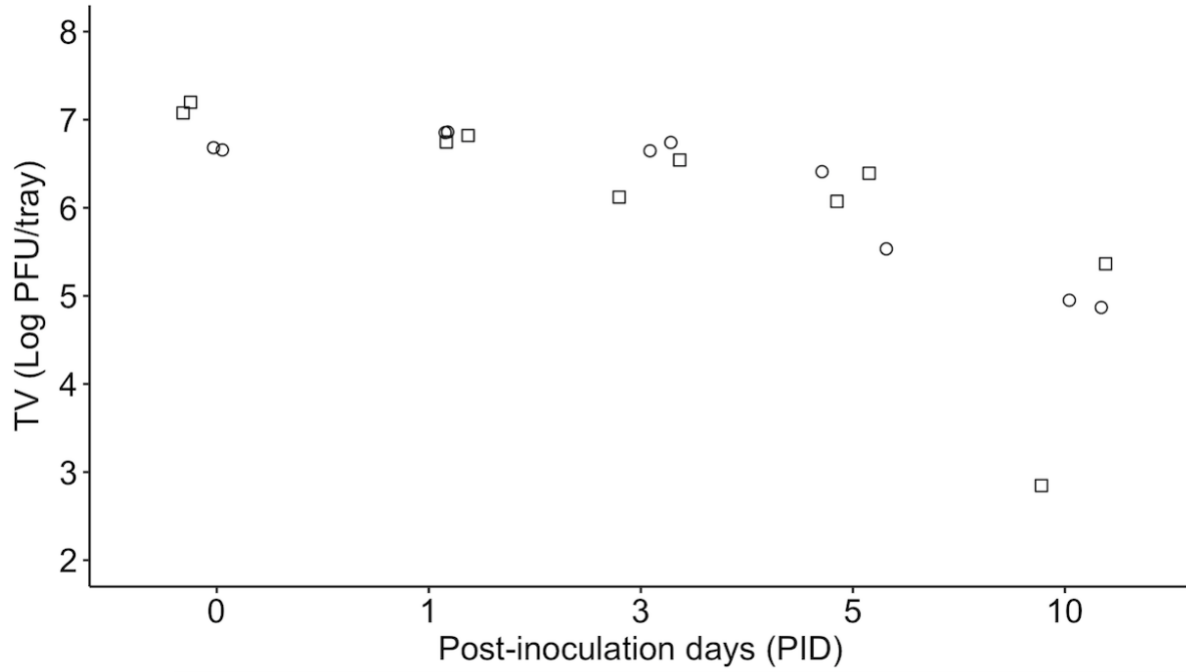


Figure 7. Tulane virus persistence in soil-free cultivation matrices without planting microgreens under indoor farming conditions over 10 days. The virus counts were log transformed and calculated as total virus counts per tray. The SFCM peat (open circle) and biostrate (open square) were plotted against PID. Each treatment was replicated twice.

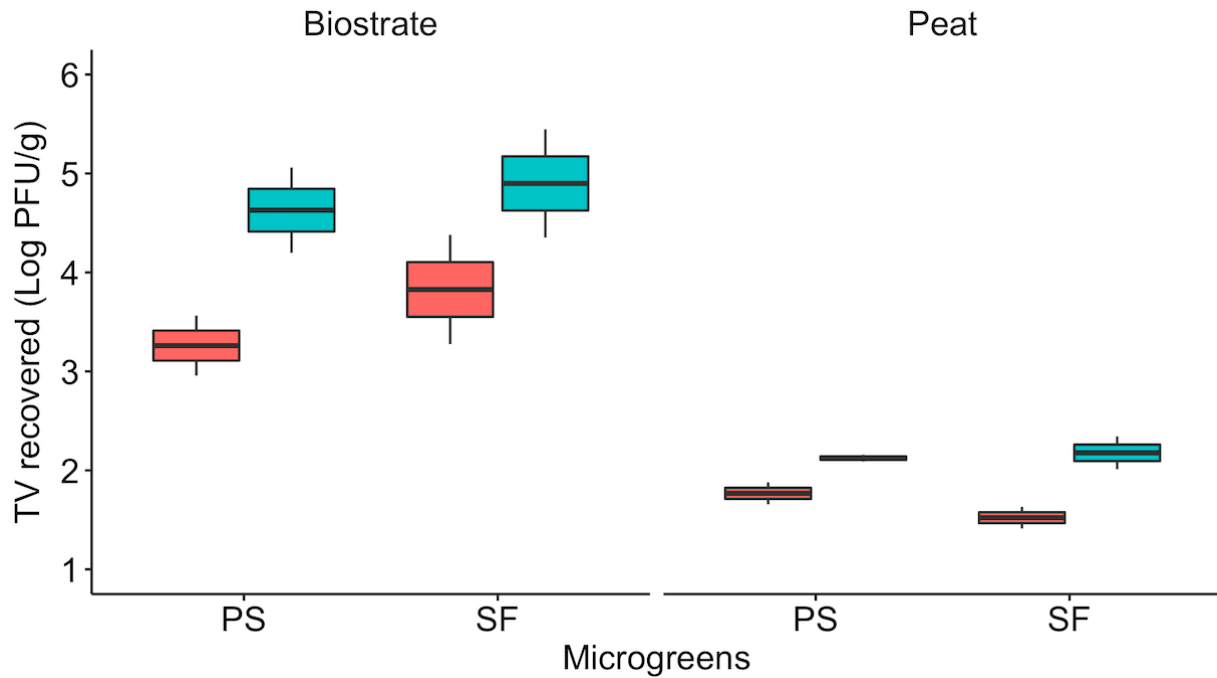


Figure 8. TV recovered in two types of SFCM for microgreen cultivation. The virus titer in planted (red) and unplanted (blue) areas of each tray were plotted against microgreen varieties. PS represent pea shoots and SF represents sunflower. The data for biostrate and peat were plotted separately. Each treatment level contains a replication of two.

