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ARTIFICIAL PHYLLOPLANES: A NOVEL TOOL TO STUDY PARAMETERS LINKED TO BACTERIAL AND VIRAL CONTAMINATION OF FRESH PRODUCE

BY

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DISSERTATION

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ABSTRACT

Every year, one-in-six Americans suffer from a food-related illness caused by bacteria, viruses, or parasites. Since 2010, fresh produce has been linked to seven foodborne outbreaks caused by *Escherichia coli* species alone. Produce surface properties, such as surface hydrophobicity and surface roughness play a key role determining the attachment of bacteria and viruses to and their removal from produce. A few previous studies have investigated the effect of surface roughness and surface hydrophobicity on the attachment and removal of bacteria and viruses from food and food contact surfaces. However, since produce surface properties undergo constant changes as a function of time and environmental factors, reports have shown inconsistent results for the same produce type with regard to bacteria attachment and removal.

Researchers have realized the need to construct artificial plant surfaces to retain the surface characteristics of natural plant surfaces during sanitation tests. A few attempts have reported the use of polymers, stainless steel, zinc substrates, or alumina to fabricate surrogate surfaces that resemble food or food contact surfaces, with varying degrees of success. Nevertheless, even the most successful one among the pervious surrogate surfaces can only replicate the topographical characteristics of natural fresh produce surfaces, but not the chemical properties of the plant surfaces. Furthermore, most of the previous surrogate surfaces lack reusability due to the nature of the fabrication material. In a microbial attachment or removal study, the surrogate surface will be subjected to mechanical forces because they need to be placed in a stomacher to do the emulation; thus, the surrogate surfaces made from soft material will be damaged. The overall goal of this study is to develop a new method for the fabrication of reusable and reproducible artificial phylloplanes that replicate the three-dimensional topological features of natural produce leaves, thus having surface hydrophobicity, roughness values, and epicuticular composition resembling

those of two selected fresh produce varieties. To achieve the goal, three inter-related studies were performed.

In the first study, the effects of physiochemical characteristics, including produce leaf surface roughness, epicuticular wax composition, and produce and bacteria surface hydrophobicity on attachment/removal of *E. coli* K12 to/from plant surfaces was investigated. The attachment and removal of *E. coli* K12 was affected by multiple factors including produce genotype, produce surface roughness, and wax composition. Rougher surfaces resulted in higher attachment of *E. coli* and less removal. In addition, the removal of *E. coli* K12 was positively correlated with alkanes, ketones, and total wax content on the leaf surfaces.

In study two, a method to create polydimethylsiloxane (PDMS)-based artificial phylloplane surface to resemble the topographical, chemical, and epicuticular characteristics of 'Outredgeous' romaine lettuce and 'Carmel' spinach to a high fidelity was developed. The artificial produce leaf surfaces were utilized to study the effect of surface hydrophobicity on the attachment of *E. coli* O157:H7 and *Listeria innocua*. The PDMS- artificial phylloplanes are reusable, economical, and recyclable. They can thus be used as a platform to investigate the interactions between bacteria and produce, and to develop new or enhanced fresh produce decontamination strategies.

In study three, the newly developed artificial phylloplane surfaces were utilized to study the effect of produce leaf physiochemical characteristics on the attachment and removal of porcine rotavirus (PRV), strain OSU, and tulane virus (TV), a surrogate of human norovirus. In addition, the artificial phylloplanes were used to screen commercially available and new sanitizers and to study the use of ultrasonication as an enhancer of viral detachment in the washing step. No significant differences in attachment of PRV and TV inoculated to fresh leaves of 'Outredgeous'

romaine lettuce and 'Carmel' spinach and their artificial phylloplanes were observed. In sanitation tests, the removal of virus attached to natural and artificial surfaces was virus type, sanitizer type, and produce cultivar dependent.

In summary, the newly developed artificial phylloplanes establish a platform with constant surface properties for studying the interactions between bacteria and produce leaf surfaces. The new surfaces overcome the biological variations of produce surfaces originated from changes during preharvest, transportation, and post-harvest processing/storage, which oftentimes result in inconsistent sanitation results. The newly developed artificial phylloplanes provide a faithful replication of the surface characteristics of fresh produce in that they 1) resemble the 3D topological features of natural produce leaf surfaces, 2) have a similar surface hydrophobicity, 3) have similar epicuticular chemical composition, mainly epicuticular wax composition, 4) produce a similar bacterial attachment pattern, and 5) are reproducible and reusable, including autoclave-able and compatible with stomacher.

With love to my family

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CHAPTER 1

INTRODUCTION

Fresh produce including lettuce and spinach were among the top 20 vegetables sold in the US in 2017 (Packer 2017). In recent years, due to a trend toward healthier lifestyles, salad bars have become increasingly available in restaurants, leading to an increase in the consumption of lettuce and other leafy green vegetables in the U.S. (Buck, Walcott et al. 2003, Palma-Salgado 2013, VanFrank, Onufrak et al. 2018). Unlike other vegetables that require a cooking step, fresh produce is often consumed raw or with minimal post-harvest processing, raising public concerns about its potential association with foodborne illnesses (Beuchat and Ryu 1997, Herdt and Feng 2009). In the US between 2010 and 2018, leafy greens and fresh produce were involved in seven different foodborne outbreaks and food recalls due to contamination with *Escherichia coli* spp. (CDC 2017). It is evident that there is a lack of understanding about which factors are key during the contamination of fresh produce with pathogenic microorganisms, and which approaches might work best to keep the food safe. Furthermore, in 2013, the cost of foodborne illness was $271 million from *Escherichia coli* O157and $2.25 billion from noroviruses (ERS 2014). From the evidence stated above, it is clear that foodborne outbreaks are a recurrent issue in the US, and they represent a financial burden on the victims and the food company involved.

Leaf surfaces can be ecosystems for a variety of microorganisms including plant and human pathogens (Hirano and Upper 2000); since fresh produce and leafy greens are consumed raw, they can easily become a vehicle for the transmission of disease-causing bacteria (Berger, Sodha et al. 2010). Leaf surfaces can become contaminated with pathogenic bacteria at any stage during growth, preharvesting or sanitization process (Park, Navratil et al. 2013). Although different sanitation protocols and chemical agents are in use at processing facilities, their low

efficacy is evidenced by the recurring outbreaks related to the consumption of fresh produce and leafy greens (Koseki 2014, CDC 2017). It is well known that the efficacy of certain chemical sanitizers can be affected by the presence of organic matter as well as by the leakage of produce electrolytes that deplete the sanitizer (Luo, Ingram et al. 2014, Palma-Salgado, Pearlstein et al. 2014). However, researchers working in fresh-produce safety have also realized that topological characteristics of the plant surfaces, such as roughness, hydrophobicity and epicuticular composition, can also affect the efficacy of sanitizing washes in the removal of microorganisms (Bunpot Sirinutsomboon 2007, Lazouskaya, Sun et al. 2016). Furthermore, most studies on the sanitization of fresh produce seek to test new combinations of sanitizers and washing conditions, and only a few have taken into consideration all three of these topological characteristics (Fuzawa, Ku et al. 2016)

Although researchers have realized the importance of surface topography on the attachment and removal of bacteria and viruses from fresh produce, the evidence presented by research studies has been inconclusive. For instance, some studies have reported a positive correlation between surface roughness and the attachment of *Escherichia coli* to various food surfaces (Wang, Feng et al. 2009, Fransisca and Feng 2012). On the other hand, others have reported no significant correlation between the roughness of food contact surfaces (stainless steel) and the promotion of bacterial attachment (Flint, Brooks et al. 2000), or between the surface roughness of leafy greens and viral adherence (Lu, Ku et al. 2015). The inconsistency of the results evidences a lack of control of the surface conditions during the studies of topography and sanitization, which has resulted in the development of synthetic microstructures to use as surrogate platforms for studies of bacteria-fresh-produce interactions. Even though research groups have been successful at mimicking plant surface shapes, these patterns oftentimes only replicate food contact-surface

topology, because they are fabricated using polymers, stainless steel, zinc substrates, and alumina (Medilanski, Kaufmann et al. 2002, Lazouskaya, Sun et al. 2016). Furthermore, those that were successful at reproducing the topographical characteristics of real fresh produce were capable of only including surface roughness and hydrophobicity. To the best of our knowledge, no research group has been able to control all three produce-surface characteristics simultaneously, i.e., roughness, hydrophobicity, and epicuticular composition when developing a plant surrogate to study plant-bacteria or plant-virus interactions.

The overall objective of this project was to conduct a comprehensive investigation to develop a new method for the fabrication of artificial phylloplanes that mimic the three-dimensional topological features of natural produce leaves, and have surface hydrophobicity, roughness values and epicuticular composition resembling those of the most commonly consumed fresh produce. Furthermore, these "leaves" should be reproducible and reusable. The specific objectives were as follows:

- Investigate the adhesion and removal of *Escherichia coli* K12 as affected by the epicuticular wax composition, surface roughness, and hydrophobicity features of the produce, and the hydrophobicity of the bacterial surface.
- Develop a rapid-replication "double-casting" method to produce these artificial phylloplanes resembling the physicochemical characteristics of selected fresh produce and examine their use in bacterial attachment/removal studies.
- Investigate the attachment and removal of porcine rotavirus (OSU) and tulane virus from fresh and artificial leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach as affected by ultrasonication in combination with oxidant- or surfactant-based sanitizers.

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CHAPTER 2

LITERATURE REVIEW

2.1 FRESH PRODUCE IN THE US

2.1.1 Fresh-produce consumption and foodborne outbreaks

Fresh produce, including lettuce, spinach, prepackaged leafy greens and other leafy greens were among the top 20 vegetables sold in 2017 in the US. (Packer 2017). In 2015, the estimated total retail sales for fresh produce in the US were $46.91 billion (Statista 2016). The increase in consumption of fresh produce in the US can be attributed to its nutritional value, being recognized as a good source of fiber, vitamins, and health-beneficial bioactive compounds; also, the convenience and versatility of the prepacking of fresh-cut produce, and the implementation of clean labels are factors that have driven the increase in consumption (Nielsen and PMA 2015, Kim, Moon et al. 2016, Kim, Shang et al. 2018). However, with the increase in consumption, an increase in the association of fresh produce with foodborne outbreaks has also been observed. In the US during the period 2010-2018, fresh produce was involved in seven different foodborne outbreaks (Table 2.1) with pathogenic *Escherichia coli* spp. alone (CDC 2018).

Table 2.1. List of *Escherichia coli* spp. outbreaks linked to fresh produce period 2010-2018

YEAR	FOOD ITEM	PATHOGEN
2018	Romaine lettuce	*Escherichia coli* O157:H7
2017	Leafy greens	*Escherichia coli* O157:H7
2016	Alfalfa sprouts	*Escherichia coli* O157:H7
2014	Raw clover sprouts	*Escherichia coli* O121
2012	Spinach and spring mix	*Escherichia coli* O157:H7
2012	Raw clover sprouts	*Escherichia coli* O26
2011	Romaine lettuce	*Escherichia coli* O157:H7

Source (CDC, 2017)

2.2 FRESH-PRODUCE DISINFECTION

Unlike vegetables that are cooked by the consumer, the only inactivation step to which fresh-cut produce is exposed prior to consumption is sanitization at the processing plants (Sapers 2014). The primary purpose of sanitization is to reduce microbial populations and remove soil debris and pesticide residues (Palma-Salgado, Pearlstein et al. 2014). Leaf surfaces can become habitats for a diverse assemblage of microorganisms, including epiphytic bacteria, as well as human and plant pathogens (Hirano and Upper 2000); moreover, it is known produce sanitization is not able to remove all harmful bacteria (Banach, Sampers et al. 2015). Therefore, the fresh-produce industry is constantly developing and proposing chemical and physical approaches for the control of microbiological contamination on fresh produce (Pascal and Susan 2012). These different approaches are considered in more detail below.

2.2.1 Chlorine

Chlorine is the most common sanitizer used by the fresh-produce industry (Herdt and Feng 2009, Luo, Nou et al. 2011) The efficacy of chlorine in killing the pathogenic bacteria and viruses present in fresh produce has been studied extensively (Luo, Nou et al. 2012, Palma-Salgado, Pearlstein et al. 2014, Fuzawa, Ku et al. 2016). One advantage is its low cost and convenience (Beuchat and Ryu 1997). However, one of the drawbacks of utilizing chlorine during the sanitization of fresh produce is the depletion of the free chlorine due to its interaction with organic matter (Palma-Salgado, Pearlstein et al. 2014); this has resulted in the utilization of higher initial chlorine doses and replenish during produce washing so that a minimum level of free chlorine (FC) is available for microbial inactivation (Gómez-López, Lannoo et al. 2014).

One issue in utilizing a higher initial dose of chlorine is the potential for creating harmful by-products called trihalomethanes (THMs), which have been classified as possible human carcinogens (Amy, Bull et al. 2000, Van Haute, Sampers et al. 2013). Multiple studies have been carried out to identify the minimum free-chlorine concentration required to eliminate the human pathogens from fresh produce. Gómez-López, Lannoo et al. (2014), investigated the simulated washing conditions used by the fresh-cut industry to sanitize spinach contaminated with *Escherichia coli* O157:H7 using chlorine. They reported that when the free-chlorine concentration was kept between 5-7 mg/L the washing tank remained free of *Escherichia coli* O157:H7; however, they observed that high trihalomethane levels (1000 mg/L) were generated in the process. In addition, Luo, Zhou et al. (2018), investigated the minimum free-chlorine concentration required to remove the bacteria present in fresh produce simulating the commercial washing of chopped romaine lettuce, shredded Iceberg lettuce, and diced cabbage. They reported that maintaining a minimum of 10 mg/L of free chlorine reduced the incidence of bacteria survival and minimized the likelihood of the cross-contamination of washed produce.

2.2.2 Peroxyacetic acid

Peroxyacetic acid (PAA) is a strong oxidizing agent used as a sanitizer by the food, dairy and beverage industries. It is commercially available as a solution of peracetic acid, hydrogen peroxide, acetic acid, and water (USDA 2016). PAA is approved to be used as a process-water additive in the fresh-produce industry at concentrations no higher than 80 mg/L. The primary mode of action for peracetic acid is oxidation, while chlorine's is the destruction of microorganisms by the interaction of hypochlorous acid with components in the organisms' cell protoplasm (Herdt and Feng 2009). Among the advantages of utilizing PAA as an antimicrobial agent are that it does

not require adjustment of the pH of the water source, produces no harmful by-products (it decomposes into acetic acid, oxygen and hydrogen peroxide), and most importantly PAA is not degraded by organic matter (S. Stampi 2002). In a study conducted by Palma-Salgado, Pearlstein et al. (2014), the degradation of peroxyacetic-acid (60 mg/L) and free-chlorine (60 mg/L) concentrations was monitored during the sanitization of lettuce under washing conditions like those of the fresh-produce industry. Following a 1-minute contact with lettuce, the peroxyacetic-acid concentration remained constant, in contrast to the chlorine, which decreased 35-65% in the presence of organic matter.

Furthermore, multiple studies have been carried out to evaluate peroxyacetic acid's ability to reduce pathogenic microorganisms present in fresh-produce leaves. In one study performed by Baert, Vandekinderen et al. (2009), the efficacy of peroxyacetic acid (250 mg/L) in removing the murine norovirus, *Listeria monocytogenes* and *Escherichia coli* O157:H7 present in iceberg lettuce was evaluated. They reported that when the lettuce was sanitized with peroxyacetic acid (250 mg/L), the microbial loads of *L. monocytogenes*, *Escherichia coli* O157:H7 and murine norovirus were lowered by 1.03, 1.30 log CFU/g, and 1.0 Log PFU/g respectively. In addition, Fuzawa, Ku et al. (2016), reported 1-3 log reductions in porcine rotavirus inoculated on three leafy-green cultivars ("Totem" Belgian endive, "Starbor" kale and "Red Russian" kale) after a disinfection step with peroxyacetic acid (50 mg/L).

2.2.3 Organic acids in combination with surfactant-based sanitizers

Natural and synthetic surfactants are widely used by the food industry as emulsifying and wetting agents in egg whites, fruit juices, vegetable oils, and gelatins (Kralova and Sjöblom 2009). In addition, synthetic surfactants are used as additives to enhance herbicide performance (Stock

and Holloway Peter 1993). Recently, the combination of organic acids and surfactants as additives for the washing of fresh produce has gained interest (Sapers 2009). Many surfactants are not antimicrobial agents, they work to sanitize fresh produce by detaching the microorganisms and increasing the wettability of its surfaces (Palma-Salgado 2013, Warriner and Namvar 2014).

A number of studies have evaluated the efficacy of organic acids (lactic acid, Levulinic acid) in combination with surfactants (sodium dodecyl sulfate) for the removal or inactivation of pathogenic microorganisms (*Escherichia coli* spp and *Salmonella* spp) from beef trims (Zhao, Zhao et al. 2014), deli-meat slicers (Chen, Zhao et al. 2014), cantaloupes (Webb, Erickson et al. 2015), and alfalfa seeds used for sprouting (Zhao, Zhao et al. 2010). However, there are a limited number of studies regarding the effectiveness of the organic acids and surfactant-based sanitizers utilized on leafy greens. In a study performed by Huang and Nitin (2017), the effects of adding different food-grade surfactants (Tween-20, sodium dodecyl sulfate, and lauric arginate) in removing *Escherichia coli* O157:H7, *Listeria innocua*, and the *Escherichia coli* bacteriophage T7 from the surface of fresh produce were evaluated. They reported that the addition of surfactants did not increase the removal of viruses from lettuce-leaf surfaces, while a greater removal of bacteria was achieved by the addition of 0.1% lauric arginate. In addition, a study performed by Fuzawa, Ku et al. (2016) examined the effectiveness of malic acid in combination with the surfactant thiamine dilaurylsulfate (TDS) in removing porcine rotavirus during the sanitization of leafy greens (“Totem” Belgian endive, “Starbor” kale, “Red Russian” kale). They reported that using a surfactant-based sanitizer resulted in a greater removal of porcine rotavirus from Belgian endive leaves (3.0 $\log_{10}$ reduction) compared to the oxidant-based sanitizer peroxyacetic acid, which achieved 1.0 $\log_{10}$ reduction.

2.2.4 Ultrasonication as a physical treatment for the sanitation of fresh produce

Ultrasound is a form of energy generated by sound waves at frequencies higher than the upper audible limit of human hearing (São José, Andrade et al. 2014). Ultrasound is generated by electrical energy supplied to a piezoelectric material referred to as a transducer; the transducer turns the electrical energy into a mechanical vibration of a particular frequency (Kentish and Feng 2014). In the food industry, two types of ultrasonic transducers are used for the treatments of foods: an ultrasonic probe, which is a cylinder in direct contact with the item being treated (Figure 2.1), and an ultrasonic tank or bath with transducers mounted to the bottom wall or stainless-steel blocks mounted on its walls (Figure 2.2) (Yu et al. 2016). In food processing applications, power ultrasound at frequencies between 20 and 100 kHz is used (Martini 2013).

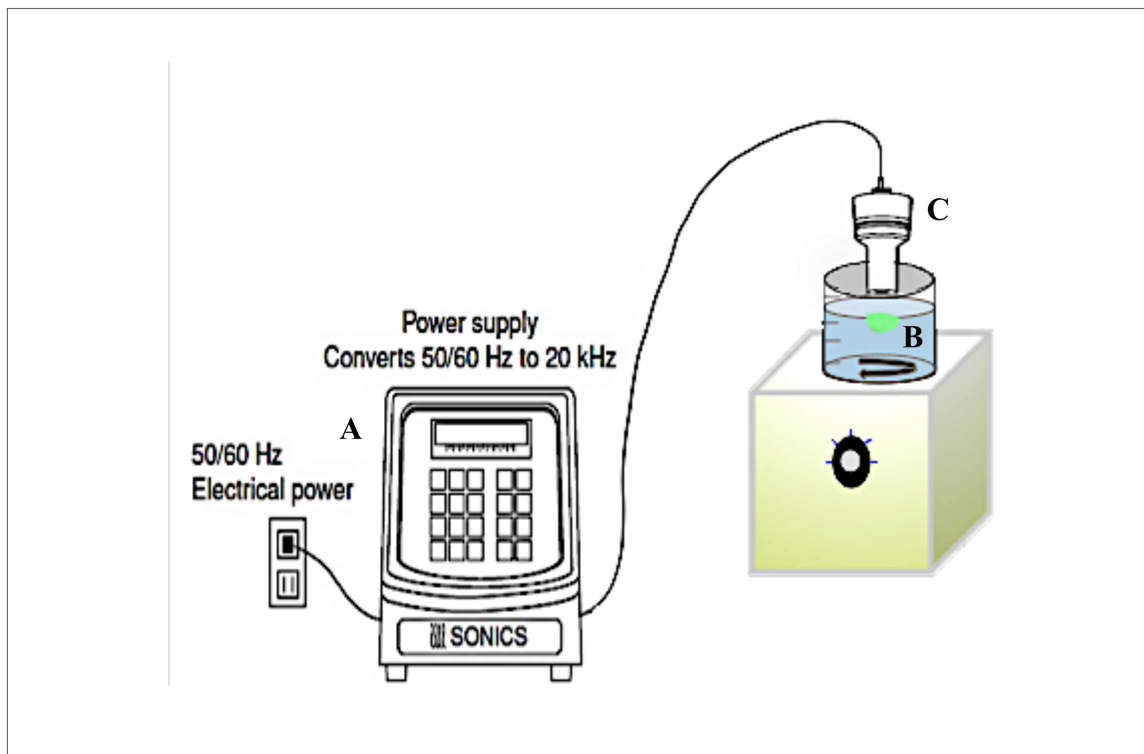

Figure 2.1. Scheme of the ultrasound probe system, (a) Generator, (b) water holder, (c) transducer probe

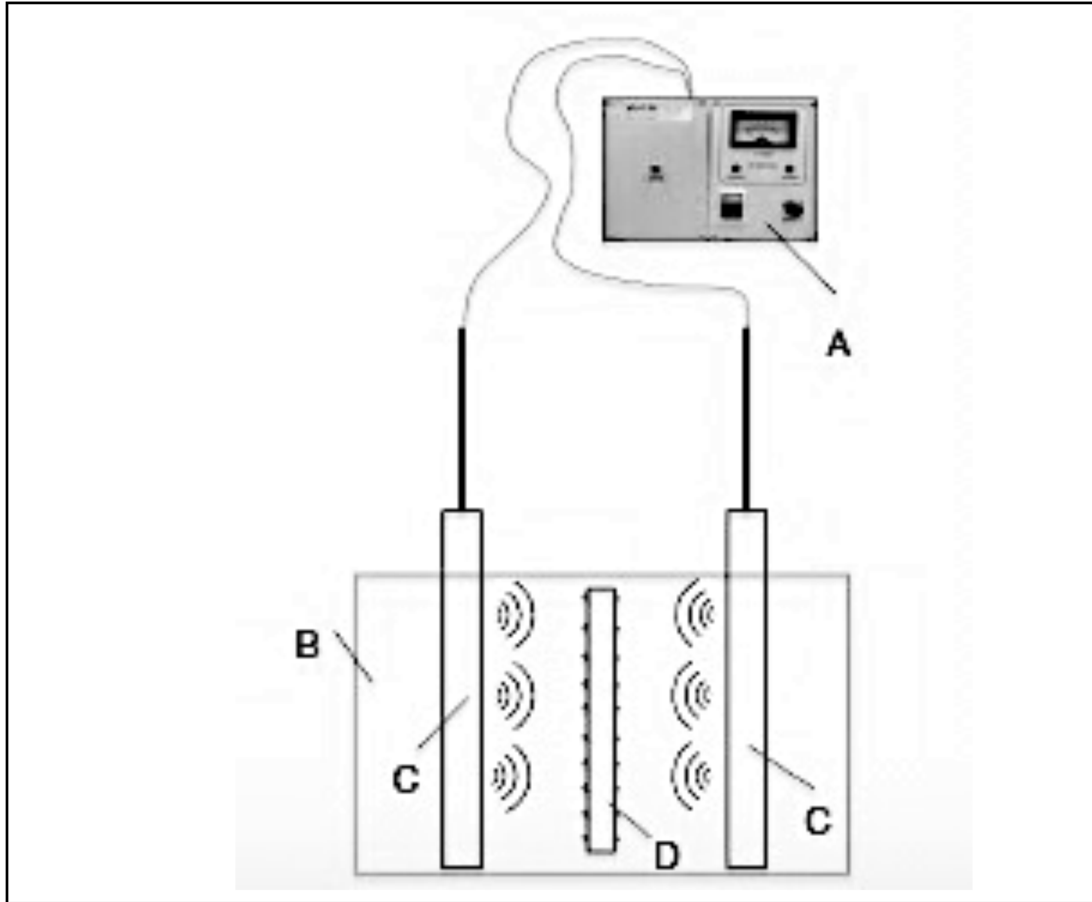

Figure 2.2. Scheme of the ultrasound tank system used in Yu et al. (2016), (a) Generator, (b) water tank, (c) transducer box, and (d) sample holder.

Power ultrasound or high-intensity ultrasound has different applications in the food industry, such as the preparation of emulsions, disruption of cells and aggregation of materials (Sango, Abela et al. 2014). Recently, the usage of ultrasound in the disinfecting of fruits, vegetables and fresh produce has gained interest. Microorganisms are attached to fresh produce are dislodged by cavitation forces created by the ultrasound (Zhou 2011). In addition, ultrasound enhances the penetration of sanitizers into micro-crevices in the produce surface and helps maintain a constant sanitizer concentration in the vicinity of the produce surface (Palma, Zhou et

al. 2017). Among the potential drawbacks of using ultrasound in the food industry are its high initial cost and potential energy consumption (Kentish and Feng 2014). Also, it has been reported that issues such as the presence of dissolved gases in the washing solution can decrease ultrasound's cavitation activity (Zhou, Feng et al. 2012) and the lack of uniformity in the acoustic-field distribution can also decrease ultrasound's efficacy during sanitization (Palma, Zhou et al. 2017).

In a study performed by Zhou et al. (2012), they evaluated the efficacy of an ultrasonic washing system in removing *Escherichia coli* O157:H7 from the surface of spinach leaves washed either individually or in batches using chlorine as a chemical sanitizer. They reported that addition of ultrasound to the traditional chlorine washing system resulted in an increased removal of 1.0 Log_{10} CFU/g when washing leaves individually, and of 0.5 Log_{10} CFU/g when washing them in batches. Similarly, Palma-Salgado et al. (2014), evaluated the efficacy of an ultrasonication washing system in the removal of *Escherichia coli* O157:H7 from the surface of iceberg lettuce treated by the traditional cutting-before-washing process used by the food industry, and compared it to lettuce treated by a newly proposed washing-before-cutting process. They reported an increased removal of 0.6-0.7 Log10 CFU/g in the samples that were washed with an ultrasound and chlorine combined method using the washing-before-cutting process.

2.3 *ESCHERICHIA COLI* O157:H7 AND FOODBORNE OUTBREAKS

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, anaerobic bacterium. It is a very small microorganism measuring 2.5 μm long by 0.8 μm in diameter and having a generation time of 15-20 minutes (Berg 2004). Most *Escherichia coli* strains are harmless, naturally colonizing the gastrointestinal tracts of healthy cattle and humans (Lim, Yoon et al. 2010, ICMSF 2018).

However, some strains are virulent and can cause serious gastrointestinal diseases in humans (Berg 2004, Pennington 2010). The most important virulence factor identified for pathogenic *Escherichia coli* strains is the production of Shiga toxins (Stx), among the most potent bacterial toxins known. Thus, these strains are referred to as the Stx-producing *Escherichia coli* (STEC) (Law 2001)

In the US, *Escherichia coli* O157:H7 is the most commonly identified STEC causing diseases in humans: it causes 3600 hospitalizations and 30 deaths every year (CDC 2017, CDC 2018). During a STEC infection, the shigatoxins can damage cells of the intestinal epithelium and cause apoptosis (Tesh 2010, Melton-Celsa 2014). In addition, one dangerous outcome of an *Escherichia coli* O157:H7 infection in humans is the possibility of developing a life-threatening kidney disease called hemolytic uremic syndrome (HUS). Although HUS can be caused by different infections, about 90% of the cases reported globally are due to Stx infections (Vanaja, Jandhyala et al. 2013). The sequelae of HUS include acute renal failure, out of which 30% develop into permanent renal damage and even death (Thorpe 2004, Spinale, Ruebner et al. 2013, CDC 2014).

It is estimated that 30% of foodborne illnesses are caused by the consumption of fresh produce contaminated with a human pathogen. *Escherichia coli* has been identified as the second leading cause of fresh-produce contamination (Painter, Hoekstra et al. 2013). Furthermore, leafy greens such as spinach and lettuce have been identified as vehicles for the transmission of STEC infections and food recalls; since 2010, a total of seven foodborne outbreaks of fresh produce contaminated with STEC have been reported in the US (CDC 2017). It has been reported as well that the attachment of *Escherichia coli* to fresh produce can occur at any stage in the farm-to-fork process (Islam, Michael P. Doyle et al. 2004, Park 2013). The possible factors that influence the

attachment of bacteria and other microorganisms to surfaces include hydrophobicity (wettability), roughness (a component of texture) (Wang, Feng et al. 2009, Fransisca and Feng 2012) and the presence of epicuticular wax (chemistry of the surface) (Lu, Ku et al. 2015).

Over the years, the United States Food and Drug Administration (FDA) and the food industry have developed and established different strategies during the post-harvest and processing of produce to minimize the incidence of contamination with pathogenic microorganisms (Herdt and Feng 2009, FDA 2017). However, these efforts sometimes fail due to many economic and environmental factors. For instance, water, which is the main tool used for sanitization, is usually reconditioned and recycled during the disinfection of fresh produce and fresh-cut produce (Gómez-López and Gogate 2018). In the same way, sodium hypochlorite, the most common chemical sanitizer used by the food industry, can react with organic matter present in the water wash and become depleted to levels at which it is no longer effective as an antimicrobial agent (López-Gálvez, Gil et al. 2010, Gómez-López, Lannoo et al. 2014). Thus, the food industry, government agencies and research groups are constantly developing chemical and non-thermal methodologies for minimizing the incidence of *Escherichia coli* and other microorganisms in fresh produce (Fransisca and Feng 2012, Tomás-Callejas, López-Gálvez et al. 2012, Bachelli, Amaral et al. 2013, Feng, Zhou et al. 2013).

2.4 NOROVIRUS AND FRESH-PRODUCE OUTBREAKS

Norovirus is a non-enveloped single stranded (+) RNA enteric virus (Figure 3A). It belongs to the caliciviridae family, and it is 26-35 nm in diameter (CDC 2009, Buesa and Rodriguez-Díaz 2016). Noroviruses are a leading cause of acute gastroenteritis, not only in the US but worldwide (Teunis Peter, Moe Christine et al. 2008). According to the CDC norovirus causes 19-21 million

cases of acute gastroenteritis, 56,000 hospitalizations and 570-800 deaths every year. In addition, due to the numerous genotypes of norovirus circulating in the environment, humans can get sick with the virus multiple times during their lifespan (CDC 2016).

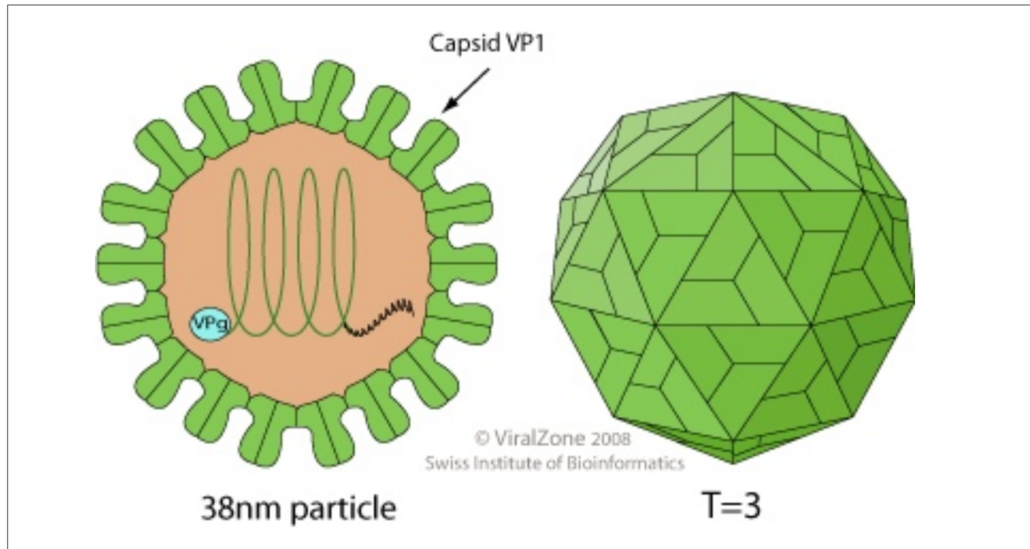

Figure 2.3. Structure of norovirus virion. Source:(SIB n.d.)

Like many other enteric viruses, noroviruses are strongly resistant to different environmental stresses and can persist on inanimate surfaces for up to seven days and still remain infectious (Kramer, Schwebke et al. 2006). Contamination with a norovirus usually occurs through a fecal-oral route, but indirect contamination through foods, fomites or contact with vomit can occur also (Kramer, Schwebke et al. 2006, Seymour and Appleton 2008, de Graaf, Villabruna et al. 2017). Since 2010, a total of 39 norovirus outbreaks related to consumption of spinach, romaine lettuce, leafy greens, and pre-packaged leafy greens have been reported in the US. The highest frequency was observed in 2012 with 5 outbreaks related to the consumption of romaine lettuce and 4 related to the consumption of spinach (Figure 2.4).

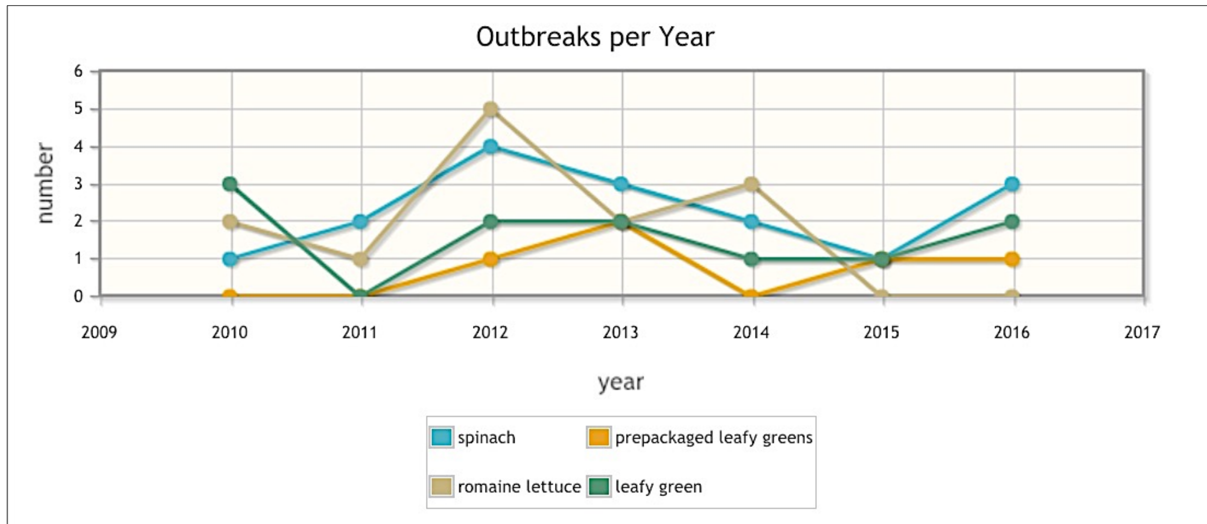

Figure 2.4. Norovirus outbreaks reported based on food items. Source: (NORS 2018)

Because of its small size and unstable genetic material, human norovirus is difficult to grow routinely in cell cultures in laboratory settings (Esseili, Saif et al. 2015) Researchers have resorted to using surrogate viruses from the same family (*caliciviridae*) to study the persistence of noroviruses on a variety of surfaces and their transfer to foods including fresh produce surfaces (D'Souza, Sair et al. 2006, Esseili, Saif et al. 2015). For instance, murine norovirus has been used as a human norovirus surrogate to study cross-contamination from water to produce and vice versa during the sanitization of fresh-cut lettuce (Holvoet, De Keuckelaere et al. 2014). In addition, the tulane virus has been studied as a norovirus surrogate to investigate the effects of viral inoculum level and type of fresh produce on the rate of viral internalization (Yang, Chambers et al. 2018). Lastly, porcine sapovirus has been studied as a surrogate to investigate different physicochemical treatments such as heat, oxidant-based sanitizers, and ethanol-based sanitizers applied during the disinfection of fresh lettuce (Wang, Zhang et al. 2012).

2.5 ROTAVIRUS AND FRESH-PRODUCE OUTBREAKS

Rotavirus is double-stranded RNA (dsRNA) enteric virus with a genome made up of 11 segments (Figure 2.5) belonging to the reoviridae family, its size is 60-80 nm (Ramig 1997). Rotavirus is the leading cause for foodborne illnesses in children under 5 years of age (Parashar, Hummelman et al. 2003, WHO 2016). In the US, rotavirus infections have been responsible for causing 50,000-60,000 hospitalizations and 30-60 deaths of young infants yearly (Malek, Curns et al. 2006). Furthermore, due to the multiple strains circulating in the environment, children, even when protected by a vaccination, can get rotavirus infections more than once before age 5 (CDC 2018).

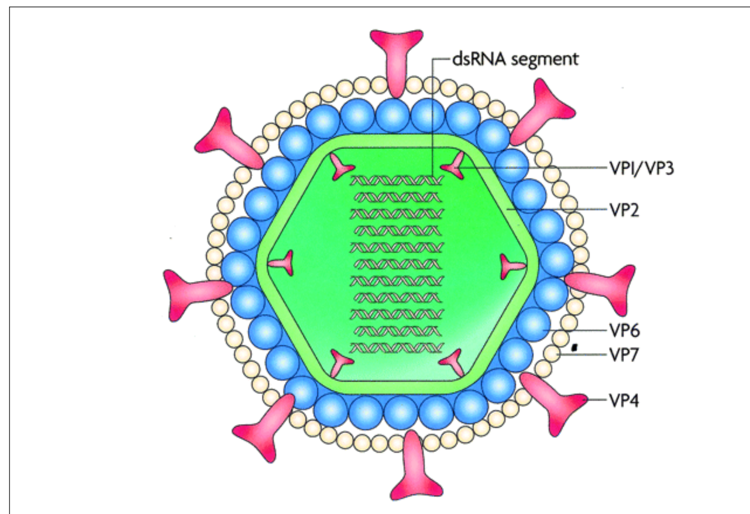

Figure 2.5. Structure of norovirus virion. Source: (Dennehy 2008)

Rotavirus gastroenteritis is not a reportable disease in the United States (CDC 2015); thus, it is challenging to quantify how many foodborne outbreaks and recalls have been linked to the consumption of contaminated foods. To fill in this gap, research groups have surveyed fresh-cut leafy greens, fruits and oysters, and have applied sequencing techniques to be able to identify the contamination in commercially available foods (Quiroz-Santiago, Vazquez-Salinas et al.

2014, Aw, Wengert et al. 2016, Fernandez-Cassi, Timoneda et al. 2017). In the same way, other research groups have investigated the efficacy of different sanitizers commonly used by the fresh-produce industry to reduce rotavirus in fresh produce (Fuzawa, Ku et al. 2016)

2.6 TOPOGRAPHICAL CHARACTERISTICS OF PLANT LEAVES THAT PROMOTE THE ATTACHMENT OF MICROORGANISMS

Numerous studies have focused on the colonization, attachment and removal of microorganisms (bacteria, viruses, yeasts and molds) to food surfaces over the past few decades (Wang, Feng et al. 2009, Palma-Salgado, Pearlstein et al. 2014, Lu, Ku et al. 2015, Lazouskaya, Sun et al. 2016). Due to inconsistencies and inconclusiveness in the rates of bacterial colonies attaching to identical food matrices, an increase in studies for identifying the physiochemical factors that promote the attachment of microorganisms to food surfaces has been observed (Lu, Ku et al. 2015, Park and Kang 2017). For inanimate surfaces, surface hydrophobicity and surface roughness are the two main parameters that have been identified as factors that promote attachment (Li and Logan 2004, Crawford, Webb et al. 2012, Preedy, Perni et al. 2014). For leafy greens and other fresh-produce items, epicuticular composition has been identified as a third parameter that promotes the attachment of microorganisms (Hunter, Shaw et al. 2015, Fuzawa, Ku et al. 2016). These different parameters are considered in more detail below.

2.6.1 Surface roughness

Surface roughness is defined as the arithmetic average of the absolute values of the surface height deviations measured from the mean plane (R_a) [1.1] (DeGarmo, Black et al. 1997). It is a parameter used as a measurement of surface finish and is a component of surface texture (Wang,

Zhou et al. 2012). The roughness values of a surface can be affected by deviations of the size scales caused by the height of features above and below the mean surface level (peaks and valleys), and by the lateral separations of these heights (Bennett 2007). The surface roughness is quantified by the vertical deviations of a real surface from its ideal form. If these deviations are large, a surface is considered rough, and if they are small, the surface is considered smooth (Wang, Zhou et al. 2012). R_a is given in equation 1.1.

$$R_a = \frac{1}{n}\sum_{i=1}^{n}|y_i| \qquad [1.1]$$

where

R_a: arithmetic average roughness

n: number of observations

y_i: absolute value of peak height

The roughness of the epidermal layer of a leaf can be affected by many of its components, such as micro asperities formed by the epidermal cells, and by the presence of hierarchical structures such as three-dimensional epicuticular wax crystals (Koch and Barthlott 2009, Bhushan 2012). Roughness is an important parameter to take into consideration during disinfection studies. According to (Bhushan 2012), surface roughness can influence wettability, which is undesirable during the disinfection of food surfaces. (Fransisca and Feng 2012) evaluated the effect of the surface roughness of three types of seeds used for sprouting (alfalfa, broccoli, and radish) on the removal of *Escherichia coli* O157:H7 using different sanitizers. They reported that the rougher surfaces had less removal of bacteria; furthermore, they found that the surface roughness values were dependent on the cultivar tested; thus, they concluded that the combination sanitizer-seed should not be translated to all cultivars tested.

2.6.2 Surface hydrophobicity

Surface hydrophobicity is a parameter used to describe the wettability of a surface (Nosonovsky 2008). It is determined using a method called the contact-angle method: a known volume of a liquid is placed on a sample surface, while a high-resolution camera captures the profile of the liquid/solid interface, and the contact angle is determined using an image-analysis software (Figure 2.6) (Law and Zhao 2015). The software uses Young's equation (Good 1992) [1.2]. Important considerations are necessary for this measurement: the liquid and surface have to be nonreactive physically and chemically; usually the liquid is water, and it has to be free of contaminants; the value obtained is referred to as the water-contact angle (WCA) and is expressed in degrees (°) (Wang, Zhou et al. 2012, Yuan and Lee 2013, Law and Zhao 2015). Depending on the wetting behavior and contact angle exhibited, surfaces are classified as either hydrophilic, when their contact angle (θ) < 90°, or hydrophobic, when their contact angle > 90° (Figure 2.7); furthermore, if they exhibit a contact angle between 120-150°, they are called superhydrophobic, and possess self-cleaning properties (Yuan and Lee 2013). θ is given in equation 1.2

$$\gamma lv \cos \theta Y = \gamma sv - \gamma sl \qquad [1.2]$$

where

θY: contact angle.

γlv: liquid-vapor interfacial tension

γsv: solid-vapor interfacial tension

γsl: solid-liquid interfacial tension

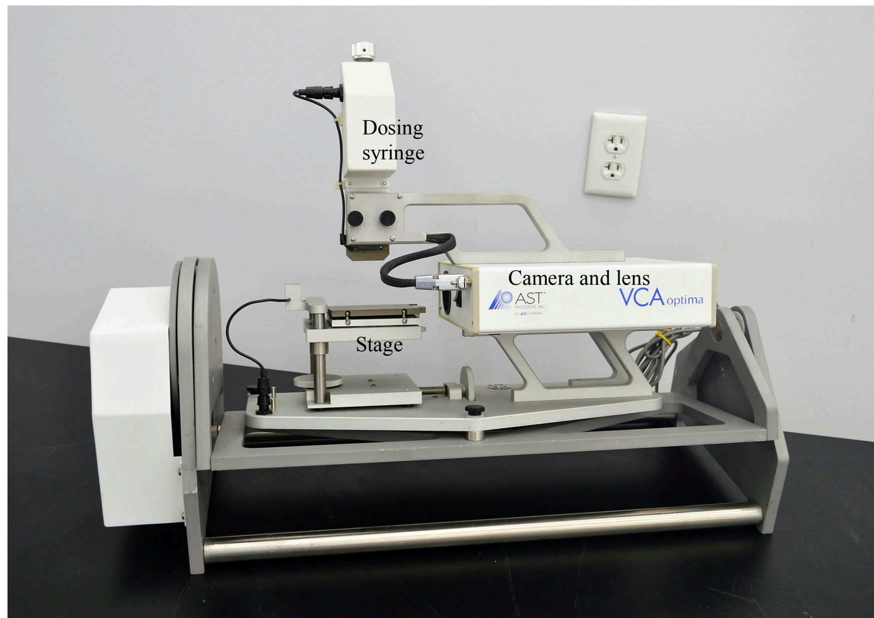

Figure 2.6. Photograph of goniometer VCA Optima (AST n.d.)