VIII. Tables

Table 4. The mean comparisons using Tukey's test for viruses recovered from biostrate among different PID.

PID	Means	SE	DF	Lower. CL	Upper. CL	Group
10	4.11	0.43	10	3.15	5.06	1
5	6.23	0.43	10	5.27	7.19	2
3	6.33	0.43	10	5.37	7.29	2
1	6.79	0.43	10	5.83	7.74	2
0	7.14	0.43	10	6.18	8.10	2

* Post-hoc analysis following a one-way ANOVA to further determine the group of means that are significantly different from others. The means are obtained from replication (n=2). SE stands for standard errors; DF stands for degree of freedom; Lower. CL and upper. CL stands for lower and higher confidence intervals. The different numbers under group represent significant differences.

Table 5. The mean comparisons using Tukey's test for viruses recovered from peat among different PID.

PID	Means	SE	DF	Lower. CL	Upper. CL	Group
10	4.91	0.43	10	3.95	5.87	1
5	5.97	0.43	10	5.01	6.93	1
0	6.67	0.43	10	5.71	7.63	1
3	6.70	0.43	10	5.74	7.65	1
1	6.86	0.43	10	5.90	7.81	1

* Post-hoc analysis following a one-way ANOVA to further determine the group of means that are significantly different from others. The means are obtained from replication (n=2). SE stands for standard errors; DF stands for degree of freedom; Lower. CL and upper. CL stands for lower and higher confidence intervals. The different numbers under group represent significant differences.

Chapter 4: Human Norovirus Surrogate Persistence during the Late Growth Stage Contamination in Microgreen Production

I. Abstract

Human norovirus (hNoV) is a pathogenic agent that is frequently associated with foodborne disease outbreaks linked to fresh produce. In the emerging microgreen production system, the understanding of virus transmission routes and persistence is limited. Virus contamination, particularly during the pre-harvest production phase, can result in contamination that lasts until the consumption stage. Virus contamination caused by farm workers and irrigation water were mimicked in this study. To understand the virus persistence on microgreen leaf surfaces, approximately 5 log PFU of Tulane virus (TV)—a hNoV surrogate—was inoculated on sunflower (SF) and pea shoot (PS) leaves at 7-day age. The virus reduction on SF was significantly higher than PS ($p=0.00015$). On day 10, the viral reductions for SF and PS were 4.50 and 2.52 log PFU/plant, respectively. In addition, the ability of TV to transfer from two types of soil-free cultivation matrix (SFCM) to microgreens was studied. On day 7, 7.6 log PFU total were added to growing trays with SF and PS grown on biostrate and peat. On day 10, the harvested SF and PS were analyzed for TV presence in the whole plant (i.e., surficial and internalized) and internalized in the tissue. However, no virus was detected from PS and SF in whole plant, indicating the absence of TV transmission from SFCM to microgreen. On day 10, TV in SFCM only reduced 0.78 and 1.06 log PFU/g in biostrate and peat, respectively. Overall, this study revealed that TV persistence on microgreen leaves is dependent on plant variety. Virus transmission from SFCM to microgreen was not observed. The findings help to further understand potential hNoV transmission routes in a microgreen production system and to develop effective preventive measures.

II. Introduction

Microgreens are small salad greens with unique color, texture, visual appeal, as well as nutritional value (Renna and Paradiso, 2020; Verlinden, 2020). Starting in 1996 as embellishments in cuisine, the microgreens industry has emerged and increased rapidly in recent years (Misra and Gibson, 2021; Turner et al., 2020). As required by the U.S. Food and Drug Administration (FDA) (2020), the production of microgreens is subject to the Food Safety Modernization Act Part 112 “Standards for the Growing, Harvesting, Packing and Holding of Produce for Human Consumption”, except for subpart M related to sprouts. Although both are harvested at an immature stage, microgreens are different from sprouts since microgreens are harvested when the first pair of true leaves emerge (Di Gioia et al., 2017; Galieni et al., 2020). Microgreens have not been linked to any foodborne disease outbreaks so far, but in the past few years, there have been several recalls of microgreen products in Canada and the United States due to the potential contamination of *Salmonella* and *Listeria monocytogenes* (Canadian Food Inspection Agency, 2020; Turner et al., 2020; FDA, 2018).

Human noroviruses (hNoV) are the most common viral pathogen found in fresh produce (Chatziprodromidou et al., 2018). The transmission routes of hNoV on farm are complex. A primary route is likely the contamination of irrigation water with human sources of fecal pollution. For instance, the feces of hNoV patients contain on average 10^5 - 10^9 genomic copies/g viruses and the shedding on average lasts 8 to 60 days (Teunis et al., 2015). The irrigation water for crops may come from groundwater (spring and well), surface water (rivers, lakes, reservoirs), reclaimed water, or a combination of sources (Centers for Disease Control and Prevention, 2016). In addition to agricultural water, the farm workers, harvesting equipment and tools, and harvest containers may also lead to hNoV contamination of fresh produce (Jung et al., 2014).

The study of viruses within microgreen production has previously been carried out using a hydroponic system (Wang and Kniel, 2016). However, commercial microgreen production more commonly utilizes trays which are arranged on stacked shelves, channels, and benches (Gioia et al., 2017; Misra and Gibson, 2021; Teng et al., 2021). The growing substrates for trays and similar containers are usually soil or soil-free cultivation matrices (SFCM), including perlite, peat moss, vermiculites, coconut coir, and fiber mats. Previously, bacterial pathogens have been investigated within microgreen production systems using SFCM, but no studies have been published on viruses in these systems. In addition, sunflower (SF) and pea shoots (PS) belong to the top three most frequently produced microgreens species in commercial farms, and the virus risk in these two microgreen species has yet to be evaluated (Misra and Gibson, 2021). In this study, the SFCM biostrate and peat were selected for characterization of virus persistence and transmission to SF and PS microgreens. Also, virus persistence on the leaf surfaces of SF and PS grown from peat were compared. Here, hNoV surrogate Tulane virus (TV) was employed for the virus persistence and transfer studies (Drouaz et al., 2015).

III. Material and method

i. Mammalian cell culture and virus propagation

Cell culture, virus propagation, and titration followed the protocol as described previously (Arthur and Gibson, 2015). Tulane virus was kindly provided by Dr. Jason Jiang from Cincinnati Children's Hospital Medical Center in Cincinnati, OH. LLC-MK2 cells (ATCC CCL-7; American Type Culture Collection, Manassas, VA) were cultured in M199 medium with L-glutamine and Earle's salts (Corning, VA, USA) with 10% Fetal Bovine Serum (FBS) (Cytiva,

MA, USA), 1% Penicillin-Streptomycin (100 U/mL, 100 µg/mL; Cytiva) and 1% Amphotericin B (Corning) supplementation. Cells were incubated at 37°C, 5% CO₂.

For virus production, MK2 cells were infected with TV at a multiplicity of infection (MOI) of 0.1, rocking at 37°C, 5% CO₂ for 1h. Following rocking, 20mL of maintenance medium (Opti-MEM with 2 % FBS) (Gibco Life Technology, Scotland, UK) were added and the flasks were further incubated until complete cytopathic effect was achieved which usually takes 48h. The flasks were transferred to -80°C and underwent three freeze and thaw cycles. The cell debris were pelleted at 3000×g 4°C for 15 min. The virus supernatant was purified by filtering with 0.45µm cellulose acetate membrane filter (Corning).

For virus quantification, 2mL of MK2 cells were seeded in 6 well plates at a concentration of 8×10⁵ cell/well and incubated at 37°C, 5% CO₂ overnight. TV samples were serially diluted in maintenance medium. After aspiration of cell growth medium, 500µL of prepared sample was added per well, followed by 1h rocking at 37°C. At the end of rocking, virus samples were removed, and the cell monolayer was covered by 2mL of a 1:1 mix of 3% low melting agarose (VWR, PA, USA) and maintenance medium. Following a 5-day incubation at 37°C, the cells were stained with 0.01% neutral red (Sigma, MO, USA) for 1h to visualize plaque forming units (PFU).

ii. Microgreen cultivation before TV inoculation

Soil-free cultivation matrix were prepared before planting. Biostrate[®] Felt 185gsm microgreen growing mat (Grow-Tech, South Portland, ME, USA) was cut into 10-inch by 10-inch square pieces to fit the growing tray (True Leaf Market, UT, USA). Three-hundred grams of Canadian *sphagnum* peat and vermiculite mix (Jiffy-Mix[®], Jiffy Growing Solutions, NL) were used to fill

additional growing trays. For biostrate and peat, 200mL and 500mL of deionized (DI) water was added, respectively, and distributed homogenously. The biostrate trays were tilted at different angles to allow the water to evenly saturate the mat. The peat in tray was uniformly mixed with the DI water by sterilized gloved hands.

Twenty-five grams of organic black oil sunflower seeds (Tiensvold Farms, NE, USA) and 40g of field pea shoot seeds (Tiensvold Farms) were soaked in sterile DI water for 6h in the dark. When finished soaking, the seeds were drained in sterilized sieves, and approximately 25g of sunflower or 40g of pea shoots seeds were planted per tray. Seeds were planted in four rows, and space was left among rows to allow for future TV inoculation. During the first two days, the trays were covered with a black lid and were only misted daily to keep moisture until seeds germinated. Once lids were removed, the growing trays were transferred to a shelf with installed growing lights (GrowBright, 4-foot, T5 6400K, HTG supply, PA, USA). On each layer of the shelf, a humidifier was sitting next to trays. The biostrate and peat trays were irrigated overhead with 100mL and 150mL of water, respectively, per day as well as 3 to 4 sprays of water mist. The indoor temperature and relative humidity (RH) were maintained within a range of 70-75°F and 55-60%. Sunflower and pea shoots were grown under the aforementioned conditions until the day of experiments.

iii. Virus inoculation on pre-harvest microgreen leaf surface

Sunflower and pea shoots were grown on peat as described in Chapter 4 Section III-ii. On day 7, 50µl of TV inoculum containing approximately 10^5 PFU TV were inoculated onto microgreen leaf surfaces by pipetting 10µL droplets (Figure 9). Both sunflower and pea shoots were inoculated on the abaxial side of leaf surface. The inoculated sunflowers were labeled by a

red sticker on the abaxial surface of the leaf while pea shoots were labeled on the peas. The leaves were allowed to air-dry (approx. 2 to 3 hours) at room temperature. From day 7 to 10, the microgreens were irrigated with the same water volume (150mL/day) as previous days by serological pipetting instead of overhead irrigation. The row-by-row pipetting irrigation prevented the inoculated viruses from being washed off the leaves.

On post-inoculation day (PID) 0, 1, 2 and 3 (i.e., plant age day 7, 8, 9 and 10), one random sunflower and pea shoot microgreen were cut off by sterile scissors. The plant was placed in 50mL tubes containing 5mL of PBS. The tubes were vortexed (VWR Analog Vortex Mixer) at maximum speed for 1 min to recover the leaf surface viruses. The eluent was passed through 0.22µm PVDF filters (Foxy Life Science, NH, USA) to remove bacteria and any plant debris. The samples were then serially diluted in maintenance medium and quantified in plaque assay.

iv. TV contaminated irrigation water in late growth stage

a *TV inoculation and sampling*

Sunflower and pea shoots were grown on peat and biostrate as described previously until day 6. Irrigation water on day 7 for both types of SFCM was pre-mixed with 4×10^7 PFU TV in a biosafety cabinet. The SFCM were irrigated with TV containing water using serological pipettes (Figure 10). The pipetting tip was 1 to 2 cm above the SFCM and care was taken not to inadvertently touch the microgreens. TV were evenly inoculated row by row on each tray. The biostrate trays were slowly tilted in different directions to ensure the inoculum uniformly distributed. Immediately after the inoculation, each tray was sampled for peat or biostrate to quantify the starting concentrations. Peat was sampled using a sterile spoon across four different

random locations in the tray and collected in a 50mL centrifuge tube. Biostrate samples (around 2×2cm) were held with forceps and cut at a random location by sterile scissors, then transferred to a 50mL tube.