Surface hydrophobicity can be affected by roughness, asperities and impurities present on the surface (Bhushan 2012). In a study reported by (Wang, Feng et al. 2009), the surface hydrophobicity of four fruits (apples, avocados, cantaloupes, and oranges) was determined. Large deviations were found, mainly due to biological factors such as ripeness of the fruit, which can affect the texture, and external factors such as the location of the water droplet during the contact-angle measurement. Although the effect of surface hydrophobicity on the attachment of organisms to inanimate surfaces has been a topic of interest in many studies, only a few studies have been carried out regarding the effect of the surface hydrophobicity of food surfaces on the attachment of microorganisms. In one study, (Wang, Feng et al. 2009) compared the attachment of *Escherichia coli* O157:H7 to two fruit surfaces that had similar surface hydrophobicities but different roughnesses and found that hydrophobicity had no role in the attachment of bacteria. In the same way, (Lu, Ku et al. 2015) evaluated the effect of the hydrophobicity of 24 leafy-green

cultivars on allowing or preventing the attachment of porcine rotavirus. They concluded that hydrophobicity expressed as WCA played no significant role in the attachment of virions to fresh produce.

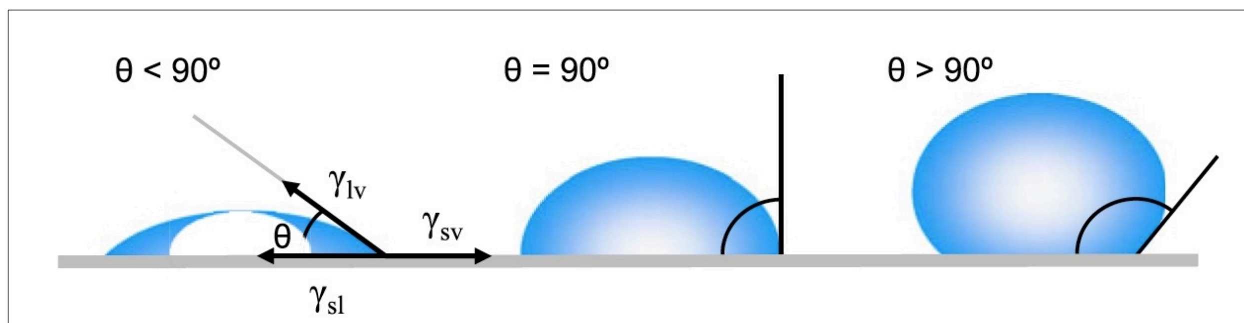

Figure 2.7. Illustration of wetting behavior of a smooth surface based on contact angle (Yuan and Lee 2013)

2.6.3 Epicuticular composition

Epicuticular wax refers to a coating made out of soluble lipids that covers the outer cuticle of a plant (Kolattukudy 1987). In many species, epicuticular waxes consists of either a smooth thin monolayer or a mixture of a thin monolayer and higher hierarchical structures called three-dimensional wax crystals (Gniwotta, Vogg et al. 2005). In addition, within these three-dimensional wax crystals, a wide range of forms can occur, e.g. platelets, ribbons, and rodlets (Barthlott, Neinhuis et al. 1998). The chemical composition of epicuticular wax is usually a mixture of aliphatic or cyclic components including hydrocarbons, long-chain fatty acids, aldehydes, ketones and primary and secondary alcohols (Buschhaus and Jetter 2011). The principal roles of epicuticular wax are to prevent water loss via evaporation, electrolyte leakage from the leaf

interior, and control foliar absorption (Stevens Peter and Baker Edward 1987, Bargel, Barthlott et al. 2004).

Research regarding the effect of epicuticular waxes on the attachment of food-borne pathogens to food surfaces and fresh-produce leaves is limited compared to that on other topographical characteristics such as surface roughness and hydrophobicity. In a study performed by Hunter et al. (2015), an investigation was done of the effect of the epicuticular wax of older vs. younger lettuce leaves on the attachment of *Salmonella enterica* ser. Seftenberg. They found a positive correlation between epicuticular wax and *Salmonella* attachment. On the other hand, Lu, Ku et al. (2015) evaluated the effect of epicuticular wax on the attachment of a porcine rotavirus (strain OSU) to 24 leafy-green cultivars. They reported that the presence of crystalline wax structures inhibited the adhesion of porcine rotavirus to surfaces. Thus, it is hard to define the role of epicuticular wax in attachment of bacteria and viruses to plant surfaces.

2.7 USE OF ARTIFICIAL LEAF SURFACES TO STUDY BACTERIA-PLANT INTERACTIONS

The consumption of fresh produce and vegetables is part of a healthy human diet (VanFrank, Onufrak et al. 2018). Over the past eight years, fresh produce, specifically leafy greens, has been involved in eight foodborne outbreaks caused by *contamination* by *Escherichia coli* spp (CDC 2017). Because prevention is key, many strategies, including the FDA Food Safety Modernization Act (FSMA), use of chemical sanitizers to water-wash as well as physical intervention technologies have been developed to prevent bacterial attachment to the surfaces of leafy greens (FDA 2017, Palma, Zhou et al. 2017). Due to their biological nature, plant leaves are diverse in structure, morphology and chemical composition, depending on growing conditions as

well as stage of maturity (Bhushan 2009, Zhang, Luo et al. 2014). Due to the variability of both the chemistry and topography of natural plant surfaces, it is challenging to deduce the plant-bacteria interactions in attachment studies (Bunpot Sirinutsomboon 2007), and it is equally challenging to replicate or generalize the results to other plant species (Fransisca and Feng 2012).

One approach that researchers have taken to overcome biological variability is the development of artificial surfaces with an ultimate goal of mimicking either the physical, biological, or chemical characteristics of plant surfaces. The most common techniques utilized for the development of these artificial surfaces include microfabrication technology called *soft lithography*, and double-casting replica molding (Doan and Leveau 2015). In both cases silicon is used as the base material to create a mold of the surface to be replicated, and a polymer material such as polydimethylsiloxane (PDMS) or soft agar is utilized as the base for the artificial surface. One of the issues of the artificial-surfaces is that they are exploited primarily to study only one factor (physical, chemical or topographical characteristics) for plant-bacteria interactions; however, plant surfaces are complex microbial habitats where physical, chemical, and biological factors can interact in bacterial attachment.

In a study carried by Sirinutsomboon et al. (2007), an artificial plant surface was developed using soft lithography (Figure 2.8). The microstructures of fresh-produce leaves were mimicked, and the researchers tried to recreate the epicuticular plant composition using thin films of paraffin wax and cellulose acetate. One of the accomplishments of this study was that they were able to standardize surface topographical characteristics such as surface roughness to study bacteria-plant interaction; however, the microstructures resembled pyramids (Figure 2.8 A) and plant cell wall ridges (Figure 2.8 B), while real plant surfaces are constituted of structures of higher complexity such as stomas; nor were they able to recreate epicuticular wax crystals. Furthermore, depending

on the source, paraffin wax composition might differ from the chemical composition of natural plant waxes (Asinger and Steiner 2016). In the same way, Zhang et al. (2014), developed artificial leaves to study bacteria-plant interactions using a combination of soft lithography and replica molding (Figure 2.9). In this study, they were able to replicate the plant surface morphology, as well as the surface roughness, and even surface hydrophobicity similar to that of fresh leaves. However, given that they used agarose (Figure 2.9 B) as the base for the artificial leaves, the plants lacked reusability; furthermore, the nature of agarose makes it difficult to use it for disinfection studies.

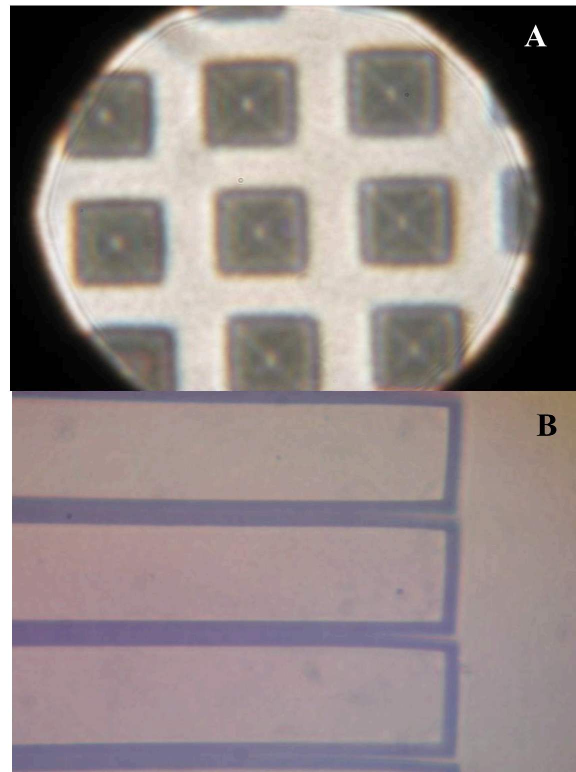

Figure 2.8. Artificial Plant surface (A)Micro-pyramids mimicking roughness of natural plant surface, (B) PDMS ridges mimicking ridges between plant surface cells. Source (Bunpot Sirinutsomboon 2007).

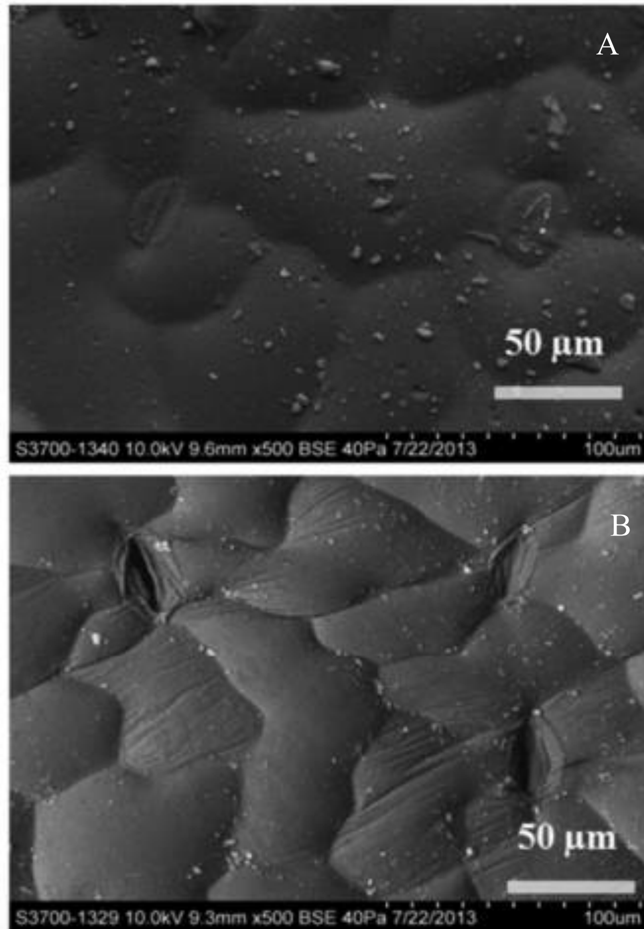

Figure 2.9. Biomimetically Patterned Surfaces of spinach and fresh spinach. (A) PDMS-agarose-based (AGAR) biomimetically patterned surfaces, (B) spinach leaf. Source (Zhang, Luo et al. 2014)

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CHAPTER 3

ADHESION AND REMOVAL OF *ESCHERICHIA COLI* K12 AS AFFECTED BY PRODUCE EPICUTICULAR WAX COMPOSITION, SURFACE ROUGHNESS, AND PRODUCE AND BACTERIAL SURFACE HYDROPHOBICITY

3.1 ABSTRACT

Contaminated leafy vegetables have been associated with high-profile outbreaks causing severe illnesses. A good understanding of the interactions between human pathogen and produce is essential for developing improved food safety control strategies. Currently, the role played by produce surface physiochemical characteristics in such interactions is not well-understood. This work was performed to examine the effects of produce physiochemical characteristics, including surface roughness, epicuticular wax composition, and produce and bacteria surface hydrophobicity on attachment and removal of vegetative bacteria. *Escherichia coli* K12 was used as a model microorganism, and its attachment to and removal from five leafy green vegetables after washing with selected sanitizers were evaluated. A detailed epicuticular wax component analysis was conducted, and the changes of the wax composition after sanitation were also evaluated. The results showed that *Escherichia coli* K12 removal is positively correlated with alkanes, ketones, and total wax content on leaf surfaces. Vegetables with high surface wax content had less rough leaf surfaces and more bacterial removal than the low wax produce. Produce surface roughness positively correlated to *Escherichia coli* K12 adhesion and negatively correlated to removal. The cells preferentially attached to cut vegetable surfaces, with up to 8.3 times more attachment than on leaf adaxial surfaces.

Keywords: Bacteria adhesion; hydrophobicity; surface roughness; epicuticular wax; sanitation; plant surface physiochemical.

3.2 INTRODUCTION

Consumption of fresh produce is essential for humans to maintain a healthy diet. In the United States, an increase in consumption of fresh produce through purchases from local growers, retailers, and consumed in restaurants and food service facilities has been observed (Olaimat and Holley, 2012). As the consumption of fresh produce increases, however, the risk of exposing consumers to foodborne pathogens and subsequent human gastroenteritis has also increased. Over the past two decades, an increase in the incidence of foodborne diseases related to consumption of fresh produce has been reported (Harris et al., 2003; Doyle and Erickson, 2008; CDC, 2013 & 2015). The most common vectors of foodborne diseases related to fresh produce are zoonotic pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp., among others (Jensen and Baggesen, 2014).

Despite of the production system used, fresh produce is grown in a manner prone to be colonized by human pathogens (Lindow and Brandl, 2003; Jochumsen, 2014). The commonly recognized routes of produce contamination include animals (and their feces), water, soil, human handling, seeds, and air (dust) (Harris et al., 2003; Barak and Schroeder, 2012). In any case, human enteric pathogens must come into contact with plants to initiate colonization. Understanding the bacterial and natural/synthetic surface interactions thus becomes important. There are currently two approaches to study bacterial and surface interactions: biochemical and surface thermodynamic methods (Chen et al., 2011). The biochemical method examines the effect of bacteria flagella, fimbriae (curli in *Escherichia coli*), O-antigen, and extracellular polysaccharides on adhesion and colonization (Lugtenberg and Dekkers, 1999; Critzer and Doyle, 2010). The surface thermodynamic analysis utilizes contact angles and zeta potentials of the interacting surfaces to determine the thermodynamic conditions for adhesion and the nature of the adhesion

forces that mediate attachment (Absolom et al., 1983; Chen et al., 2011). An important but often omitted aspect in bacteria adhesion study is the topology of the surface, especially those of plant surfaces. Considering the sizes of bacteria, e.g. 1.16 ± 0.31 μm in diameter for *Escherichia coli* O157:H7 (Bradford et al., 2006), produce leave surfaces are rough with “valleys” and “mountains,” which provide sites for enriched moisture and nutrients thus favoring bacterial attachment and colonization (Wang et al., 2012).

Compared with the many efforts to elucidate the mechanism of bacteria adhesion to plant surfaces, few studies have examined the role played by produce and bacteria surface physio-chemical properties in a bacteria removal, i.e. disinfection process. In the case of surface roughness, for instance, this is partially caused by a lack of reliable method to quantify roughness of soft and deformable plant surfaces. The early work of Wang et al. (2007 & 2009) introduced a non-contact confocal laser scanning microscopy (CLSM) method to reconstruct 3-D surface topography of produce and extract surface roughness information, which was used to examine the effect of surface roughness on removal of *Escherichia coli* O157:H7 from fruit and seed surfaces (Fransisca and Feng, 2012). In recent years, CLSM and similar instruments started to be equipped with capacity to analyze surface topography and estimate surface roughness, leading to a few recent reports on the effect of surface roughness on attachment and removal of viruses and pathogens to/from produce surfaces (Lu et al., 2015; Lazouskaya et al., 2016; Park and Kang, 2017).

The outermost layer of plant surfaces is largely composed of biopolyester cutin and epicuticular waxes, which directly interacts with microorganisms approaching and attaching to produce surfaces. Epicuticular waxes are a complex mixture of compounds, such as long-chain fatty acids, alcohols, alkanes, ketones, esters or triterpenoids (Jetter et al., 2006). A few studies

into plant leaf-associated bacterial interaction have examined the effect of leaf surface waxes on colonization of plant pathogens (Marcell and Beattie, 2002). Such studies often focus on comparison of waxy vs. non-waxy surfaces or the effect of cuticular mutants on colonization (O'Brien and Lindow 1989; Reisberg et al., 2013). The role that epicuticular waxes played in human pathogen and produce surface interactions is largely unknown. Our recent publications (Lu et al., 2015) reported that epicuticular waxes could act as a physical barrier to prevent infection by porcine rotavirus, and surface properties of fresh produce may affect the efficacy of virus disinfection (Fuzawa et al., 2016). However, there is no information on how the attachment of vegetative human pathogens to and their removal from vegetable leaf surfaces are affected by epicuticular wax compositions.

Leafy greens' involvement with human foodborne outbreaks highlights the need to provide new insight into human pathogen and plant surface interactions and their implications in microbial safety of fresh produce. This work was performed with a hope to shed light on the effects of physiochemical characteristics, including plant surface roughness, epicuticular wax composition, and produce and bacteria surface hydrophobicity, of plant surfaces on attachment and removal of vegetative human pathogen to/from plant surfaces. *Escherichia coli* K12 was used as a model microorganism, and its attachment and removal to/from five leafy green vegetables, e.g. 'Two star' lettuce, 'Totem' Belgian endive, 'Southern Giant Curled' mustard, 'Red Russian' kale, and 'Starbor' kale after washing with selected sanitizers were evaluated. A detailed epicuticular wax component analysis was conducted and the changes of wax composition after sanitation were also evaluated.

3.3 MATERIALS AND METHODS

3.3.1 Sample preparation

Five leafy green cultivars with surface hydrophobicity ranging from 58.3˚ to 128.7˚ and surface roughness of 3.2 to 10.0 μm (Table 1) were selected based on preliminary studies performed to twenty-four leafy green cultivars (APPENDIX 1). All seeds were purchased from Johnny's Selected Seeds (Winslow, ME, USA). Seeds of each cultivar were germinated in 32-cell plant plug trays filled with sunshine LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Urbana-Champaign under the conditions of 25 °C/17 °C and 14 h/10 h: day/night temperature regimen with supplemental lighting. Twenty days after germination, the seedlings were transferred to 4 L pots and harvested following the protocol used by Lu et al. (2015) to meet market maturity requirements. At the harvest time, a total of nine leaves were collected (three each from three plants) and immediately transported to a processing facility, stored at 5 ± 1 °C, and used within 72 hours of collection. A sterile 1.6-cm-diameter cork borer was used to cut three disks from each leaf prior to further analysis. Each set of leaves in this study, consisted of three biological replicates.

3.3.2 Bacterial strain preparation

Escherichia coli K12 from the food microbiology culture collection at the University of Illinois at Urbana-Champaign was used in the experiments. The bacterial strain was prepared by repeated sub-culturing on a Tryptic soy agar plate (Sigma Aldrich, St. Louis, MO. USA). Cultures of *Escherichia coli* K12 were grown in a tryptic soy broth (Sigma Aldrich, St. Louis, MO. USA) for 24 h at 37 °C with agitation at 180 RPM. Cells were harvested by centrifugation at 5 °C ± 1 °C and 4,332g for 8 minutes and washed twice in a sterile phosphate buffered saline solution. The

recovered *Escherichia coli* K12 precipitate was reconstituted in 100 ml of sterile 0.1 % peptone water. The initial inoculum solution was ten-fold serially diluted with 0.1% peptone water; 100 µl of the selected dilutions were spread plated in duplicate over tryptic soy agar plates and incubated at 37 °C for 24 h. The colony-forming units were counted manually, and it was determined that the initial inoculation level was 5.0×10^8 CFU/ml.

3.3.3 Bacterial cell surface hydrophobicity

Bacterial surface hydrophobicity was determined with two methods: the microbial adhesion to hydrocarbons (MATH) method (Rosenberg *et al.*, 1980), and the sessile drop method (goniometer) (van Loosdrecht *et al.*, 1987), both with slight modifications. Bacterial strain was grown as indicated in § 3.3.2. The recovered *Escherichia coli* K12 precipitate was reconstituted in 10 ml and 100 ml of sterile distilled water, reaching an initial inoculation level of 3.2×10^9 CFU/ml and 1.6×10^8 CFU/ml for the sessile drop and MATH method, respectively. The procedure for each method is considered in more detail below.

3.3.3.1 Sessile drop (goniometer) method.

Contact angles of bacteria surfaces were determined on bacteria lawns produced by spreading 100 µl of reconstituted *Escherichia coli* K12 (initial inoculum 3.2×10^9 CFU/ml) onto tryptic soy agar plates and incubated at 37 °C for 24 h. At the end of the incubation, the plates containing *Escherichia coli* K12 colonies were placed in a laminar-flow purifier PCR enclosure with a vertical airflow of 0.3-0.4 m/s (Labconco ®, Kansas City, MO, USA) and allowed to dry for 1 hour at 22 °C to remove excess moisture. The dried agar plates containing cell lawns were cut into squares of 1 cm^2 and taped (3M, Minnesota, USA) to a microscope slide. *Escherichia coli*

K12 cell surface contact angles were measured by placing 1μl of distilled water on top of the cell surface and determining water contact angle using a VCA Optima contact angle instrument and VCA Optima XE software ® (AST Products Inc. Billerica, MA. USA).