On day 10, clean stomacher bags were weighed for net weight. Sunflower and pea shoots were sampled into bags and weighed. While sampling, forceps were used to hold the top of microgreen and scissors were used to cut the stem 1cm above SFCM. About 5 to 10 microgreen plants (ranged between 2 to 10g) were collected per bag. SFCM sampling for day 10 was the same as day 7.

b Microgreen and SFCM sample recovery

Microgreen samples were either pre-treated to eliminate surface associated viruses or non-treated. To characterize the internalized virus only, the surfaces of microgreens were disinfected by immersing plants in 1000 ppm chlorine for 5 seconds. The plants were then transferred to a clean stomacher bags containing 200mL of DI water to rinse off chlorine. After three times rinsing, the microgreens were dried using paper towels, and immersed in 0.25M sodium thiosulfate to neutralize any residual chlorine. Afterwards, the microgreens were again rinsed three times with 200mL water and lastly dried on paper towel. The treated microgreens were transferred to new stomacher bags and processed for virus recovery following the same procedure as the non-treated group. Five mL PBS were added to each stomacher bag, then gently smashed with a hammer, and stomached at 230 rpm for 2 minutes. The eluent was pipetted into a 15mL tube and centrifuged at 3000×g for 5min. Afterwards, the supernatant was slowly pipetted into a new tube. The supernatant was further filtered sequentially with 1 μm and 0.22 μm filters to remove any remaining plant debris and bacteria. The samples were serially diluted and quantified in plaque assays.

For SFCM samples from day 7 and 10, the TV recovery followed the same procedures. Five and 10mL of PBS of were added to biostrate and peat samples, respectively. Biostrate was vortexed at maximum speed for 1min, while peat was vortexed at intermediate speed for 30sec. Eluent was pipetted into a clean 15mL tube and centrifuged at 3000 ×g for 5min to spin down the bacteria. The supernatant then went through a 1µm nylon filter and a 0.22µm PVDF filter to remove remaining bacteria. One mL of Penicillin-Streptomycin (100 U/mL, 100 µg/mL) was added to each tube and vortexed. For peat, after vortexing, the sample was centrifuged at 800 rpm for 5min. The supernatant with still visible floating particles was poured into a funnel with filter paper (VWR Grade 417, Avantor), then further filtered through a 1µm nylon filter and a 0.22µm PVDF filter. The remaining peat on filter paper was scraped off and collected with pelleted peat in tube. SFCM samples were measured dry weight after 48h incubation in 80°C oven. The infectious viruses were quantified by plaque assay, and PFU/g (dry basis) was calculated.

v. Statistical analysis

Data were first logarithm transformed from PFU/g and PFU/plant to Log PFU/g and Log PFU/plant. The TV survival on microgreen leaf surface was summarized in boxplot. The effect of microgreen type and plant age on virus persistence were fit in the two-way analysis of variance (ANOVA) in RStudio (version 1.4.1106, implementing R version 4.0.4) (<https://www.rstudio.com>). For the TV titer in SFCM with day 7 inoculation, a three-way ANOVA was used. In all analysis, $p < 0.05$ were set as significant level.

IV. Results

i. TV survival on microgreen surface

To analyze the effect of different microgreen varieties on TV survival on leaf surfaces, TV was monitored for reduction from microgreen age day 7 to 10 (Figure 11). Starting from approximately 4.6 log PFU/plant on day 7, the virus reductions on day 10 were 4.50 and 2.52 log PFU/plant for SF and PS, respectively. Tulane virus reduction on PS surface was significantly less than on SF ($p=0.00015$). Moreover, on day 10, TV was no longer detectable on SF (limit of detection 0.8 log PFU/plant) while PS titer was still at 2.18 Log PFU/plant.

For each microgreen variety, the number of days significantly impacted the virus titer on leaf surfaces. For PS, the TV recovered titer on day 7 was significantly different from the rest of days. This indicates that the majority of virus reduction occurred between days 7 and 8. The TV titer on day 8, 9, and 10 continued to reduce, although the titers among these days were not significantly different. For SF, the same reduction pattern was observed as for PS, while the day-to-day reduction in SF was higher than PS.

ii. Internalization of TV from late stage inoculated SFCM to microgreens

On day 7, TV of 7.6 log PFU/tray were inoculated to SFCM to observe the virus transfer to SF and PS. The total virus transferred to the edible part of microgreens was analyzed without pre-treatment, while the internalized virus in tissue was detected after plant surface disinfection. On day 10, TV was not detected in microgreen edible part regardless of microgreen pre-treatment or not, for PS and SF grown in either type of SFCM.

Meanwhile, SFCM and microgreen roots mixture were sampled for day 7 and 10 (Figure 12). The microgreen variety did not affect the virus titers in SFCM ($p>0.05$). Starting at 6.07 log

PFU/g in biostrate, the virus only reduced 0.78 log PFU/g by day 10. The post-hoc analysis showed that virus titer on day 10 was not significantly different from day 7 ($p>0.05$). While for peat, TV was an average of 5.09 log PFU/g on day 7, and the virus on day 10 had reduced an average of 1.06 log PFU/g ($p= 0.008$).

V. Discussion

In fresh produce production, viral contamination may be introduced by the irrigation water, plant medium, or farm workers (Machado-Moreira et al., 2019). In this study, TV persistence on microgreen leaf surface mimicked the situation of virus contamination by overhead irrigation or the hand touch by hNoV shedding workers. The second experiment investigated a single contamination event of SFCM from virus contaminated irrigation water.

The TV on preharvest microgreen leaves were significantly higher in PS than SF, indicating that the virus persistence pattern is potentially plant variety-dependent. Two possible theories may explain this observation. First, the topographical differences of leaves between SF and PS might lead to the difference in virus persistence (Doan et al., 2020). According to a comparison of *Escherichia coli* O157:H7 survival among spinach cultivars Emilia, Lazio, Space and Waitiki, the leaf blade roughness and stoma density had significant impact on the bacterial survival (Macarisin et al., 2013). The *E. coli* population was 0.4 log CFU higher ($p<0.05$) on the highest leaf roughness cultivar Waitiki. Also, the number of stomata on leaves showed a positive relationship with the recovered number of *E. coli* (Macarisin et al., 2013). Another possibility is that the leaf exudates profile on SF and PS contributed to the difference in virus persistence. Rowe et al. (2012) reported that mature sunflower leaves produce a mixture of secondary metabolites into the glandular trichomes located on the leaf surface. The main components of

sunflower trichome secretion were sesquiterpene lactones (STL). Recent studies found that one STL called brevilin A—isolated from medicinal herb *Centipeda minima*—had antiviral activity against Influenza A virus H1N1, H3N2, and H9N2. Bervilin A inhibited the virus replication under both *in vitro* and *in vivo* conditions (Zhang et al., 2019, 2018). The sunflower in the present study is at the microgreen stage, so the production of STL and the specific types remains unclear. Further characterization of the sunflower microgreen surface exudates is needed.

Previous studies on bacteria and virus on fresh produce have observed that the inoculation levels affected the efficiency of bacterial and viral internalization from growth substrate (Cooley et al., 2003; Yang et al., 2018). Also, based on our previous finding in Chapter 3, the day 0 virus inoculation to SFCM did not lead to the virus transfer into microgreens on day 10 (data not shown). This study utilized a later growth stage inoculation into SFCM to ensure a high inoculation level of TV when harvesting on day 10. The virus reduction from day 7 to day 10 was on average 0.92 log PFU/g in SFCM. Nevertheless, the TV was still not detected in microgreens edible tissue, indicating that the inoculation dose in this case was probably not the reason for the failure of virus internalization.

So far only one published paper studied hNoV surrogate internalization from growing media to microgreens. In Wang and Kniel (2016), murine norovirus (MNV) was inoculated into the nutrient film technique hydroponic system on day 8 and subsequent internalization into kale and mustard was observed. Within 2h the MNV had internalized into edible tissue, and viruses were detectable until day 12 harvesting. The present study is not comparable with their research due to differences in cultivation system, virus types, microgreen varieties, and even plant age may affect the result (Hirneisen et al., 2012; Pu et al., 2009). The effect of cultivation method and plant variety on TV internalization has been described by Yang et al. (2018). The study by

Yang and co-authors was carried out in one-month old radish and two-month old onion and lettuce. They found that TV in nutrient solution successfully internalized in hydroponically grown onion and radishes, but not in the soil grown system. While for lettuce, the TV internalization occurred in both hydroponic and soil systems.

Despite the absence of TV internalization in microgreen edible tissue in the present study, the long persistence of virus in SFCM is noteworthy. In commercial production, the virus containing SFCM can easily cross-contaminate harvesting machines or workers' hands, potentially leading to a spread of contamination. On the other hand, due to the highly perishable nature once harvested, some microgreens are sold as a living produce in plastic tray containing growth medium to extend the shelf life (Renna et al., 2017). In this case, the virus in SFCM may further cause cross-contaminations when consumers harvest the microgreens in kitchen.

There are some limitations in this study. First, during the virus persistence assay on leaf surface, the potential internalization of TV into leaves through stomata was not considered. *Salmonella enterica* were found to aggregate on the stomata of lettuce leaves, further penetrate and invade the tissue (Kroupitski et al., 2009). Norovirus-like particles were previously reported to aggregate in romaine lettuce stomata (Esseili et al., 2012a). However, it is unknown whether hNoV would behave the same way on leaf surface and even internalize into tissue or not. A scanning electron microscope (SEM) on kale, arugula, lettuce and mizuna microgreens showed that their stomata were slightly longer than the mature leaves (Park et al., 2013; Turner et al., 2020). Thus, in the present study, the potential of TV internalization in microgreen tissue through stomata is unclear. Second, there was a greater standard error for virus recovered on day 7 from biostrate. This was due to the fact that the water content among biostrate trays was not

exactly the same. As a result, when inoculating the same amount of TV, viruses were more diluted in higher water content biostrate trays.

This study revealed that in certain microgreen varieties, the virus can persist longer. This raises the question regarding whether the washing steps before consumption can effectively remove the viruses. To answer the question, a thorough understanding on how virus attach or bind to microgreen leaves is required. A previous study showed that washing step for lettuce leaf only reduce viruses by less than 1 log PFU (Bae et al., 2011). Human norovirus is known to specifically bind to the histo-blood group antigen (HBGA) like carbohydrate moiety on lettuce leaves (Esseili et al., 2019). Therefore, in future work on microgreens, the virus attaching mechanism should be explored. On the other hand, the present study did not observe the virus internalization from tested SFCM into SF and PS. More microgreen varieties should be tested for virus internalization on SFCM. For instance, the mature lettuces have been well studied for hNoV and surrogate virus internalization in both hydroponics and soil cultivation matrix (Esseili et al., 2012b; Yang et al., 2018). However, the virus internalization into the lettuce microgreen has not been characterized (Weber, 2016).

Overall, the present study characterized virus contamination of microgreens when introduced to the microgreen system at close-to-harvest timing. The leaf surface virus persistence was found to be plant variety-dependent. While the virus persisted in SFCM, it is not internalized into the edible tissue based on the tested two microgreen species. In addition, this study provided a set of reliable virus recovery methods from different types of microgreen SFCM which can be used for future virus studies on crops.