3.3.3.2 Microbial adhesion to hydrocarbons (MATH) method

For the determination of cell surface hydrophobicity using the adhesion to hydrocarbons technique, 4 ml of *Escherichia coli* K12 (initial inoculum level 1.6×10^8 CFU/ml) were placed in round-bottom glass tubes containing 1 ml of *n*-hexadecane (Sigma Aldrich, St. Louis, MO. USA). The tubes were vortexed (Fisher Scientific, PA, USA) for 2 minutes followed by 15 minutes resting to allow for phase separation. Afterward, the aqueous phase was carefully removed with a transfer pipet (Fisher Scientific, PA, USA) and transferred to a 1 ml cuvette (Fisher Scientific, PA, USA) to determine the extent of bacterial cell partitioning between n-hexadecane and *Escherichia coli* K12 cell solution. The light absorbance of the aqueous phase was measured at 400 nanometers using a LAMBDA 1050® UV/Vis/NIR Spectrophotometer (Perkin Elmer, Waltham, MA. USA). Hydrophobicity was calculated using the following equation (Rosenberg et al., 1980):

$$\%\,\mathrm{hydrophobicity} = 100 * (\mathrm{Acontrol} - \mathrm{AMATH}) / (\mathrm{Acontrol}) \qquad [3.1]$$

Where;

Acontrol: % absorbance of the control culture not subjected to the MATH test,

AMATH: % absorbance of bacterial hydrocarbon mixture aqueous phase.

Strains with % hydrophobicity of ≥ 70%, 50-70% and < 50% were classified as high, moderate and low hydrophobicity, respectively (Qiao, 2012).

3.3.4 *Escherichia coli* K12 adherence assay

Each set of leaves in this study, consisting of three biological replicates of five leafy green cultivars were collected at commercial maturity from the greenhouse, stored at 5 °C ± 1 °C, and used within 72 hours of collection. The collected leaves were rinsed once with sterile distilled water at 25 °C ± 1 °C to remove soil debris on surface, and dried using Kimwipes® (Kimberly-Clark, Irving, TX. USA) with gentle patting to minimize leaf surface morphological disturbance. A sterile cork borer of 1.6 cm diameter was used to cut three disks (approximate area of each disk was 2.01 cm^2) from each leaf. Each disk was submerged with adaxial surface facing down into well plate cells containing 2 ml of *Escherichia coli* K12 inoculum. Plant disks were incubated at 8 °C ± 1 °C for 2 hours. After inoculation, the plant disks were transferred to a laminar-flow purifier PCR enclosure 22 °C ± 1 °C with a vertical airflow of 0.3-0.4 m/s (Labconco®, Kansas City, MO. USA) for one hour to allow further bacterial attachment. To remove loosely attached bacteria, the inoculated disks were transferred to a beaker containing sterilized distilled water at 25 °C, rinsed for one minute, and agitated at 150 RPM at a produce to water ratio of 1:1000 (w/w). To eliminate the possible bacteria internalization through the cut surfaces of the disks, prior to the disinfecting procedure, the edge of each disk was removed with a sterile cork borer of 1.2 cm diameter. The surface area of each inner disk was about 1.13 cm^2 after cutting that were used in sanitation treatments.

3.3.5 Sanitation procedure

Nine disks from each vegetable cultivar were used for sanitation tests. The disks were inoculated and prepared for sanitation as stated in § 3.3.2. The disks were treated for 1 minute at a ratio of 1:150 (produce/sanitizer solution, w/w) at room temperature in one of the following

solutions: distilled water, chlorine, peroxyacetic acid, and malic acid with thiamine dilauryl sulfate (TDS). The chlorine solutions were prepared by dilution of Pure Bright germicidal bleach® (active ingredient: sodium hypochlorite, 6.0%) to 100 mg/L of free chlorine. The peroxyacetic acid solutions were prepared by dilution of Tsunami-100® (active ingredient: peroxyacetic acid) to 80 mg/L of acid concentration. Malic acid + TDS solution were prepared by dilution of malic acid and TDS to 0.5% w/v of acid and 0.05 % w/v of TDS. The sanitizing solutions were transferred to sterile beakers containing a metal stirring bar, and after addition of the vegetable disks, agitated at 150 RPM for one minute to sanitize the disks.

3.3.6 Enumeration of *Escherichia coli* K12

The *Escherichia coli* K12 count on the outer rings and 1.13 cm^2 inner disks was enumerated separately. The outer rings and the inner disks before and after sanitation were separately transferred to sampling bags (Fisher Scientific, MA, USA) containing 0.1% peptone water, and supplemented with 0.1% sodium thiosulfate to neutralize chlorine and stop chlorine associated reactions. For treatment with the peroxyacetic acid and malic acid + TDS, phosphate buffered saline was added to neutralize and stop the reaction. Samples were pummeled for 2 minutes, followed by a two-minute resting to allow any existing foam to dissolve. The pummeled samples were ten-fold serially diluted with 0.1% peptone water; 100 µl of the selected dilutions was spread plated in duplicate over Tryptic Soy Agar plates and incubated at 37 °C for 24 hours. The colony-forming units were counted manually.

3.3.7 Plant surface roughness and hydrophobicity

Surface roughness of five produce phenotypes was determined using the laser confocal scanning microscopy non-contact method, following the procedure of Lu et al. (2015). The arithmetic average of the absolute values of the surface height deviations measured from the mean plane was used as the roughness (R_a) of the plant surfaces. For produce surface hydrophobicity measurement, nine disks (approximate area of each disk was 2.01 cm^2) from each vegetable cultivar were left untreated and nine were sanitized with a produce to sanitizer ratio of 1:40 (w/w) in water or one of the sanitizer solutions mentioned in section 2.5. The treated disks were dried using Kimwipes® (Kimberly-Clark, Irving TX. USA) with gentle patting and then taped (3M, Minnesota, USA) to a microscope glass slide with the adaxial surface of leaves facing up. The glass slides containing the leaf disks were covered with moistened paper towel and aluminum foil to prevent dehydration. Water contact angle was obtained using a goniometer (KSV Instruments, Stockholm, Sweden) model CAM 200. Using a calibrated pipette, 5 μl of deionized water was placed at the center of each disk and within 20 seconds five contact angle readings were measured.

3.3.8 Scanning Electron Microscopy

Surface characterization was carried using a Scanning Electron Microscope. Images of the epicuticular surface were taken using a FEI Quanta FEG 450 ESEM (Hillsboro, OR, USA). The images were captured under low vacuum at 20 kV and at 400X, 800X, and 1200 X resolution from at least three different samples.

3.3.9 Statistical analysis.

The experiments were performed with a complete randomized design (CRD) with each treatment conducted three times. The *Escherichia coli* K12 population counts were subjected to logarithmic transformation before statistical analysis. Data were analyzed using a general linear model available in SAS version 9.1 (SAS Institute, Raleigh, NC, USA). Significant differences and separation was determined using Student's T-test and Tukey's test with $\alpha = 0.05$.

3.4 RESULTS AND DISCUSSION

3.4.1 Role of produce and *Escherichia coli* K12 cell surface hydrophobicity on adhesion

The contact angle obtained with the sessile drop method for *Escherichia coli* K12 was 22°, indicating that the *Escherichia coli* K12 cell surfaces were hydrophilic. Similarly, the percentage of *Escherichia coli* K12 cells adhering to hydrocarbons was 18%, also showing a hydrophilic nature. Since generally bacteria with hydrophilic cell surfaces prefer hydrophilic surfaces (An and Friedman, 1998), we expected *Escherichia coli* K12 cells would attach more to hydrophilic produce surfaces. As can be seen in Table 3.1, this was indeed the case for 'Two star' lettuce with a WCA of 62.1^o and the highest attachment (6.8 ± 0.3 Log CFU/cm^2), whereas for 'Red Russian' kale and 'Starbor' kale with hydrophobic surfaces (WCA = 125.6^o and 128.7^o, respectively) they had the second lowest attachment among the 5 vegetables (Table 1). 'Totem' Belgian endive was an exception to which the least attachment (5.7 ± 0.2 Log CFU/cm^2) of *Escherichia coli* K12 was recorded. It is known that for some bacteria and surface pairs, the cell charge and hydrophobicity do not play a significant role in the attachment. For instance, Boyer et al. (2007) reported that no association between the *Escherichia coli* O157:H7 cells' hydrophobic characteristics and lettuce attachment among curli-producing and deficient strains. Therefore, hydrophobicity of the produce

and the bacterial cell surface alone explained adhesion trend for ‘Red Russian’ kale and ‘Starbor’ kale but not for ‘Southern Giant Curled’ mustard. However, other factors should be considered.

3.4.2 *Escherichia coli* K12 adhesion to intact and cut produce surfaces

The adaxial surface roughness and contact angle of 5 plant phenotypes, together with *Escherichia coli* K12 adhesion are shown in Table 3.1. The *Escherichia coli* K12 attachment to adaxial of the inner disks of ‘Two star’ lettuce (6.8 ± 0.3 Log CFU/cm^2) was the highest among five phenotypes while the attachment to ‘Totem’ Belgian Endive (5.7 ± 0.2 Log CFU/cm^2) was the lowest. Produce with low surface roughness, such as ‘Starbor’ (1.8 μm) and ‘Red Russian’ kale (2.7 μm), had a less attachment except ‘Two star’ lettuce (3.2 μm) which had a hydrophilic surface and the highest attachment (6.8 ± 0.3 Log CFU/cm^2). The ‘Southern Giant Curled’ mustard with the roughest surface facilitated an *Escherichia coli* K12 attachment of 6.6 ± 0.2 Log CFU/cm^2, higher than all other produce phenotypes except ‘Two star’ lettuce. The hydrophobic plant surfaces (WCA < 90°) helped to reduce affinity of *Escherichia coli* K12 thus having an attachment lower than most other produce except ‘Totem’ Belgian endive. The contribution of surface roughness and contact angle to *Escherichia coli* K12 attachment seems to be intertwined for certain produce phenotypes, such as in the case of ‘Totem’ Belgian endive, which had the most hydrophilic surface and moderate roughness (4.9 μm) but the least attachment.

The attachment of *Escherichia coli* spp. to organic and inorganic surfaces relies more than one mechanism, including electrostatic interactions, hydrogen bonds, plant wetting properties related to its structure, as well as composition of bacterial cellular extracellular polysaccharides and the hydrophobicity of the bacterial cell wall (Wagner et al., 2003; Hassan and Frank, 2004; Goulter et al., 2009; Bazaka et al., 2011). According to Shaw et al. (2008), non O157 EHEC strains

adhere to salad vegetables via structural translocators called EspA, which also plays an important role in colonization of humans and bovine hosts. Our results generally support the concept that hydrophobic and less rough produce surfaces promote less bacterial attachment. However, this does not apply to ‘Totem’ Belgian endive with regard to hydrophobicity and ‘Two star’ lettuce regarding surface roughness. This indicates that bacterial adhesion to different plant surfaces is mediated by multiple factors for each bacteria-plant surface pair, and care must be taken to avoid extrapolating the finding of one bacteria-produce surface interaction to other plant surfaces.

The average thickness of the vegetable disks was 0.04 to 0.05 mm for all 5 cultivars as measured with a caliper. By assuming the same *Escherichia coli* cell density $\sigma_{adaxial}$ on the adaxial surface of the outer rings as on the inner disks, we used the average disk thickness 0.045 mm to estimate the *Escherichia coli* K12 attachment to cut edges of the vegetable disks. The (attached) cell density on the cut edge (σ_{edge}) was estimated with the following relations:

$$\sigma_{ring} \cdot A = \sigma_{adaxial} \cdot A + \sigma_{edge} \cdot B \text{ or } \sigma_{edge} = \frac{(\sigma_{ring} - \sigma_{adaxial}) \cdot A}{B} \quad [3.2]$$

where A is the adaxial area of the outer ring, B is the area of cut edge, and σ_{ring} (Log/cm^2) is a lumped attachment count of the cut ring (ring adaxial + cut edge) based on ring adaxial surface. As can be seen in Table 1, the attachment of *Escherichia coli* K12 to cut edge was much higher than that on the adaxial of the vegetable disks ($\sigma_{edge} > \sigma_{adaxial}$), except for that of ‘Southern Giant Curled’ mustard. For instance, the cells attached on the cut edge of ‘Totem’ Belgian endive was 8.3 times higher than that attached to its adaxial surface ($\sigma_{edge}/\sigma_{adaxial} = 8.3$). Previous studies have reported preferential attachment of human pathogens to cut and injured produce surfaces than intact surfaces (Han et al., 2000; Takeuchi et a., 2000). The findings in this study not only

confirmed the previous observations but also provided quantification on the difference in attachment. Our results also showed that the differential attachment to intact and cut surface is produce type specific. This may be caused by a number of reasons, such as the differences in the produce physicochemical properties and cut surface topography. The cells may attach to the cut surfaces due to leaking organic matter or may just be entrapped in the cut open cell structures and intercellular spaces (Solomon and Matthews, 2005).

3.4.3 *Escherichia coli* K12 removal from plant surfaces

The removal of *Escherichia coli* K12 from five produce surfaces after a sanitation treatment is shown in Figure 3.1. In all produce samples, the treatments with sanitizer removed more *Escherichia coli* K12 cells than washing with just water. The enhancement due to sanitation treatment is plant phenotype specific. The *Escherichia coli* K12 cell removal was more pronounced from relatively smooth produce surfaces, such as from 'Starbor' kale (1.8 μm, 2.0-2.6 Log CFU/cm^2) and 'Red Russian' kale (2.7 μm, 1.4-2.2 Log CFU/cm^2) while the removal from 'Southern Giant Curled' that had the roughest surface (10.0 μm) was the lowest (0.8-1.0 Log CFU/cm^2). This observation is in good agreement with a few previous studies that there was a negative correlation between surface roughness and bacterial removal. The work of Wang et al., (2009) examined removal of *Escherichia coli* O157:H7 from four fruits with surface roughness of 1.43 to 14.18 μm, and aluminum stubs with surface roughness of 0.30 to 8.41 μm. They observed an increase in cell attachment and a decrease in cell removal during a sanitation treatment with an increasing surface roughness for both fruits and aluminum stubs. A similar correlation was reported for removal of *Escherichia coli* O157:H7 from seed surfaces (Fransisca and Feng, 2012), and for a chlorine dioxide gas treatment of seven fruits and vegetables and seven organic and

inorganic surfaces for removal of *Escherichia coli* O157:H7 *Salmonella typhimurium*, and *Listeria monocytogenes* (Park and Kang, 2017). The results from this study and previous studies showed that sanitation was more effective for produce with smoother surface.

Among the sanitizers used in this study, the traditional chlorine treatment was more effective for disinfection of 'Two star' lettuce than the other two sanitizers. The peroxyacetic acid and the malic acid + TDS treatment obtained the greatest *Escherichia coli* K12 survival count reduction on 'Red Russian' and 'Starbor' kale, respectively. No sanitizer treatment was able to remove more *Escherichia coli* cells from all five vegetable surfaces than others, highlighting the complex nature of bacterial removal from plant surfaces. Besides surface roughness and hydrophobicity, the chemical homogeneity on produce surfaces may also be important. For instance, epicuticular wax crystals found on plant surfaces can create micro-roughness inducing non-wetting and water repellency conditions and impacting the flow and distribution of sanitizer on the surface thus impacting bacterial removal (Wagner et al., 2003; Wang et al., 2009).

3.4.4 Comparison of bacterial removal between low and high epicuticular wax plants.

The total wax content of two kales (67.7 – 85.0 μg/cm^2) was much greater than the other vegetables (3.04-9.44 μg/cm^2). We compared these 2 groups of plants with regard to wax composition to examine its correlation with bacterial removal (Table 3.2). There were significantly different epicuticular wax concentrations between kales and other leaf vegetables. The kale plants have significantly higher alkane, fatty acid, and ketone concentrations than average values from the other leaf vegetables ('Totem' Belgian endive, 'Two star' lettuce, and 'Southern giant curled' mustard). The averaged total wax concentration of two kales was 16.1-fold higher than other selected leaf vegetables. The increased wax concentration affected contact angle (about a 1.9-fold

difference). The higher wax containing plants tend to have less surface roughness. This can also be seen from the SEM microimages (Figure 3.2). The surfaces of the kales are covered with web-type epicuticular wax with no visible valleys and hills (cuticular folds), while the surfaces of the other 3 vegetable leaves showed stomata and cuticular folds. Note that the epicuticular wax on the kale cultivars was partially destroyed after fixation of *Escherichia coli* K12. They usually do not show any stomata because of thick covering waxes (intact SEM images are available in a previous publication, Lu et al., 2015). With regard to the size of *Escherichia coli*, the low wax plants with an average roughness of 6.0 μm may provide sheltering for cells harbored at the "valleys" of the surface during a produce washing/sanitation process. That might be part of the reason for the significantly less removal (1.0 ± 0.4 Log CFU/cm^2) in low wax samples compared to the removal from high wax produce surfaces (1.6 ± 0.7 Log CFU/cm^2).

3.4.5 Changes in plant hydrophobicity as affected by sanitation.

The changes in produce surface hydrophobicity after sanitation may have implications when considering re-contamination or cross-contamination of a produce that has went through a previous sanitation process. As shown in Figure 3.3, changes in plant adaxial hydrophobicity were observed after a sanitation treatment. The surface hydrophobicity of the treated produce surfaces can be higher or lower than the untreated counterpart, depending on the produce genotype and sanitizer. 'Starbor' kale treated with a combination of malic acid + TDS had a hydrophobicity about 30% lower than the untreated, while the reduction for 'Totem' Belgian endive and 'Two star' lettuce was 16% and 24%, respectively. Some treatments, such as chlorine, increased the surface hydrophobicity. The chlorine wash resulted in a nearly 30% increase of the surface hydrophobicity of 'Totem' Belgian endive. Although the mechanism behind the observed changes

in hydrophobicity is not clear, we hypothesized that the combination of organic acid (malic acid) and a surfactant (TDS) can detach or destroy the tridimensional epicuticular wax crystal structure changing it to a thin layer with no visible structure thus increasing wettability (more hydrophilic) (Tamura et al., 2001; Behnke et al., 2012).

3.5 CONCLUSIONS

Attachment and removal of *Escherichia coli* K12 to/from five vegetable cultivars were affected by multiple factors including produce genotype, produce surface roughness, and wax composition. *Escherichia coli* K12 cells preferentially attached to cut surfaces, with up to 8.3 times more attachment than to leaf adaxial surfaces. Leaf surface roughness was found to be positively correlated to *Escherichia coli* K12 adhesion and negatively correlated to its removal. For the first time, we reported the effect of epicuticular wax composition on removal of vegetative bacteria from vegetable leaves, e.g., the removal of *Escherichia coli* K12 was positively correlated with alkanes, ketones, and total wax content on leaf surfaces. In addition, vegetables with greater surface wax have less rough leaf surfaces and more bacterial removal than low wax produce.

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3.7 FIGURES AND TABLES

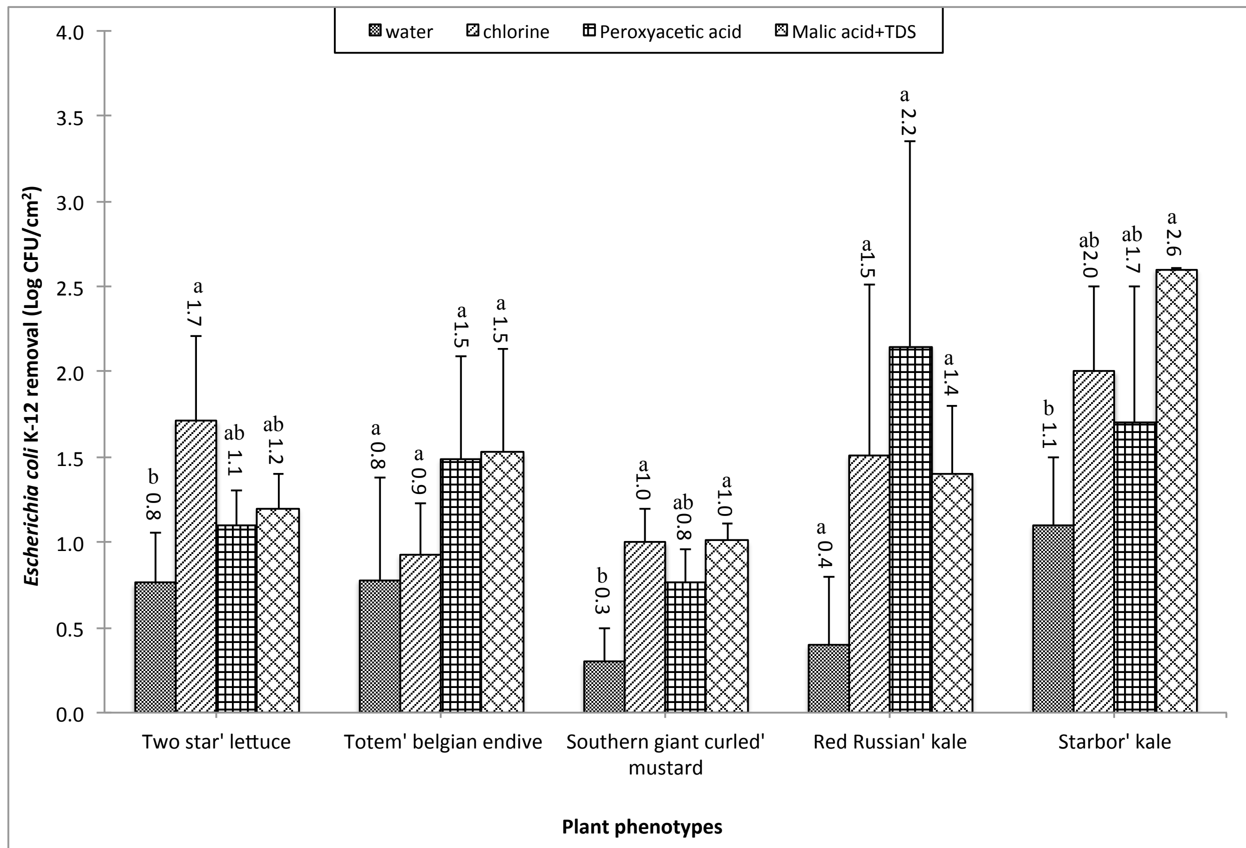

Figure 3.1. *Escherichia coli* K12 removal from plant surfaces, using selected sanitizers.

a-b: Different letters on bar (sanitizer) indicate significantly different *Escherichia coli* K12 removal within the same vegetable cultivar by Tukey's test (α= 0.05).