VI. References

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VII. Figures

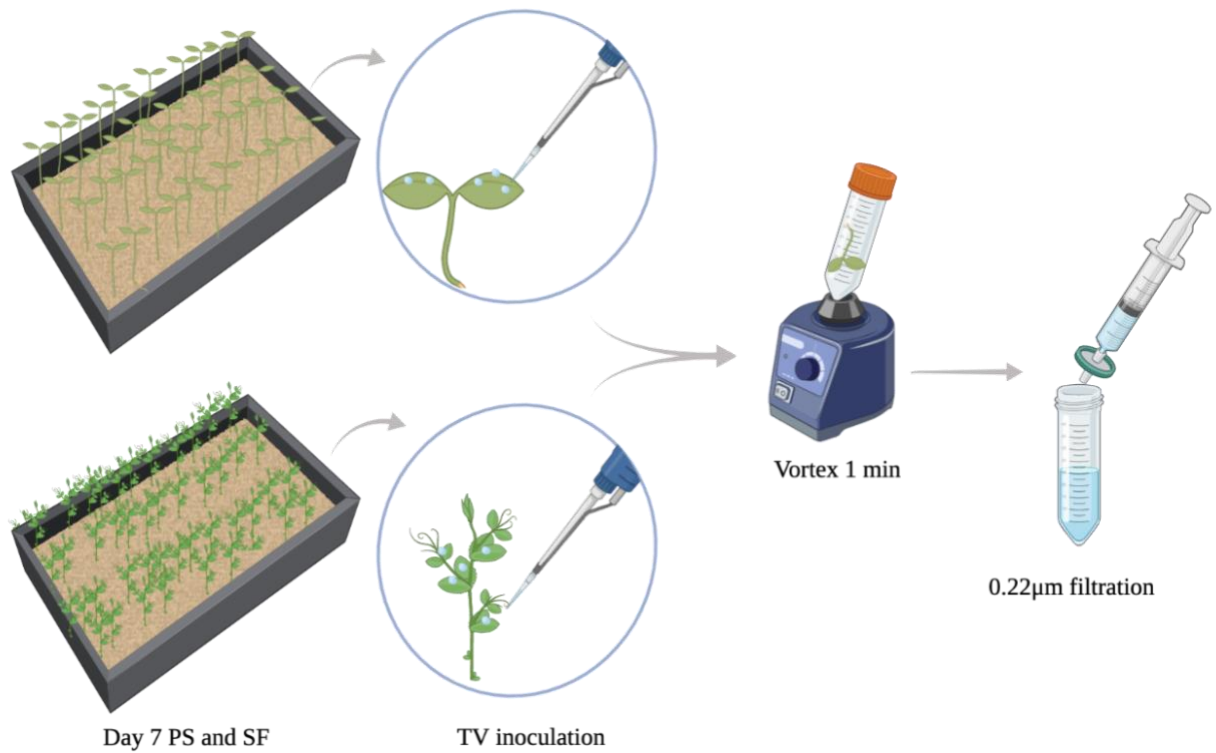


Figure 9. Flow diagram of TV inoculation on day 7 microgreen leaf surfaces and virus recovery. The 10µL droplets of TV inoculum on SF and PS leaves are shown in the diagram. After drying in RT, the microgreens were recovered for virus concentration.

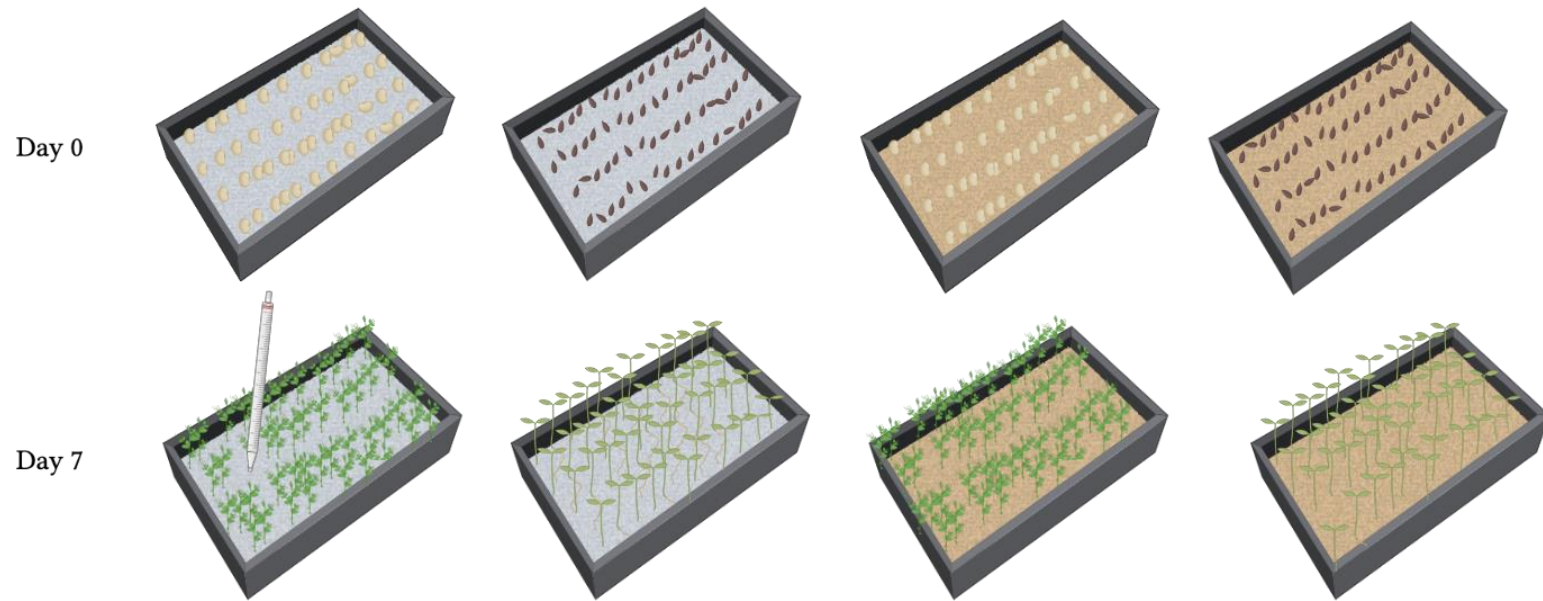


Figure 10. The layout of plant seeds on day 0 and the inoculation of TV on day 7. On day 0, the sowing of seeds was in a row-by-row pattern on biostrate and peat. On day 7, TV was inoculated into SFCM by a serological pipette in spaces between rows without contact with above ground edible tissues.

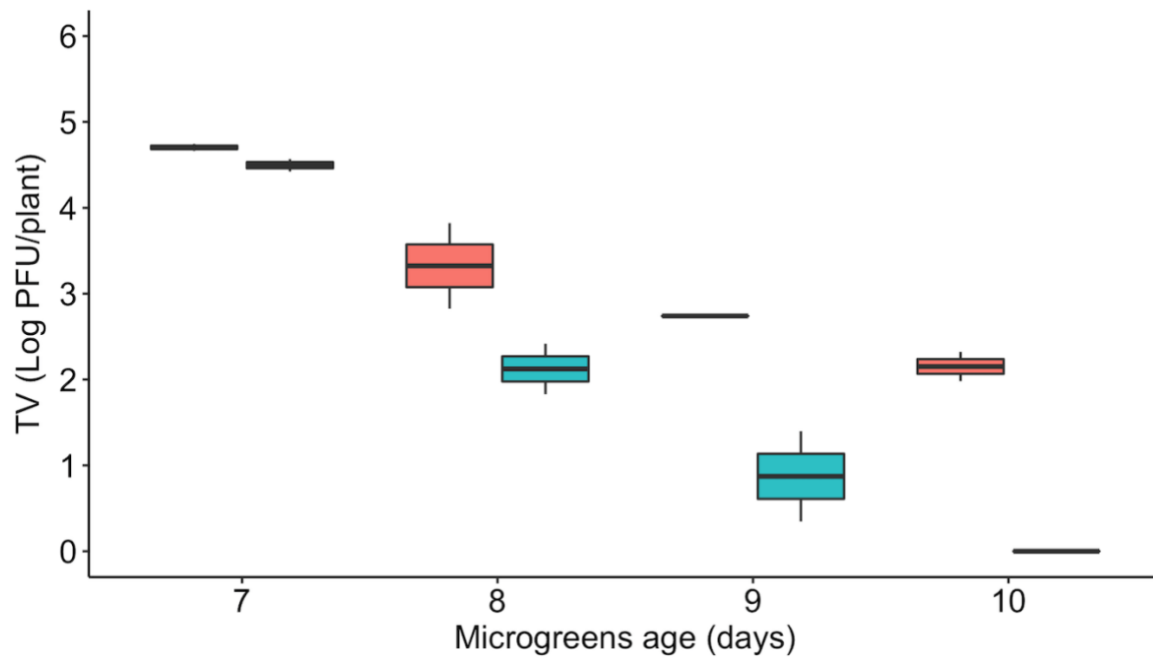


Figure 11. The survival of TV on sunflower and pea shoots leaf surface. The virus titers (log PFU/plant) on microgreen leaves surfaces were plotted against plant age (days). Sunflower (blue) and pea shoots (red) surface virus counts were based on two biological and two technical duplicates. The limit of detection (LOD) was 0.8 log PFU/plant, the below LOD data points were considered as 0 log PFU/plant in graph.