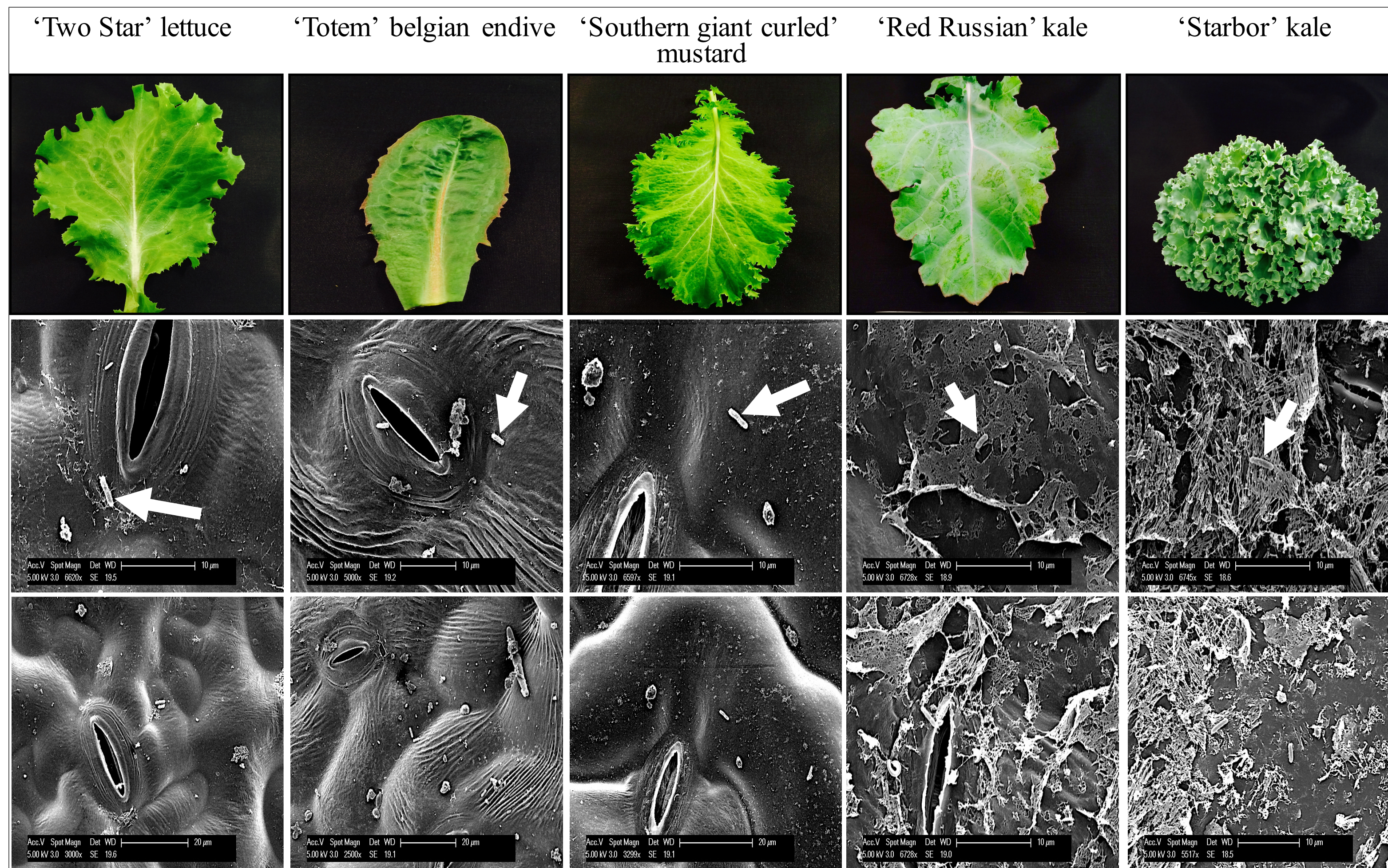

Figure 3.2. Images of produce cultivar phenotypes and SEM micro-images of *Escherichia coli* K12 attachment to epicuticular surface.

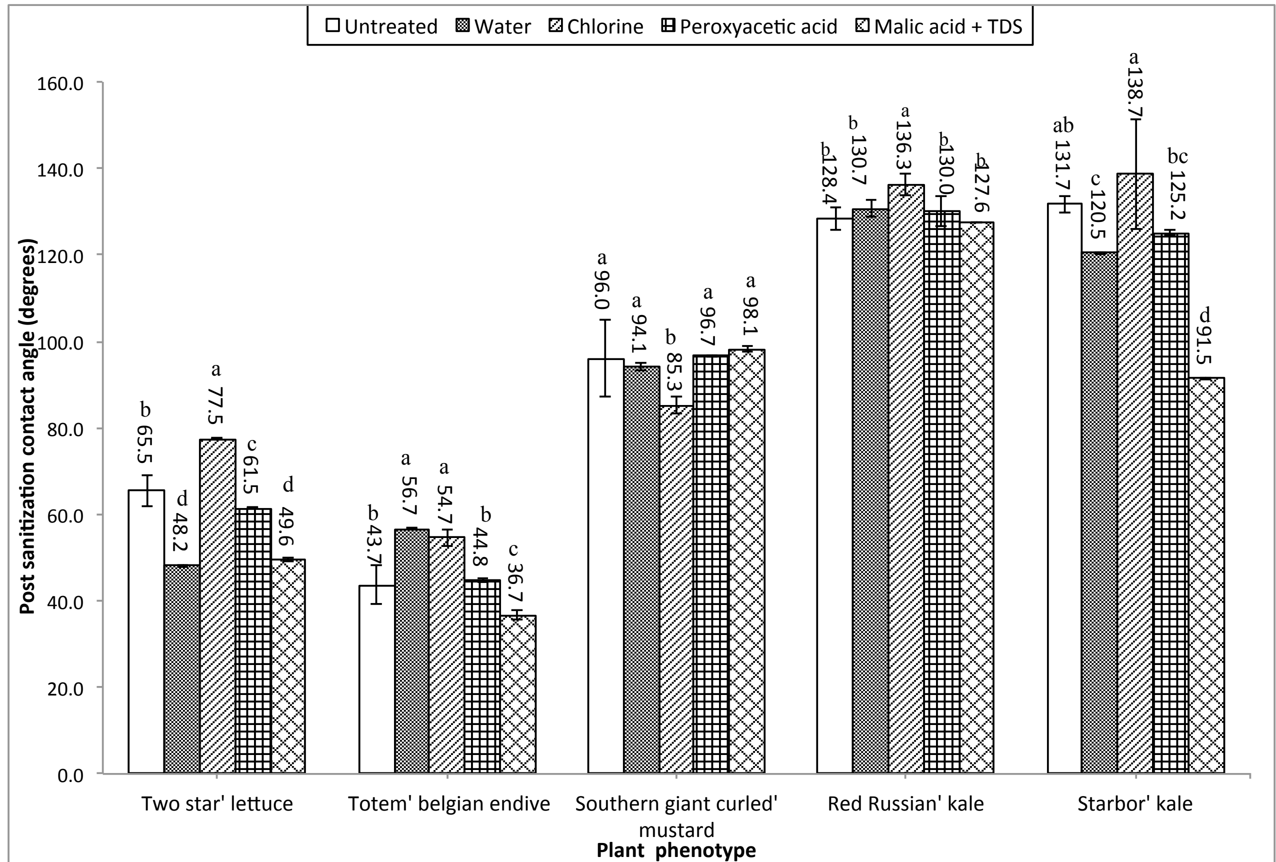

Figure 3.3. Changes in plant surface hydrophobicity as effected by sanitization.

a-d: Different letters on bar (sanitizer) indicate significantly different contact angle within the same vegetable cultivar by Tukey's test (α= 0.05).

Table 3.1 *Escherichia coli* K12 attachment to selected plant surfaces.

PRODUCE	CONTACT ANGLE (°) (ADAXIAL)	SURFACE ROUGHNESS (µm) (ADAXIAL)	MEAN CELL ATTACHMENT LOG CFU/CM^2			$\sigma_{EDGE}/\sigma_{ADAXIAL}$
			Inner Disk ($\sigma_{adaxial}$) (adaxial) (A*: 1.13 cm^2)	Outer Ring (σ_{ring}) (adaxial) (A: 0.88 cm^2)	Cut Edge (σ_{edge}) (A: 0.02 cm^2)	
'Two star' lettuce	62.1	3.2	6.8 ± 0.3 $^{a(x)}$	7.4 ± 0.6 $^{a(x)}$	21.0	3.1
'Totem' Belgian endive	58.3	4.9	5.7 ± 0.2 $^{b(y)}$	6.9 ± 0.5 $^{a(x)}$	47.1	8.3
'Southern giant curled' mustard	107.8	10.0	6.6 ± 0.4 $^{ab(x)}$	6.6 ± 0.4 $^{a(x)}$	1.6	0.2
'Red russian' kale	125.6	2.7	6.3 ± 0.7 $^{ab(x)}$	6.9 ± 0.1 $^{a(x)}$	23.7	3.8
'Starbor' kale	128.7	1.8	6.3 ± 0.1 $^{ab(y)}$	6.6 ± 0.1 $^{a(x)}$	10.1	1.6
LSD	–	–	1.07	1.09	–	–

A*: Area of each surface

a-b: Means for cell attachment within produce type (column) with different letter are significantly different (α= 0.05)

x-y: Means for cell attachment within inner disk and outer ring (rows) with different letter are significantly different (α= 0.05)

Table 3.2 Comparison between high wax containing plants (average of kales) and low wax containing plants (average of other leafy vegetables).

Plant classification	Alkanes (μg/cm²)	Alcohols (μg/cm²)	Fatty acids (μg/cm²)	Ketones (μg/cm²)	Total wax (μg/cm²)	Contact angle (degree)	Roughness (μm)	Bacteria removal (Log/cm²)
Low wax*	1.22±0.46 b	3.17±1.72 a	1.43±0.40 b	0.20±0.18 b	6.01±1.81 b	76.1±27.5 b	6.0±3.5 a	1.0±0.4 b
High wax**	45.31±2.99 a	1.88±0.57 a	4.72±1.58 a	25.58±4.84 a	77.48±5.71 a	127.2±2.2 a	2.3±0.6 b	1.6±0.7 a

*Low wax includes ‘Totem' Belgian endive, ‘Two star' lettuce, and ‘Southern giant curled' mustard.

**High wax includes ‘Red Russian’ kale and ‘Starbor’ kale.

a-b: Mean comparison between low wax (columns) and high wax with different letter are significantly (α= 0.05)

CHAPTER 4

ARTIFICIAL PHYLLOPLANES RESEMBLING PHYSICOCHEMICAL CHARACTERISTICS OF SELECTED FRESH PRODUCE AND THEIR USE IN BACTERIA ATTACHMENT/REMOVAL STUDIES

4.1 ABSTRACT

Every year, one-in-six Americans acquires a food-related illness caused by bacteria, viruses and parasites. Leafy greens and fresh produce have been linked to most foodborne outbreaks caused by such microorganisms. The recurrence of food-borne illness outbreaks caused by consumption of fresh produce highlights the importance of a good understanding between the interactions between bacteria and produce leaf surfaces. This type of research necessities the use of a model fresh produce surface that can eliminate the variations caused by biological variants and time-dependent nature of produce surface properties. A few previous studies have reported efforts to develop an artificial plant leaf surfaces. However, almost all previous works have focused on physical replication of the leaf surface topology, while reproduction of the produce leaf surface chemistry, especially the epicuticular wax compositions remains largely untouched. To fill this gap, we proposed and developed a new method to fabricate artificial phylloplanes that mimic both the topographical and epicuticular characteristics of fresh produce. In the work reported in this document, two of the most consumed fresh produce commodities in the U.S., i.e. 'Outredgeous' romaine lettuce and 'Carmel' spinach, were used as the model surfaces. The surface hydrophobicity of the artificial phylloplanes was modified with addition of non-ionic surfactant with different HLB values to match the hydrophobicity of produce leaves. The key epicuticular wax compounds identified from the natural spinach and lettuce leaves were coated on the leaf replica to replicate the chemical composition of the natural leaf surfaces. The surrogate surfaces

were used to study the attachment *Escherichia coli* O157:H7, *Listeria innocua*. In addition, these surfaces are reusable, and have surface hydrophobicity, surface roughness values and epicuticular wax compositions similar to that of fresh produce. The new artificial phylloplanes of fresh produce can be used as a platform for studying the interactions between human pathogens with produce surfaces and for developing new produce sanitation strategies.

Keywords: Surface roughness, surface hydrophobicity, epicuticular composition, PDMS, artificial phylloplanes

4.2 INTRODUCTION

Fresh and fresh-cut produce continue to be associated with outbreaks of foodborne illness. This has promoted researchers to develop understanding of the interactions between human pathogens with produce phyllosphere (Critzer and Doyle 2010), with the purpose of providing insight into pathogens colonization and persistence on produce surfaces, route of contamination, and means to more effectively remove pathogens or decontaminate produce surfaces. Adaxial and abaxial surface of plant leaves provide habitats for a diverse assemblage of microorganisms including fungi, yeast, viruses and bacteria (Hirano and Upper 2000). Leafy greens can become contaminated with microorganisms at multiple stages in the farm-to-fork continuum. For instance, during growth, produce surfaces can be contaminated with soil, improperly processed manure, or contaminated irrigation water (Park 2013, Shenoy, Oliver et al. 2017). During sanitization and processing of fresh produce, microorganisms present on outer leaves can come into contact with inner leaves causing cross-contamination of the final product (Palma-Salgado, Pearlstein et al. 2014, Jensen, Friedrich et al. 2015, Maffei, Sant'Ana et al. 2017). In most cases, bacteria have to come into contact with produce surfaces to initiate adhesion and colonization.

The variation in chemistry and topography of plant surfaces oftentimes results in inconsistence in attachment studies (Sirinutsomboon et al. 2007). Research regarding interaction between food-borne pathogens and fresh produce leaves is limited compared to that on phytopathogens (Schreiber, Krimm et al. 2005, Seo and Matthews 2012). In most cases studies regarding bacteria-fresh produce interaction are focused on the effect of bacteria cell and/or produce surface characteristics on the attachment of bacteria to produce (Patel, Sharma et al. 2011), whereas most studies with fresh produce are aim at determining the best combination of sanitizer

concentration and processing time to remove bacteria (Sy, Murray et al. 2005, Ölmez and Kretzschmar 2009, Luo, Nou et al. 2012, Palma-Salgado, Pearlstein et al. 2014).

The properties of fresh produce phyllosphere that impact bacteria attachment and removal include produce surface roughness, surface hydrophobicity, and epicuticular composition (Koch and Ensikat 2008, Yeats and Rose 2013). Noticeably, these properties undergo constant changes for fresh produce during pre-harvest growth and during post-harvest transportation and storage, as they are living plant tissues with active metabolic activities. Produce variety and growth conditions (weather, soil, water, fertilization, etc.) also play a key role determining the produce surface properties, not to mention the variation on the different locations of the same produce leaf. Consequently, in a sanitation test, the produce surface conditions would not be the same for the same produce variety with same sanitizer when the test was performed by different research groups, or even by the same research group but at a different time. As a result, most previous sanitation tests were performed, in a sense, under uncontrolled conditions with regard to produce surface properties. Such variations introduce unknown but could be significant noises that would mask or bias the true effect of the sanitizer, leading to often observed inconsistent sanitation data reported by different research groups, or in the data of the same group. Therefore, there is a need to develop a platform for studying bacteria and fresh produce interactions, as well as produce sanitation with controllable and constant surface property phylloplanes that resemble produce surface physical, biochemical, and biological properties.

The requirements for an artificial phylloplane that can be considered as a replica of a natural produce leaf surface should thus include 1) resembling the 3D topological features of natural produce leaf surfaces, 2) having a similar surface hydrophobicity, 3) having a similar epicuticular chemical composition, mainly epicuticular wax composition, 4) producing a similar

bacterial attachment pattern, and 5) reproducible and reusable, including autoclave-able and compatible with stomacher.

In the past decade, some research groups have attempted to develop man-made microstructures that could be used as a replica or analog of natural produce surfaces, with varying degrees of success. The early work of University of California, Davis had developed plant surface structure analogs with a microfabrication method (photolithography) (Sirinutsomboon et al, 2007, 2008, 2010, 2011, Sirinutsomboon and Delwiche, 2013). To study the effects of plant surface microstructure on attachment of *Escherichia coli* O137:H41, they fabricated uniformly patterned vertical micro-pillars, pyramids, or grooves on polydimethylsiloxane (PDMS) pieces to mimic trichomes, stomata, and ridges between plant cells on produce leaf surfaces, respectively. They used the silanization method to produce hydrophobic surfaces on the silicon, resulting in hydrophobic microstructures similar to those on natural plant surfaces. As the first documented effort, their goal was to use analogs of trichomes, stomata, and intercellular grooves "with controlled shapes, sizes, and distributions to avoid uncontrolled variables that occur on natural plant surfaces." Therefore, their method only produced man-made arrays of vertical trichomes, stomata, and grooves with uniform shape and size, not the 3D topology of the produce surfaces. The initial work at University of Illinois was evolved from making simple patterns on PDMS films (Feng and Pearlstein, 2012) to fabrication of PDMS surfaces with 2D patterns of natural produce leaves produced from the SEM image of spinach leaf surface (Palma et al., 2014). They also developed a method to modify surface hydrophobicity of PDMS by mixing it with different ratios of surfactant with different hydrophile-lipophile balance (HLB) values and pouring it onto a silicon wafer mold with features resembling the surface of spinach leaves. Their method provided a means to mimic hydrophobicity of any leaf surfaces. But it cannot reproduce a 3D surface topology from

produce leaves. A simple two-stage double-casting method to transfer of 3D natural patterns on Trembling Aspen leaf surfaces was developed by McDonald et al. (2013). They used PDMS to produce negative mold of leaf surface. Then a self-assembled monolayer of 2H-perfluorodecyltrichlorosilane (FDTS) was used as an anti-adhesion agent to facilitate transfer of micro-patterns to PDMS positive replica. Slightly later, a USDA ARS group reported a method to reproduce 3D fresh produce surface topology on a PDMS film (Zhang, Luo et al. 2014). After obtaining the negative 3D image of the produce surface on PDMS, they utilized a relatively complex chemical surface modification method to coat the negative PDMS surface with a layer of Pd nanoparticles to make a PDMS negative mold. Then the positive PDMS leaf surface analog was produced using a thermal molding method (120 °C, 20 minutes) from the negative mold. The leaf surface replica after the thermal molding process happened to have a water contact angle (WCA) similar to that of spinach. Noticeably, almost all previous studies exploring biological surface replica have focused on topological or physical reproduction of the surfaces. Much less efforts have been placed on developing artificial plant surfaces with similar chemical and biological properties with natural leaves.

To fill in this gap, we conducted a comprehensive investigation to develop a PDMS-based artificial phylloplane surface to resemble the topographical, chemical, and epicuticular characteristics of romaine lettuce and ‘Carmel’ spinach to high fidelity. This standard platform enables to modify and control factors such as surface roughness, surface hydrophobicity, and epicuticular composition to values mimicking any selected leafy greens. The PDMS leaf replica was used as a substrate to evaluate attachment and removal of *Escherichia coli* O157:H7 EDL933, and *Listeria innocua.* In addition, we examined the reusability of these phylloplane surfaces by exposing them to commonly used disinfection practices in laboratory settings.

4.3 MATERIALS AND METHODS

4.3.1 Greenhouse production of leafy vegetables.

'Outredgeous' romaine lettuce (*Lactuca sativa* L.) and 'Carmel' spinach (*Spinacia oleracea* L.) were used in this study. They were grown in a greenhouse as previously described (Fuzawa, Ku et al. 2016). Briefly, lettuce and spinach seeds purchased from Johnny's Selected Seeds (Winslow, ME) were germinated in 32-cell plant plug trays filled with Sunshine LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at University of Illinois under a 25°C/17°C and 14 h/10 h day/night temperature regimen with supplemental lighting. Twenty days post-germination, the seedlings were transferred to 4-liter pots. Leaf tissues from the 'Carmel' spinach plants were harvested 40-45 days after sowing seeds and that from the 'Outredgeous' romaine lettuce plants were harvested 50-65 days after sowing seeds. For this study, leaves were harvested at market maturity.

4.3.2 Bacterial strain preparation

Escherichia coli O157:H7 and *Listeria innocua* obtained from the food microbiology culture collection at the University of Illinois at Urbana-Champaign were used in this experiment. Bacterial inoculums were prepared by repeated sub-culturing on tryptic soy agar (TSA) plates for *Listeria innocua* and TSA plates containing 50 mg/L of nalidixic acid (Sigma Aldrich, St. Louis, MO) for *Escherichia coli* O157:H7. Cultures of *Listeria innocua* were grown in Tryptic Soy Broth (TSB) and that of *Escherichia coli* O157:H7 in TSB with 50 mg/L of nalidixic acid (Sigma Aldrich, St. Louis, MO) for 22 hours at 37 °C. Cells were harvested by centrifugation at 4 °C and 2,455 *g* for 10 minutes. and washed twice in sterile 0.1% peptone water. The recovered bacterial

precipitates were diluted in 10 mL of 0.1% peptone water; the initial inoculation level was $2.1 \times 10^8\ CFU/ml$ for *Escherichia coli* O157:H7 and $2.5 \times 10^8\ CFU/ml$ for *Listeria innocua*.

4.3.3 Development of a reproducible artificial phylloplane with topography and hydrophobicity similar to 'Outredgeous' romaine lettuce and 'Carmel' spinach leaves using a double casting rapid-replication method.

4.3.3.1Preparation of polydimethylsiloxane (PDMS) elastomer.

Polydimethylsiloxane (PDMS) Sylgard® 184 Silicone Elastomer Kit (Dow Corning Corporation, MI, USA) was prepared according to manufacturer instructions. Briefly, a mixture of base/curing agent at ratio of 10:1 was prepared and thoroughly mixed for 5 minutes. The base/curing agent mixture was degassed under low vacuum until no oxygen bubbles were visibly present in the mixture. In addition, a PDMS-surfactant solution was prepared by mixing degassed PDMS and 1 to 10% (v/v) Caprol-PGE860® surfactant (Abitec, OH, USA) and thoroughly mixed for 5 minutes. The PDMS-surfactant solution was degassed under low vacuum until no oxygen bubbles were visibly present in the mixture.

4.3.3.2 Development of a master mold to reproduce artificial phylloplanes using a double casting method.

Reproducible artificial phylloplanes with physical, chemical and topographical characteristics similar to 'Outredgeous' romaine lettuce, and 'Carmel' spinach leaves were developed. First, a negative impression (master mold) was developed by collecting leaf samples of lettuce and spinach grown in a greenhouse until commercial maturity. Samples were rinsed with distilled water to remove soil and debris from the surface (§4.3.1). The plants were taped (3M,

Minnesota, USA) to the bottom of a 4-inch glass Petri Dish (Fisher scientific, NH, USA); Degassed PDMS was poured on top of the taped leaf until the petri dish was full to 75% capacity. Solidification took place by placing the sample for 8 hours under refrigeration (4 °C ± 1 °C), followed by 22 hours solidification under controlled temperature (25 °C ± 1 °C) and relative humidity (70% ± 3%). The obtained inverted PDMS master molds were treated for 10 minutes with a solution of 1% hydroxypollymethylcellulose (HPMC) (Sigma Aldrich, MO, USA) diluted in 1M phosphate-buffered saline (pH 3.0) and stored at 25 °C ± 1°C.

4.3.3.3 Development of a reproducible artificial phylloplanes with topography and hydrophobicity similar to 'Outredgeous' romaine lettuce and 'Carmel' spinach

The PDMS-surfactant solution (§4.3.3.1) was poured onto HPMC-treated PDMS molds, and the molds were cured in a hot plate for 5 minutes at 100 °C ± 5 °C. Hardened PDMS surface were removed from the PDMS master mold and stored at 25 °C until further use or was spin-coated with 200 µl of epicuticular chemical solution containing the main epicuticular wax compounds of 'Outredgeous' romaine lettuce and 'Carmel' spinach. The spin-coated samples were placed in a glass petri dish and cured under low vacuum for 15 minutes and stored in airtight sampling bags until further use.

4.3.3.4 Evaluation of safety of surfactants on *Escherichia coli* O157:H7

As explained in §4.3.3.1, different concentrations of surfactants were utilized to hydrophobicity of PDMS. *E. coli* O157:H7 87-23 was utilized to evaluate if the addition of the surfactant onto the PDMS solution would cause toxicity to microorganisms.

Two different surfactants were selected and prepared at two different concentrations. Caprol-3GO and Caprol PGE-860 both are "non-ionic, non-alkoxylated emulsifier" with hydrophile-lipophile balance (HLB) values of 7 and 11, respectively (Abitec Corp., 2014). Each surfactant was diluted in 0.1% peptone water to a final concentration of 7% or 10% (v/v) and stirred for 10 minutes in a biological cabinet until dissolved. A tenfold dilution of *Escherichia coli* O157:H7 (§4.3.2) and surfactant solution was prepared in glass test tubes and placed in an incubator equipped with a shaker. The samples were shaken at 120 RPM and a survival growth curve was determined by sampling the solution at 0, 2, 12, 24 hours. The *Escherichia coli* cells surviving on surfactant solutions were enumerated via spread plating with tryptic soy agar (TSA) plates containing 50 mg/L of nalidixic acid and incubated for 24 hours at 37 °C.

4.3.4 Preparation of epicuticular chemical solution.

Based on the information presented by (Lu, Ku et al. 2015), a wax solution containing the key epicuticular wax compounds of 'Carmel' spinach leaves were prepared by mixing chloroform with 22% (w/v) of the alkane octadecenol, 54% (w/v) of the fatty alcohol 1-hexacosanol, and 24% (w/v) of the fatty acid myristic acid. The mixture was placed in airtight containers and stirred for 1 hour in a water bath (70 °C ± 2.0 °C). A wax solution containing the key epicuticular wax compounds of 'Outredgeous' romaine lettuce leaves was prepared by mixing chloroform with 41% (w/v) of the alkane heneicosane, 20% (w/v) of the fatty alcohol 1-hexacosanol, and 39% (w/v) of the fatty acid myristic acid. The mixture was placed in airtight containers and stirred for 1 hour in a water bath (70 °C ± 2.0 °C). To prevent evaporation and precipitation of epicuticular wax, the wax solutions were kept in airtight containers and placed in a darkroom until further use.

4.3.4.1 Confirmation of deposition of epicuticular chemical solution on surfaces

Using a Pasteur pipette, approximately 200 µl of epicuticular chemical solution was placed in direct contact with attenuated total reflectance (ATR) crystal on a multibounce plate at controlled ambient temperature (25 °C). An FTIR spectrometer (more info of FTIR) connected to the software SPECTRUM ® was used during FTIR data collection. FTIR spectra were recorded from 8 scans at a resolution of 4 cm^{-1} at 4000–400 cm^{-1}. These spectra were subtracted against background air spectrum. After every scan, a new reference air background spectrum was taken. The ATR plate was carefully cleaned in situ by scrubbing with ethanol twice and dried with soft tissue paper before placing the next sample. Cleanliness was verified by collecting a background spectrum and compare to the previous one. These spectra were recorded as absorbance values at each data point in triplicate.

4.3.5. Comparison of physical, chemical and biological characteristics of the artificial phylloplanes vs leafy greens surfaces

4.3.5.1. Determination of surface hydrophobicity

Surface hydrophobicity was measured as previously described by Lu et al., 2015. Briefly, nine disks (approximate area of each disk 2.01 cm^2) from each vegetable cultivar were excised and used for determination of surface hydrophobicity. The disks were rinsed with distilled water to remove soil and debris and dried using Kimwipes® (Kimberly-Clark, Irving TX. USA) with gentle patting motions. The dried disks were then taped (3M, Minnesota, USA) to a microscope glass slide exposing the adaxial surface of leaves. The glass slides containing the leaves disks were covered with moistened paper towel and aluminum foil to prevent dehydration of leaf disks. Similarly, nine disks (approximate area of each disk 2.01 cm^2) from each artificial phylloplane

leaves with and without epicuticular chemical solution were excised and taped (3M, Minnesota, USA) to a microscope glass slide exposing the adaxial surface of leaves and covered with aluminum foil to prevent contamination with debris and dust particles. Water contact angle of all surfaces was obtained using a goniometer (KSV Instruments, Stockholm, Sweden) model CAM 200. Using a calibrated pipette, 5 µl of deionized water was placed at the center of each disk and within 20 seconds five contact angle readings were measured.

4.3.5.2 Determination of Surface roughness

Produce samples were prepared following the same procedure used for contact angle measurement and surface roughness was measured as previously described by Lu et al., 2015. A confocal microscope (NanoFocus, µSurf explorer) was used to determine 3-dimensional surface parameters. Area-average root mean square roughness (-Sq bar) was obtained from the average of a number of linear root mean square roughness Sq measured from the 3-D image reconstructed from 2-D laser confocal images over an area of 0.3 mm × 0.3 mm (0.09 mm^2). Image analysis was done using the software Mountains (Digital Surf, France)

4.3.6 Determination of *Escherichia coli* O157:H7 and *Listeria innocua* attachment to artificial phylloplanes vs leafy greens surfaces

Each set consisting of three biological replicates of romaine lettuce and fresh spinach leaves were cleaned by rinse step with sterile Milli-Q water to remove debris and patted dried using Kim wipes® (Kimberly-Clark, TX). Each artificial phylloplane was sterilized using 10 minutes of UV light. A diluted bacteria solution was prepared by diluting 1 ml of concentrated *E. coli* O157:H7 and Listeria innocua inoculum in 9 ml of 1X PBS buffer (Initial Inoculum *E. coli* = 7.0 Log_{10} PFU/ml and L. innocua = 8.4 Log_{10} PFU/ml). Using sterile tweezers each piece was

transferred to an empty sterile petri dish and 100 μl of each bacteria solution in PBS buffer was spot inoculated at 10 different spots on adaxial surface. The petri dish was loosely capped and incubated for 2 hours at 25 °C ± 1 °C in a biological cabinet. After the incubation period, the samples were transferred to a sterile container with 1X PBS buffer at ratios of 1:10 (surface: buffer solution) and were agitated for 1 minute to remove loosely attached bacteria. After the removal of loosely attached bacteria, each sample was transferred to a sterile sampling bag containing 1X PBS buffer and pummeled for 1-minute to remove all bacteria attached to the surface. The remaining supernatant was collected, spread in selective media incubated for 24 hours at 37 °C.

4.3.7 Reusability of the artificial phylloplanes

To determine PDMS-based artificial phylloplane surfaces endurance to commonly used disinfection practices, they were exposed to two different disinfection procedures. Changes in surface hydrophobicity and epicuticular composition were evaluated. The PDMS-based surface samples were immersed twice in 70% ethanol (v/v) for 36 hours, air dried inside a safety cabinet for 2 hours and stored at 25 °C for 24 hours prior analysis. In addition, PDMS-based surface samples were placed inside an autoclave at 121 °C for 30 minutes and were stored at 25 °C for 24 hours prior analysis. Surface hydrophobicity was determined following procedure in §4.3.5.1, while epicuticular composition was determined following procedure in §4.3.4.1.

4.3.8 Scanning electron microscopy and laser confocal microscopy

Surface characterization was carried using a scanning electron microscope. Images of the epicuticular surfaces were taken using a FEI Quanta FEG 450 ESEM (Hillsboro, OR, USA). The images were captured under low vacuum at 20 kV and at 400×, 800×, and 1200× resolution from

at least three different samples. In addition, surface characterization was carried by excising two disk from each leaf using a 1.65 cm diameter sterile cork borer. These disks were then taped (3M, Minnesota, USA) to a smooth microscope glass slide exposing the adaxial surface of leaves. A confocal microscope (NanoFocus, μSurf explorer) was used to do 3-dimensional surface characterization.

4.3.9 Statistical analysis

The experiments were performed with a complete randomized design (CRD) with each treatment conducted three times. Bacterial counts were subjected to log transformation before statistical analysis. Data were analyzed using a general linear model available in SAS version 9.1 (SAS Institute, Raleigh, NC, USA), and with Origin-Pro 2016 (OriginLab Corporation, MA, USA). Mean separation was determined using Tukey's test with $\alpha = 0.05$. Relationships were considered significant when $P < 0.05$.

4.4 RESULTS AND DISCUSSION

4.4.1 Determination of toxicity of matrix utilized for the development of artificial phylloplanes.

As seen in Figure 4.1, the process to develop artificial phylloplanes involved a double casting procedure utilizing PDMS as the base polymer. The PDMS-double-casting technique has been widely utilized in other applications such as replication of "high-aspect-ratio microstructures", development of components of nanophotonic devices, as well as fabrication of microfluidic devices (Gitlin, Schulze et al. 2009, Hongbin, Guangya et al. 2009, Shao, Wu et al. 2012, Seghir and Arscott 2015). One of the advantages of using PDMS as a base for double casting

process is the low cost, low labor and time involved with the procedure. However, one of the obstacles of utilizing PDMS is the high hydrophobicity of the material (WCA=120°), which is different from most fresh plant phylloplanes (Lu, Ku et al. 2015). Studies have shown that mixing non-ionic surfactants directly with PDMS can lower the hydrophobicity of PDMS (Seo and Lee 2006, Fatona, Chen et al. 2015) and thus in our case improve the wettability of the artificial phylloplanes. Nonetheless, in the food industry a common practice is to utilize surfactant as a component of chemical sanitizers for wash of fresh produce (Fransisca and Feng 2012, Palma-Salgado, Pearlstein et al. 2014, Fuzawa, Ku et al. 2016). It is therefore necessary to understand if the PDMS surfaces with surface hydrophobicity modified by a surfactant is toxic to bacteria. As seen in Figure 2, after up to 24 hours of growth in the non-ionic surfactant solutions with HLB of 7 and 11, there is no significant change (or reduction) in *Escherichia coli* O157:H7 population at each sampling time (0, 2, 12, 24 hrs.) for each of the surfactants tested. Thus, the artificial phylloplanes with addition of surfactants are not toxic to *Escherichia coli* cells.

4.4.2 Characterization of physiochemical properties of master mold and artificial phylloplanes

A qualitative comparison of the surface microstructures of the fresh produce leaf and the artificial phylloplanes, or PDMS replica is shown by CLSM and SEM micrographs. The CLSM and SEM images of the fresh leaves (A, D), master mold (B, E) and artificial phylloplanes (C, F) are presented side-by-side for the 'Outredgeous' romaine lettuce (Figure 4.3) and 'Carmel' spinach (Figure 4.4). As seen in Figures 4.3B, 4.3E, 4.4B, and 4.4E, with the replication method, the created master molds (B, E) exhibit negative impressions of the features of fresh leaves (Figures 4.3A, 4.3D, 4.4A, 4.4D). By comparing Figures 4.3D, 4.3F and Figures 4.4D, 4.4F, one can see

that the artificial phylloplanes are true replicas of fresh leaves. Features such as guard cells, open stomas, and vertical variations (peaks and valleys) of different sizes and scales were replicated.

To further examine the fidelity of the artificial phylloplanes to the fresh leaves, the surface hydrophobicity and surface roughness of the natural leaves and PDMS replicas were measured. As shown in Table 4.1, no significant differences ($P > 0.05$) were observed in the hydrophobicity (water contact angle) values between the fresh leaf of 'Outredgeous' romaine lettuce and the lettuce artificial phylloplane made with 10% surfactant. Similarly, no significant differences ($P > 0.05$) in the hydrophobicity values of the 'Carmel' spinach fresh leaf and the spinach artificial phylloplane made with 10% surfactant (values). Also, we were able to determine that a slightly hydrophobic sample could be achieved by mixing PDMS and surfactants at concentrations <5%. Moreover, as seen in Table 4.1 no significant differences were observed between the surface roughness (μm) of fresh romaine lettuce and its PDMS replica, as well as between the fresh leaf of 'Carmel' spinach and the spinach artificial phylloplane ($P > 0.05$).

4.4.3 Characterization of epicuticular composition of developed artificial phylloplanes

One important component of fresh leaves is their epicuticular wax, which acts as a barrier that prevents loss of water from the surface of the plant and as a barrier from abiotic stresses (Jetter and Riederer 1995, Wójcicka 2015). Epicuticular wax composition varies depending on the species, cultivar, age and environmental factors. On leaf surfaces, the wax usually exists in the form of a mixture of smooth amorphous layer and hierarchical structures (crystals) (Bargel, Barthlott et al. 2004, Shepherd and Wynne Griffiths 2006, Lu, Ku et al. 2015).

To faithfully replicate produce leaves, besides mimicking the leave topological properties and regulating the PDMS surface hydrophobicity to match that of natural leaves, the artificial phylloplanes should also represent the leave surface chemical composition, mainly the wax

composition. For that purpose, a chemical solution of different long-chain hydrocarbons mixed with chloroform (Table 4.1) was used to coat the artificial phylloplanes using a spin coating process. The compounds in the chemical solution were chosen to represent the key compounds of the epicuticular wax according to the work of (Lu, Ku et al. 2015). After coating, to exam if the PMDS replica surface has been coated with the compounds, a FTIR analysis was performed.

Figure 4.5 shows the infrared spectra for PDMS without wax coating, and for the artificial phylloplanes of the 'Outredgeous' romaine lettuce and 'Carmel' spinach coated with wax. Each of the infra-red (IR) active functional groups is highlighted with a band depending on the functional group region. As seen on Figure 4.5, the alkene bands are at 3090 cm^{-1}, ketone bands are at 1750 cm^{-1}, and PDMS silicone groups at 1020-1074 cm^{-1}. The appearance of the new alkene and ketone bands on the IR spectra of PDMS replica confirm that the wax compounds are deposited on PDMS surfaces. The epicuticular wax in the form of a mixture of small crystals and amorphous layer was also shown on the natural and artificial spinach leaves in the SEM images in Figure 4.6 E-F.

4.4.4 Attachment of *E. coli* O157:H7 and L. innocua to natural and artificial spinach leaves

A comparison of the attachment of *Escherichia coli* O157:H7 and *Listeria innocua* to natural surface and artificial phylloplanes of the 2 produce types was shown in Table 2. No significant difference between the attachment of *Escherichia coli* O157:H7 and *Listeria innocua* to surfaces of natural and the hydrophobic artificial phylloplanes, respectively, was found. This finding suggests that bacterial cells may have a similar interaction with the PDMS leaf replica developed in this study and that of a natural biological leaf surface, at least regarding attachment of the cells to two kinds of surfaces.

The surface hydrophobicity is shown to affect bacterial attachment. Between the 2 artificial produce surfaces, significantly more ($P < 0.05$) cells were attached to hydrophilic (WCA = 70°) surfaces than on the hydrophobic surfaces (WCA = 110°). Similarly, and between the natural leaf surface and the hydrophobic artificial surfaces, significantly more ($P < 0.05$) cells were found on the fresh produce surfaces (WCA = 74° for spinach and WCA =71° for lettuce) than on the hydrophobic PDMS replica (WCA = 110°) of them. These findings are in agreement with (Crick, Ismail et al. 2011) who evaluated the effect of hydrophobicity of various surfaces on the attachment of *E. coli* and S. aureus and found that the hydrophobicity of PDMS reduced the attachment of both types of bacteria compared to hydrophilic surfaces such as glass. The attachment of *Escherichia coli* O157:H7 on fresh and artificial spinach leaves is shown in Figures 4.6A and 4.6D. Some attached *Escherichia coli* cells can be identified in Figures 4.6A and 4.6D. No significant differences in the attachment patterns between fresh and artificial phylloplanes can be found.

4.4.5 Reusability of artificial phylloplanes

In order to prove that the artificial phylloplanes of lettuce and spinach will work as an effective low-cost platform to study factors that promote bacterial attachment, we evaluated the reusability of artificial phylloplanes after exposing them to two rounds of disinfection with ethanol and two rounds of heat sterilization using an autoclave.

As seen in Figure 4.7, no significant changes in surface hydrophobicity (water contact angle) were observed when the hydrophilic and hydrophobic spinach artificial phylloplanes were disinfected with 70% (v/v) ethanol or disinfected by two rounds of sterilization at 121 °C for 30 minutes ($P > 0.05$). Although changes in surface hydrophobicity of up to 7° ± 2° were observed for lettuce artificial phylloplane disinfected with 70% ethanol and sterilization at 121 °C for 30 minutes (P <

0.05), these changes did not cause the sample to become hydrophobic. Lastly, no significant differences in surface hydrophobicity were observed when lettuce hydrophobic artificial phylloplanes were disinfected with ethanol or sterilization ($P > 0.05$)

Furthermore, as seen in Figure 4.8, the FTIR spectra shows that after exposing the artificial phylloplanes of lettuce and spinach to two rounds of sterilization at 121 °C for 30 minutes no changes in epicuticular wax composition were observed. The signal of IR-active functional groups from the surfaces compared was still identifiable. Thus, the artificial phylloplanes can be used for at least three times for experiments of bacterial attachment.

4.5 CONCLUSIONS

In this study, a double-casting method to fabricate artificial phylloplanes that mimic to high fidelity the physical, chemical, and biological characteristics of fresh leaves of lettuce and spinach was developed with a soft polymer (PDMS). The surface hydrophobicity of the PDMS fresh produce leaf replica was manipulated with addition of non-ionic surfactant with different HLB values to match the hydrophobicity of produce leaves. A method was developed to coat the PDMS leaf replica with the main epicuticular wax compounds extracted and identified from the natural spinach and lettuce leaves to replicate the chemical composition of the natural leaf surfaces. Similarities in bacterial attachment patterns between the fresh produce leaves and artificial phylloplanes were observed. The PDMS leaf replicas are reusable, economical, and recyclable. The artificial produce leaf phylloplanes can be used as platform to investigate the interactions between bacteria and produce phylloplanes, and to develop new or enhanced fresh produce decontamination strategies.

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4.7 FIGURES AND TABLES

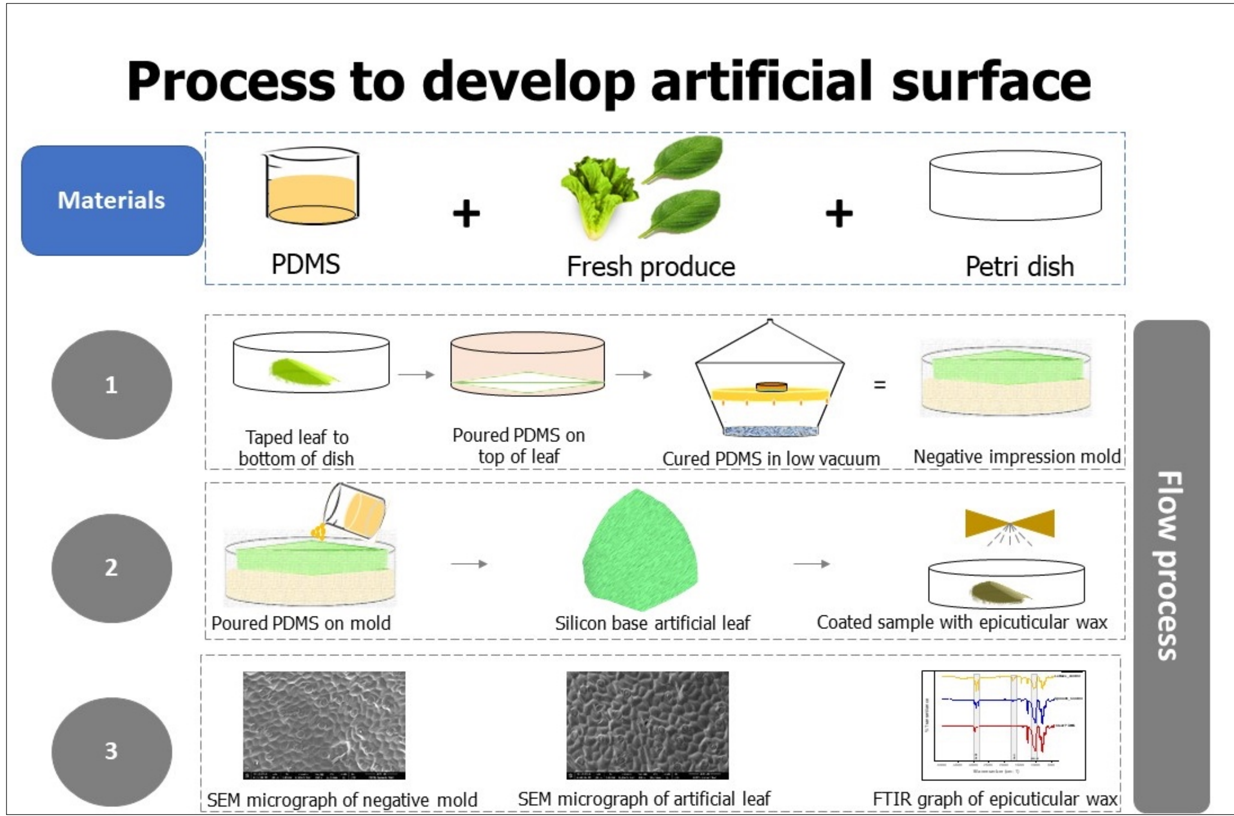

Figure 4.1 Flow process for the development of artificial surface

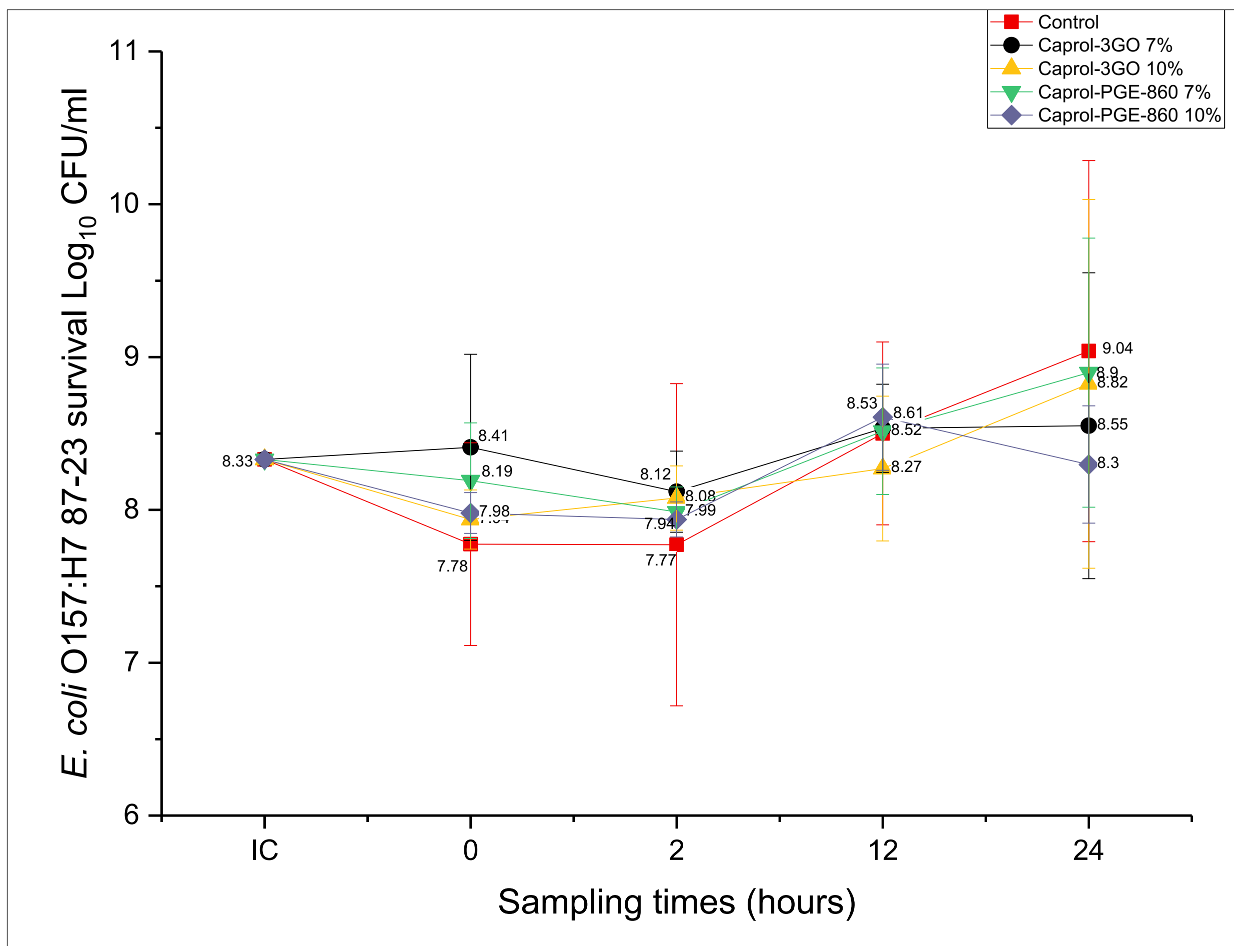

Figure 4.2. Growth rate of *Escherichia coli* O157:H7 in the presence of surfactant solutions at different incubation times.