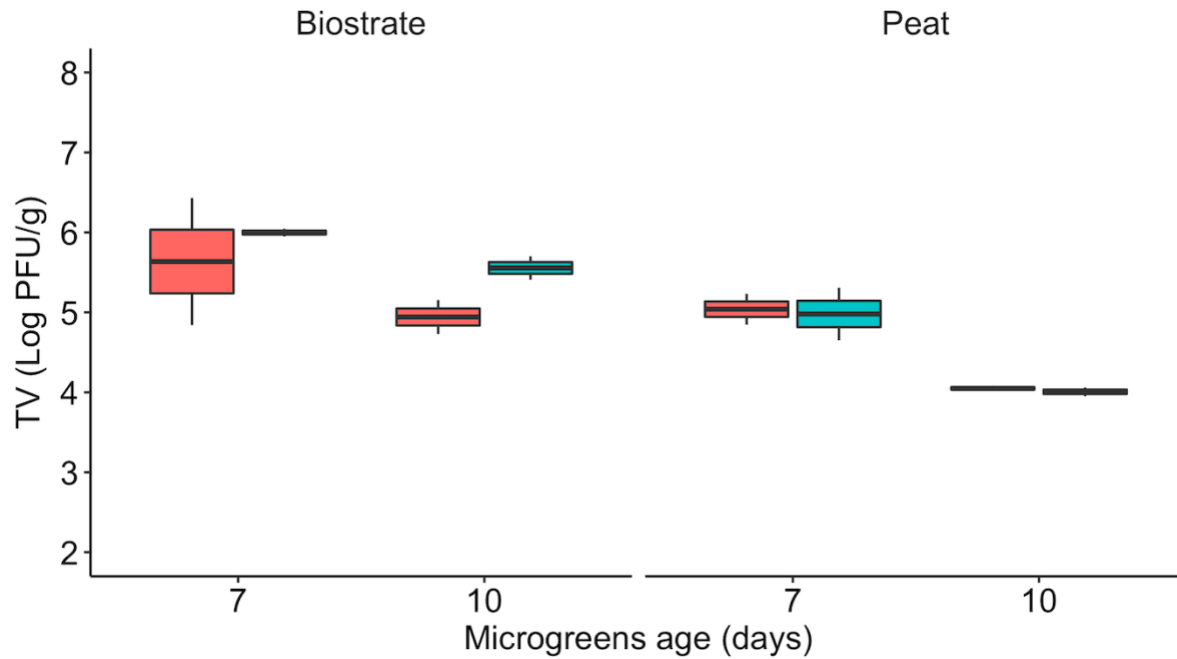


Figure 12. The TV titer in SFCM on microgreen age day 7 and 10. Day 7 was the virus inoculation day while day 10 was the microgreen harvesting day. The virus titer (Log PFU/g) was plotted against microgreen age (days). The result summarized separately for biostrate and peat, as well as for sunflower (green) and pea shoots (red). Data for each treatment includes two replicates of biological and technical, respectively.

Chapter 5: Conclusions

Leafy greens play a crucial role in a healthy balanced diet (Randhawa et al., 2015). However, reducing the microbial risks in leafy green production and preparation is a challenge (Kaczmarek et al., 2019). This dissertation aimed to understand the human noroviruses (hNoV) persistence on leafy greens (i.e. lettuces and microgreens) and the related production systems.

Lettuce is the most consumed type of leafy green with an annual consumption of 5,888g per individual in the United States (Pang et al., 2017). Therefore, pathogens that are frequently associated with contaminated lettuce have been studied intensively. Human norovirus is the primary viral pathogen found on lettuces (CDC, 2021). Here, the hNoV surrogate Tulane virus (TV) on hydroponically grown oakleaf lettuces at pre-harvest stage were studied for its persistence on leaf surface. It was found that oakleaf lettuce inoculated on 40 days age survived to day 45 when the lettuce was fully mature and ready to harvest. The major reduction of virus on leaves was observed on post inoculation day (PID) 2. Over four days of observation, TV was reduced by over 4 log PFU/leaf. The findings indicate that when virus contamination occurs close to harvest, the virus could very well persist to the post-harvest and consumption stages.

Microgreens are a group of novel leafy greens with a rich nutritional value compared to their mature plant counterparts (Choe et al., 2018). Currently, the knowledge surrounding foodborne pathogen risks within microgreen cultivation system is limited, especially in regard to the risk of contamination with human enteric viruses. This dissertation for the first time characterized the viral persistence in two types of soil-free cultivation matrix (SFCM) used in the production of microgreens—biostrate and peat. It was found that the hNoV surrogate TV survived on SFCM over 10 days with a reduction of 2.08 and 1.76 log PFU/tray for biostrate and peat, respectively. Furthermore, the microgreen sunflower (SF) and pea shoot (PS) seeds were

planted on virus contaminated SFCM and analyzed for virus transfer into edible tissue of the microgreens. No virus was found in either microgreen variety after harvest on day 10. However, an interesting finding was that in SFCM, the planted area contained significantly lower virus than the control (unplanted area). On biostrate and peat, the differences were on average 1.15 and 0.49 log PFU/g, respectively. The finding provides insights on the potential interactions between TV and rhizosphere microorganisms. Future studies are needed to illustrate the mechanisms of this observation.

Next, since the TV transfer from day 0 inoculated SFCM to edible tissue was not detected based on preliminary studies, an experiment was carried out to understand the effect of later growth stage inoculation on virus transfer. On microgreen age of 7 days, the TV was inoculated by serological pipette to biostrate and peat without contact with the growing SF and PS. Although this gave a higher TV titer in SFCM than day 0 inoculation, again no virus was detected on day 10. Besides SFCM inoculation, in a separate study, TV was also inoculated on leaves of SF and PS on day 7. It was found that TV survival on leaves was plant variety-dependent. On day 10, the reduction of virus was 4.50 log PFU/plant for SF, while only 2.52 log PFU/plant for PS.

Overall, the study in a microgreen production system indicated that TV can persist in SFCM over the entire microgreen cultivation time. However, the viral transfer from SFCM to edible microgreen tissue was undetected for SF and PS. Nevertheless, the TV inoculated on leaf surface of pre-harvest PS can survive to post-harvest, indicating that more safety attention should be paid to certain microgreen species. The findings in this dissertation will help to develop viral preventive strategies in the future with better targeting. Also, this study revealed that the practices implemented in the post-harvest stage should not only prevent the introduction of virus,

but also apply effective cleaning and sanitizing practices to eliminate the viruses potentially carried from the pre-harvest stage.

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Appendix

IBC Approval letters



Office of Research Compliance

December 7, 2018

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: Ines Pinto, Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 19012

PROTOCOL TITLE: The interactions between human norovirus surrogates and exudates of lettuce leaves

APPROVED PROJECT PERIOD: Start Date December 6, 2018 Expiration Date December 5, 2021

The Institutional Biosafety Committee (IBC) has approved Protocol 19012, "The interactions between human norovirus surrogates and exudates of lettuce leaves". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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March 14, 2019

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: Ines Pinto, Biosafety Committee Chair

RE: Protocol Renewal

PROTOCOL #: 13017

PROTOCOL TITLE: Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health

APPROVED PROJECT PERIOD: Start Date January 10, 2013 Expiration Date January 9, 2022

The Institutional Biosafety Committee (IBC) has approved your request, dated February 22, 2019, to renew IBC # 13017, "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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April 16, 2019

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: Ines Pinto, Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 19025

PROTOCOL TITLE: Transfer of human norovirus surrogates and foodborne bacterial pathogens from soil-free horticulture media to microgreens

APPROVED PROJECT PERIOD: Start Date April 11, 2019 Expiration Date April 10, 2022

The Institutional Biosafety Committee (IBC) has approved Protocol 19025, "Transfer of human norovirus surrogates and foodborne bacterial pathogens from soil-free horticulture media to microgreens". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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