There were no significant differences in the survival counts of *E. coli* O157:H7 grown in different surfactant solutions, no significant differences within the different percentages (%) used, and no significant differences at the different sampling times by Tukey's test (α=0.05)

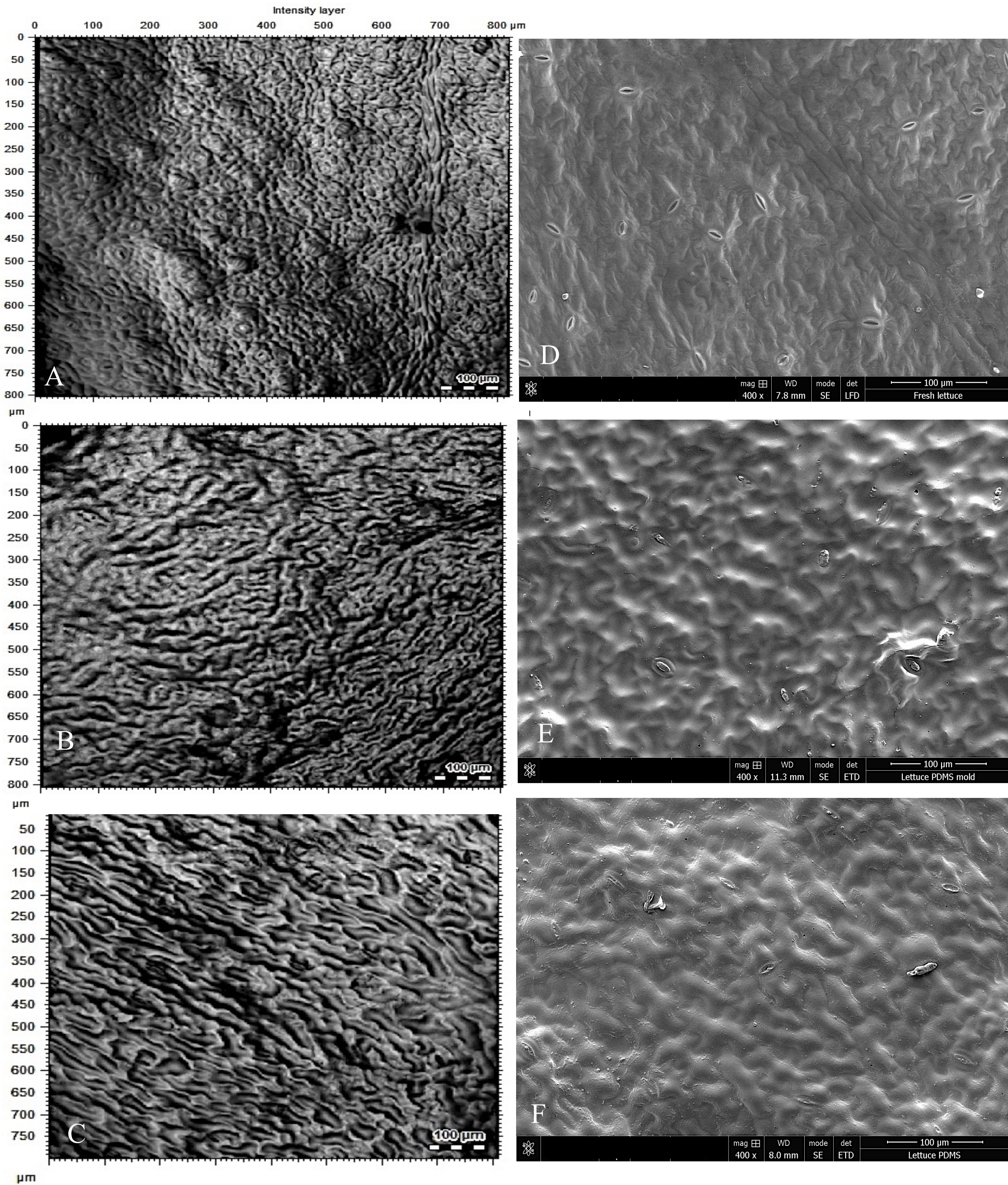

Figure 4.3. Selected lettuce micrographs using Confocal Microscope (CM) and Scanning Electron Microscope (SEM) A) CM plant leaf surface, B) CM PDMS mold C) CM artificial surface, D) SEM plant leaf surfaces, E) SEM PDMS mold F) SEM artificial surface

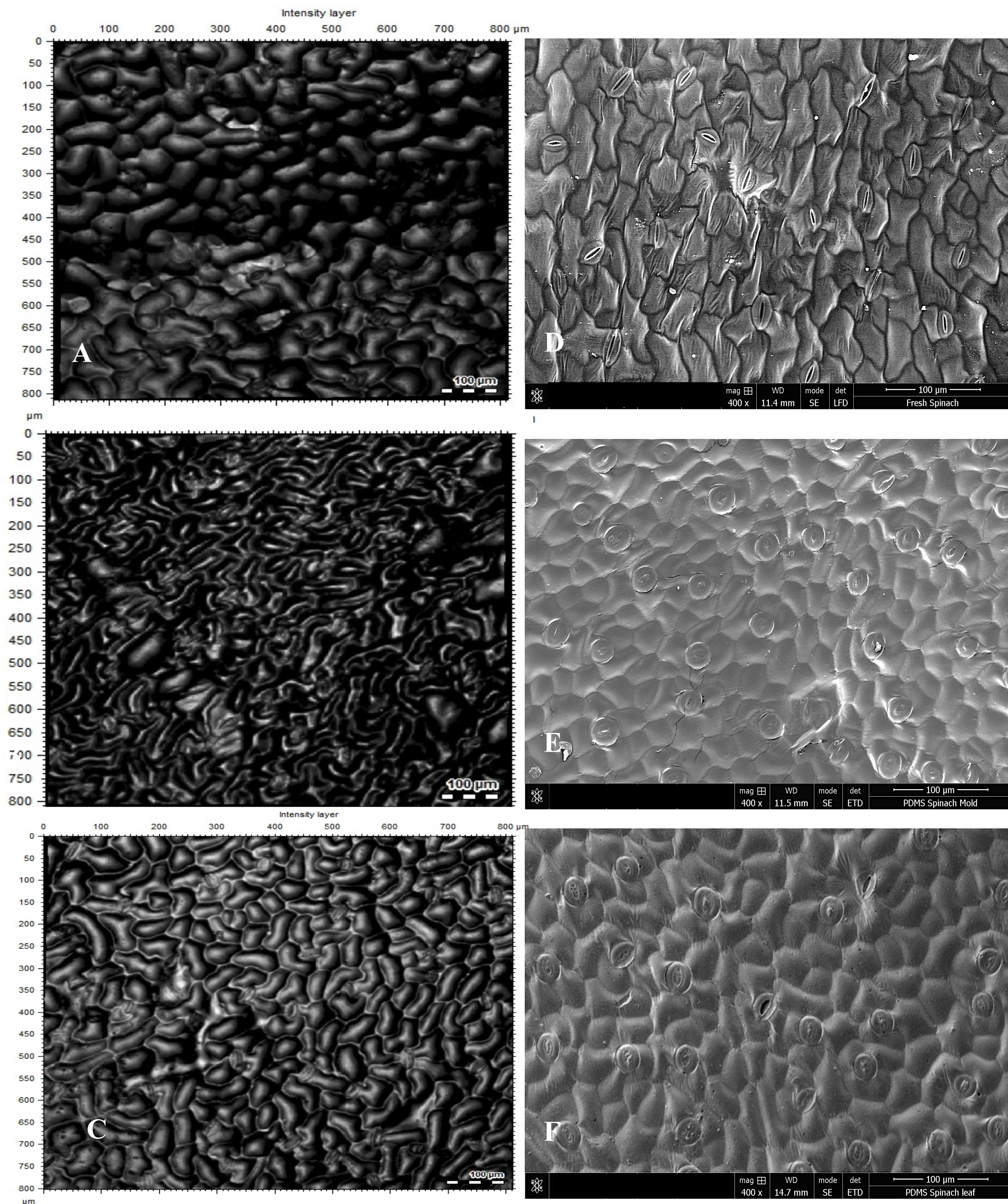

Figure 4.4. Selected Spinach micrographs using Confocal Microscope (CM) and Scanning Electron Microscope (SEM). A) CM plant leaf surface, B) CM PDMS mold C) CM artificial surface, D) SEM plant leaf surface, E) SEM PDMS mold F) SEM artificial surface

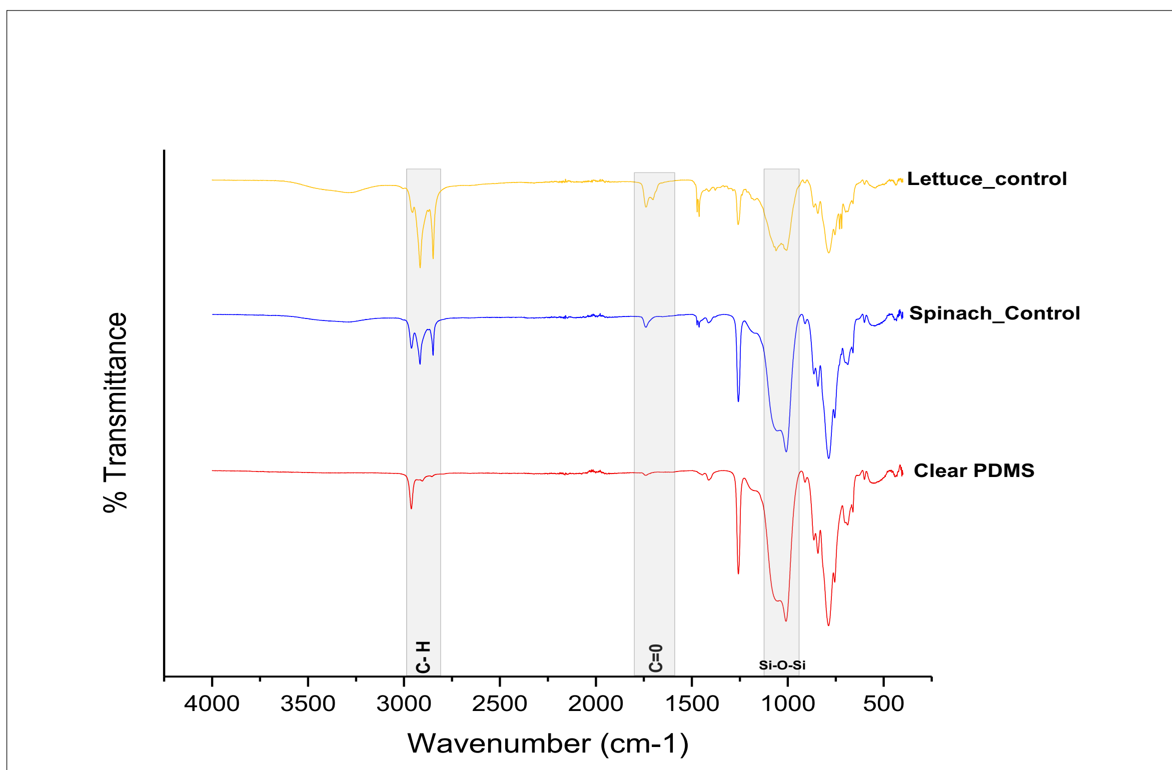

Figure 4.5. Confirmation of deposition of epicuticular wax on artificial phylloplane using FTIR

*Highlighted zones indicate the presence of IR-active functional groups. The alkene band is at 3090 cm^{-1}, The ketones band is at 1750 cm^{-1}, PDMS silicone groups at 1020-1074 cm^{-1}.

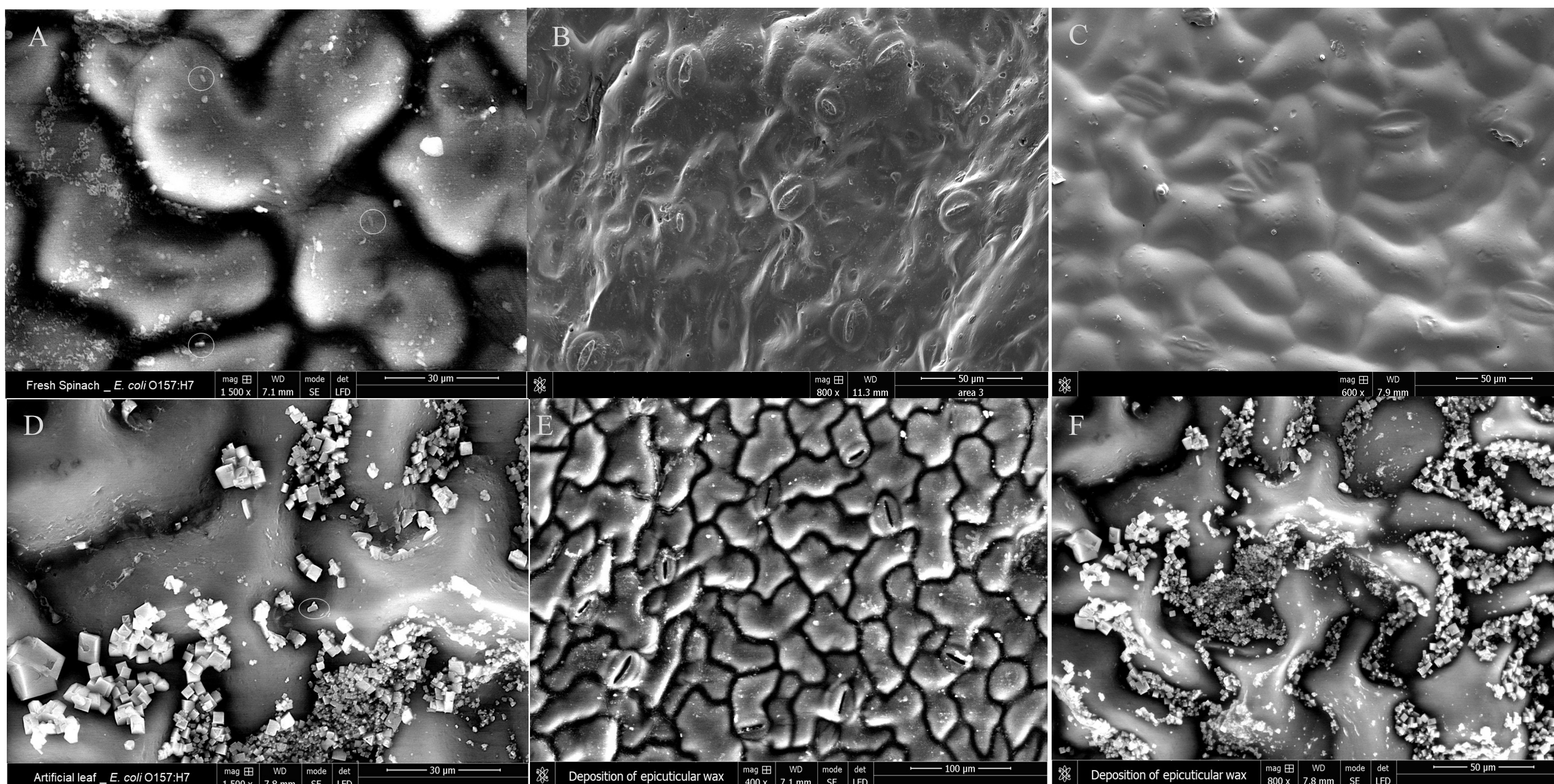

Figure 4.6. SEM micrographs for confirmation of bacterial attachment to produce surface and deposition of epicuticular wax on artificial phylloplane. (A) Fresh Spinach leaf inoculated with *E. coli* O157:H7 (B) Negative impression mold (C) Artificial leaf without wax (D) Artificial leaf with wax inoculated with *E. coli* O157:H7 (E) Artificial leaf with wax 400X (F) Artificial leaf with wax 800X. Presence of bacteria is highlighted with circle.

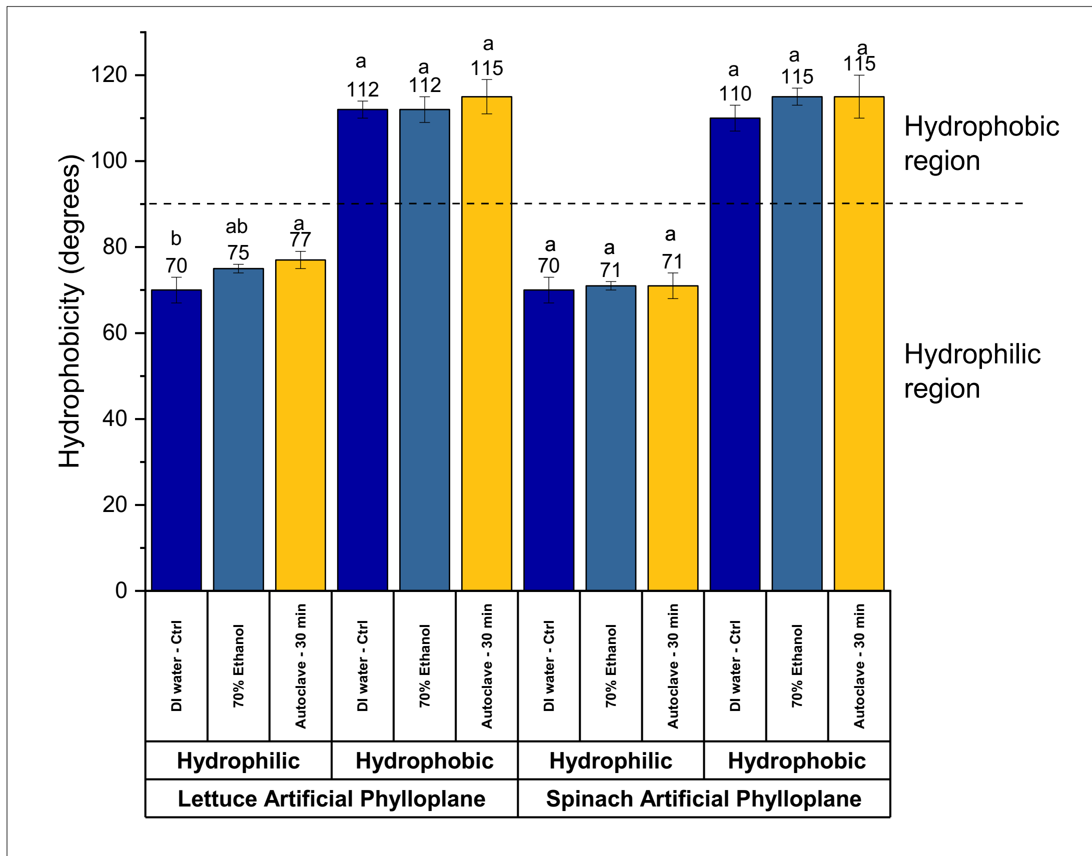

Figure 4.7. Changes in physical properties of artificial phylloplane after disinfection procedure a-b: Means (columns) with different within each condition tested (control, ethanol, autoclave) with different letter are significant different (α= 0.05)

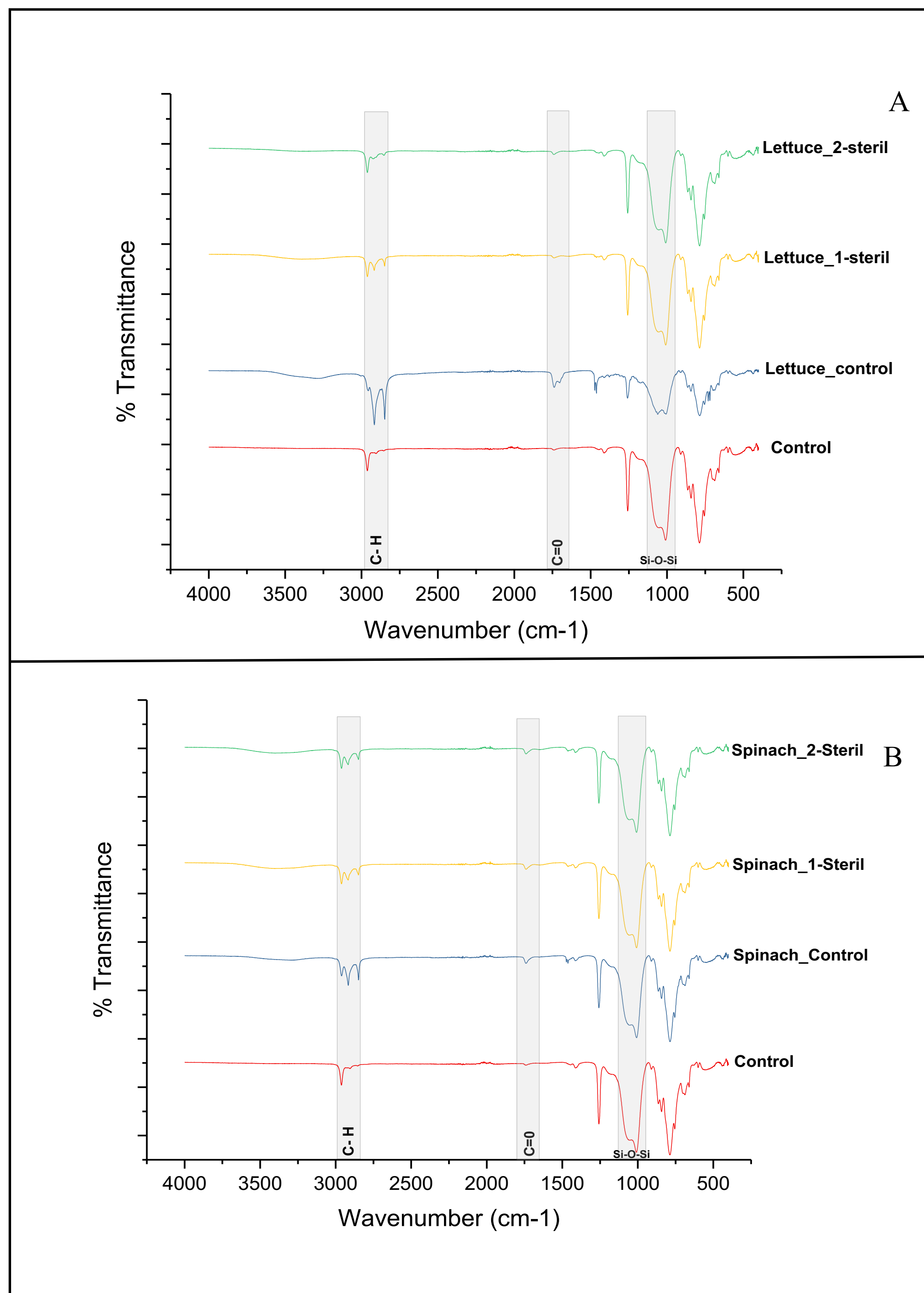

Figure 4.8. FTIR micrographs confirming the presence of epicuticular wax after sterilization with autoclave. (A) Lettuce (B) Spinach. *Highlighted zones indicate the presence of IR-active functional groups. The alkene band is at 3090 cm^{-1}, The ketones band is at 1750 cm^{-1}, PDMS silicone groups at 1020-1074 cm^{-1}.

Table 4.1 Physical and chemical properties of fresh leaves, negative impression and artificial leaves

SAMPLES	CONTACT ANGLE (Degrees) MEAN ± STD DEV	SURFACE ROUGHNESS (μm) MEAN ± STD DEV	EPICUTICULAR WAX COMPOSITION ($\mu g/cm^2$)			
			Fatty alcohol ($\mu g / cm^2$)	Alkane ($\mu g / cm^2$)	Fatty acid ($\mu g / cm^2$)	Total wax content ($\mu g / cm^2$)
'Carmel' spinach						
Fresh sample	74.0 ± 6.0 (c)	8.0 ± 1.2 (b)	NA♣	NA	NA	10.0 ± 0.7[1]
PDMS mold	87.0 ± 3.0 (b)	18.0 ± 6.0 (a)	NA	NA	NA	NA
Artificial surface made with 1% surfactant	110.0 ± 2.0 (a)	11.0 ± 3.0 (b)	2.1	4.2	4.2	10.4 ± 0.4
Artificial surface made with 10% surfactant	68.0 ± 2.0 (c)	12.0 ± 3.1 (b)	2.1	4.2	4.2	10.4 ± 0.4

[1] Reference value taken from Lu and Ku et al 2015

♣ No wax added

a-c: sample means for contact angle within each cultivar tested (column #2) with different letters are significantly different (α=0.05)

a-b: sample means for surface roughness within each cultivar tested (column #3) with different letters are significantly different (α=0.05)

Continuation of **Table 4.1**

SAMPLES	CONTACT ANGLE (Degrees) MEAN ± STD DEV	SURFACE ROUGHNESS (μm) MEAN ± STD DEV	EPICUTICULAR WAX COMPOSITION ($\mu g/cm^2$)			
			Fatty alcohol ($\mu g / cm^2$)	Alkane ($\mu g / cm^2$)	Fatty acid ($\mu g / cm^2$)	Total wax content ($\mu g / cm^2$)
'Outredgeous' romaine lettuce						
Fresh sample	71.0 ± 7.0 (c)	9.0 ± 3.0 (a)	NA	NA	NA	19.9 ± 8.2[1]
PDMS mold	92.0 ± 2.0 (b)	13.0 ± 5.0 (a)	NA	NA	NA	NA
Artificial surface made with 1% surfactant	109.0 ± 2.0 (a)	8.0 ± 1.0 (a)	10.7	4.4	4.8	19.9 ± 0.9
Artificial surface made with 10% surfactant	76.0 ± 6.0 (c)	8.0 ± 1.1 (a)	10.7	4.4	4.8	19.9 ± 0.9

[1] Reference value taken from Lu and Ku et al 2015

♣ No wax added

a-c: sample means for contact angle within each cultivar tested (column #2) with different letters are significantly different (α=0.05)

a-b: sample means for surface roughness within each cultivar tested (column #3) with different letters are significantly different (α=0.05)

Table 4.2 Attachment of *Escherichia coli* O157:H7 and *Listeria innocua* to artificial phylloplanes.

CULTIVAR	CONDITION TESTED	CONTACT ANGLE (Degrees)	*ESCHERICHIA COLI* O157:H7 EDL 933 MEAN ± STD DEV	*LISTERIA INNOCUA* MEAN ± STD DEV
'CARMEL' SPINACH	Fresh sample	74	5.0 ± 0.2 (a)	5.9 ± 0.1 (b)
	Artificial surface, 10% surfactant	70	4.7 ± 0.7 (a)	6.8 ± 0.2 (a)
	Artificial surface, 1% surfactant	110	4.5 ± 0.4 (a)	5.7 ± 0.2 (b)
'OUTREDGEOUS' ROMAINE LETTUCE	Fresh sample	71	5.2 ± 0.06 (a)	5.7 ± 0.1(b)
	Artificial surface, 10% surfactant	70	5.0 ± 0.06 (b)	6.3 ± 0.3(a)
	Artificial surface, 1% surfactant	110	4.3 ± 0.1 (c)	4.7 ± 0.3 (c)

a-c: sample means for each bacteria strain within each cultivar tested (column) with different letters are significantly different (α=0.05)

CHAPTER 5

ATTACHMENT AND REMOVAL OF PORCINE ROTAVIRUS (OSU) AND TULANE VIRUS TO FRESH AND ARTIFICIAL LEAVES OF 'OUTREDGEOUS' ROMAINE LETTUCE AND 'CARMEL' SPINACH AS AFFECTED BY ULTRASONICATION IN COMBINATION WITH OXIDANT- OR SURFACTANT-BASE SANITIZER(S)

5.1 ABSTRACT

This work examined the attachment of porcine rotavirus (PRV), strain OSU, and tulane virus (TV), a surrogate for human norovirus, to fresh and artificial surfaces of 'Outredgeous' romaine lettuce and 'Carmel' spinach. The effect of produce type, sanitizer, and ultrasound treatment on removal of PRV and TV from produce surfaces was investigated. Sanitization was performed with two oxidant-base sanitizers, e.g. chlorine and peroxyacetic acid, and 0.5% malic acid + 0.05% thiamine dilauryl sulfate (TDS), a surfactant-base sanitizer in combination with ultrasound. PRV and TV were spot inoculated to fresh and artificial produce surfaces and treated for one minute with a sanitizing solution with and without ultrasound. The removal of PRV from produce leaves treated by different sanitizers was significantly higher than that of TV. No difference in viral attachment and subsequent removal between the fresh and artificial produce surfaces was found, indicating the ability of the artificial phylloplanes to resemble real spinach and lettuce leaves. The addition of ultrasound significantly increased viral removal from both produce surfaces. The removal of virus attached to fresh and artificial surfaces was virus type, sanitizer type, and produce cultivar dependent. The artificial phylloplanes can be used as a useful tool to screen commercial sanitizers.

Keywords: Ultrasound, sanitizer, artificial phylloplane, fresh produce

5.2 INTRODUCTION

Fruits and vegetables are an essential component of a balanced diet. Due to their health benefits, such as reducing the risk of chronic diseases and help with weight management, an increase in their consumption has been observed in recent years (VanFrank, Onufrak et al. 2018). However, with the increase in the consumption of fruits and vegetables, especially those consumed raw, an increase in the incidents of foodborne illness outbreaks/recalls reported to the Center for Disease control and Prevention (CDC) has also been observed (Sivapalasingam, Friedman et al. 2004, Berger, Sodha et al. 2010, CDC 2017). According to the CDC, every year approximately 48 million cases of foodborne illnesses are reported in the U.S., resulting in 3,000 deaths. Over the period from 1998 to 2008, 22% of the foodborne illnesses were attributed to consumption of leafy vegetables, and out of those, 60% were caused by viral agents and 27% by human pathogens (Painter, Hoekstra et al. 2013). Furthermore, from 2010-2018, a total of 9 new outbreaks involving fresh produce and leafy greens (romaine lettuce, raw clover sprouts, spinach leaves, salad mixes, and alfalfa sprouts) were reported (CDC 2017), demonstrating the need for continued effort to develop effective means for securing microbial food safety of fresh produce.

The most common manifestation of foodborne illness among adults and children is acute gastroenteritis (AGE); the symptoms of gastroenteritis include inflammation of stomach, diarrhea, and vomiting (Patel, Hall et al. 2009, Yen, Wikswo et al. 2011, CDC 2016). Viruses such as norovirus and rotavirus are the leading cause of acute gastroenteritis among adults, infants, and young children, mainly due to their persistence in the environment and their low infectious doses (10 to 100 particles) (Graham, Dufour et al. 1987, Teunis Peter, Moe Christine et al. 2008, Siebenga, Vennema et al. 2009). Each year norovirus causes on average 19-21 million total illnesses in the U.S (CDC 2018). According to the CDC in the period of 2009-2013; a total of

7,700 foodborne outbreaks were reported in the U.S. out of which norovirus contributed to the largest number of associated deaths. In 2013 rotavirus caused 215,000 deaths globally among children under the age of 5 years (Hall, Lopman et al. 2013, Painter, Hoekstra et al. 2013, MMWR 2015, Fuzawa, Ku et al. 2016, CDC 2018, WHO 2018)

The "farm to fork" supply chain of fresh produce is composed of multi-steps with many of them susceptible to produce contamination with pathogenic microorganisms. For instance, during growth, fresh produce can be exposed to contaminated soils, dust, irrigation water, bird droppings, or improperly processed manure (Islam, Michael P. Doyle et al. 2004). Common harvesting practices such as infield cutting, shredding, and rinsing of produce may also lead to contamination of the product. Studies have shown that microorganisms harbored on the outer layers of fresh produce can contaminate the inner layers of the produce during cutting, and the cutting may also promote internalization of microorganisms through mechanically-induced open wounds (Deering, Mauer et al. 2012, Shenoy, Oliver et al. 2017). In addition, shredding prior sanitization promotes the release of organic matter causing a reduction of sanitizer concentration (Luo, Nou et al. 2011, Luo, Nou et al. 2012, Gómez-López, Lannoo et al. 2014, Palma-Salgado, Pearlstein et al. 2014). The Food and Drug Administration (FDA) has developed a series of initiatives to reduce the risk of fresh produce contamination with human pathogenic microorganisms; nonetheless, at least one foodborne illness outbreak caused by contaminated fresh produce is reported annually in the US (CDC 2017, CDC 2018).

In recent years, researchers have tried to identify factors that promote attachment of human virus to fresh produce and hinder disinfection efforts. (Wang, Zhang et al. 2012) investigated the effect of leafy green surface chemistry, specifically surface pH on the attachment of porcine sapovirus (SaV) to lettuce leaves. They concluded that higher binding of SaV to lettuce leaves

occurred at pH 5 and the SaV was capable of surviving on the leaves for up to 7 days when stored at 4 °C. The effect of surface hydrophobicity, surface roughness, and composition of epicuticular wax of 24 cultivars of leafy greens on the attachment of porcine rotavirus (strain OSU) was examined by (Lu, Ku et al. 2015). They reported that concentration of alkanes in the leafy green epicuticular wax, as well as surface hydrophobicity and surface roughness, played an essential role in the variation in viral adsorption to leaf surfaces. Recently, (Fuzawa, Ku et al. 2016) investigated the efficacy of different sanitizers on removal of viral particles from three contaminated produce cultivars ("Red Rusian" kale, "Starbor" kale, and "Totem" belgian endive). They found that oxidant-base sanitizers were less effective in removing porcine rotavirus attached to produce than the surfactant-base counterpart, possibly due to strong interactions between the leaf and the viral particles.

Although many studies have been performed to understand fresh produce disinfection process, one of the main issues associated with produce sanitation studies is a lack of repeatability among different research groups even for wash of the same produce type using the same sanitizer. This is caused by the nature of fresh produce as they are living plant tissues constantly undergoing metabolic processes. The biological variation among produce items of same genus and species is another important reason for the often-observed variation in bacterial removal. For instance, one lettuce leaf may have different surface roughness, hydrophobicity, and surface wax with another, and even the different location of the similar lettuce leaf may have different surface properties. Methods need to be developed to minimize the error and uncertainty caused by the above-mentioned variations, to provide a better understanding of the effect of surface roughness, hydrophobicity, and epicuticular composition on attachment and removal of viral organisms from fresh produce. In this study, we utilized newly developed artificial plant leaf surfaces as a platform

to study the effect of produce leaf physiochemical characteristics on the attachment and removal of porcine rotavirus (PRV), strain OSU, and tulane virus (TV) - a surrogate of human norovirus. The artificial leaf surface was also used to screen commercially available and new sanitizers and to study the use of ultrasonication as an enhancer of viral detachment in the washing step.

5.3 MATERIALS AND METHODS

5.3.1 Sanitizers

Two oxidant-base sanitizers (peroxyacetic acid and sodium hypochlorite) and one surfactant-base sanitizer (malic acid + thiamine dilauryl sulfate (TDS)) were used in this study. Peroxyacetic acid (Tsunami 100 ®) was purchased from Ecolab (Saint Paul, MN) and sodium hypochlorite (bleach) was from a local supermarket. For the surfactant-based sanitizer, malic acid and TDS were purchased from Sigma Aldrich (St. Louis, Mo) and Sanigen Co. Ltd (Juan-dong, South Korea), respectively.

5.3.2 Fresh and artificial produce washing protocol

Washing of fresh and artificial produce surfaces was carried in a double jacket glass beaker (500 ml), equipped with a stir bar. The ultrasound treatment was performed by submerging an ultrasonic probe (10.2 cm diameter) 5 cm into a sanitizing solution; an ultrasound generator (25 kHz, Quality Sonic Products, EZ, SOEST, Netherlands) was used to drive the probe. Prior to each test, the glass beaker was filled with 100-250 ml of each sanitizing solution (4 °C ± 1 °C) for washing without ultrasound or subsequently degassed for 1 minute for ultrasound enhanced wash to remove dissolved gases and improve ultrasound efficacy. A leaf-to-sanitizer-solution ratio of 1:100 (by mass) was used in all washing tests. The sodium hypochlorite solutions were prepared

by dilution of Clorox® (active ingredient: 6.15% sodium hypochlorite) in Milli-Q water to 20 mg/L of free chlorine (pH = 5.0 ± 0.01). The peroxyacetic acid solutions were prepared by dilution of Tsunami-100® (active ingredient: Peroxyacetic acid) in Milli-Q water to 80 mg/L of acid concentration (pH = 2.6 ± 0.01). The malic acid + TDS solutions (pH = 2.5 ± 0.03) were prepared by dilution of malic acid and thiamine dilauryl sulfate (TDS) solution (dissolved in ethanol) in Milli-Q water to 5% and 0.05% of acid concentration (w/v), respectively. The free chlorine concentration was measured using a free chlorine standard kit (Hach Company, Loveland, CO, USA). The concentration of the peroxyacetic acid was determined by titration using a Peroxyacetic / Peroxide #311 test kit provided by Ecolab (St Paul, MN, USA)

5.3.3 Sample preparation and inoculation

5.3.3.1 Viral strain preparation.

Porcine rotavirus (PRV) strain OSU and tulane virus (TV) were used in this study. Both virus types were propagated in a monkey MA104 cell line (ATCC, VA) and maintained at 37 °C in a 5% CO_2 incubator with Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS). They were then propagated using confluent cells in 150 cm^2 flasks; the cells were washed three times with pre-warmed Hank's Balanced Salt Solution buffer (HBSS). The PRV and TV were activated with trypsin at a concentration of 1 μg/ml for 30 minutes at 37 °C followed by the addition of serum-free MEM. The trypsin-activated rotavirus solution was added to the confluent cells in 150 cm^2 flasks and incubated at 37 °C for 60 minutes in a 5% CO_2 incubator (Thermo Scientific, MA USA), then serum-free MEM was added to the flask and incubated for 4 to 5 days at 37 °C in the presence of trypsin until most of the cells were detached. The rotavirus solutions were sequentially frozen at -80 °C and thawed three times. The PRV and TV solutions were

centrifuged at 1,000 *g* for 12 minutes at 25 °C ± 1 °C and filtered through a 0.22 μm-pore-size filter to remove cell debris. Afterward, the viral solutions were purified by centrifuging them with a 40% sucrose solution at 1,000 *g* for 3 hours at 4 °C. The PRV and TV solutions were resuspended in 1X phosphate buffered saline (PBS) plus 0.1mM $CaCl_2$ and stored at -80 °C until further use. Virus titers were quantified by using the plaque-forming unit (PFU) assay. The identification of both PRV and TV after propagation was confirmed by sequencing and matching with GenBank the fragments of OSU and TV after RT-PCR.

5.3.3.2 Preparation of fresh and artificial produce leaves

Each set of leaves in this study, consisting of four biological replicates of spinach leaves and romaine lettuce heads, were purchased from a local supermarket and immediately transported to a processing laboratory where they were stored at 5 °C ± 1 °C and used within 72 hours of purchase. For the romaine lettuce, the three outermost leaves of each head were removed and discarded, and a sterile kitchen knife was used to slice the head lettuce into pieces of approximately 1 in^2 (6.45 cm^2). Whole spinach leaves were used in this experiment. Artificial surfaces, consisting of four replicates, were created following procedure in Chapter 4 §4.3.3. by preparing polydimethylsiloxane (PDMS) according to the manufacturer protocol. To modify the surface hydrophobicity of the PDMS surfaces, 1 or 10% (v/v) of surfactant Caprol PGE-860 (HLB value = 11) was added in PDMS followed by degassing under vacuum (3,330 Pa) for 2 hours. The PDMS surfaces with addition of 1% Caprol PGE-860 were hydrophilic (WCA= 70°) while those with 10% surfactant were hydrophobic (WCA= 110°). The degassed PDMS mixture was poured onto PDMS molds and baked at 100 °C for 3 minutes. The hardened PDMS surfaces were spin-coated with epicuticular wax solutions following the procedure in Chapter 4 §4.3.4 (Lu et al., 2015).

5.3.3.3 Leaf inoculation

Prior to inoculation, the adaxial surfaces of fresh romaine lettuce and spinach leaves were cleaned for 1 minute with a gentle stream of sterile Milli-Q water to remove debris and patted dry using Kim wipes® (Kimberly-Clark, TX). Each artificial surface was sterilized by exposing it to UV light for 10 minutes. Diluted PRV and TV stock solutions were prepared by diluting 0.5 ml of pure rotavirus in 0.5 ml of 1*X* PBS buffer + 1mM $CaCl_2$. The initial viral stock inoculum was TV= 7.3 ± 0.3 Log_{10} PFU/ml and PRV = 8.4 ± 0.4 Log_{10} PFU/ml. Using a sterile tweezer each leaf was transferred to an empty sterile petri dish and 100 µl of rotavirus solution in PBS buffer was spot inoculated at 10 different spots on the adaxial surface of the leaves. The petri dish was then loosely capped and incubated for 2 hours at 25 °C ± 1 °C in a biological cabinet (Labconco Purifier™ Logic+™ Class II, Fisher Scientific, Waltham, MA). After incubation, each set of leaves was transferred to a sterile container with 1*X* PBS buffer using a leaf-to-solution ratio of 1:10 (by mass) and agitated for 1 minute to remove loosely attached virus.

5.3.4 Sanitization tests

The inoculated fresh and artificial produce leaves were sanitized using a leaf-to-sanitizer-solution ratio of 1:100 (by mass), with each treatment conducted four times. The sanitizing solutions used included: 1X PBS buffer (water wash, control), sodium hypochlorite (20 mg/L free chlorine), peroxyacetic acid (80 mg/L free peroxyacetic acid), and 0.5% malic acid + 0.05% TDS surfactant. Each leaf was submerged in 100 – 250 ml of washing solution (4 °C ± 1 °C) and washed for 1 minute with agitation (150 RPM) with or without ultrasound (25 kHz). The reaction of the free chlorine was stopped by using 10% sodium thiosulfate diluted in 1X PBS buffer, and the reaction of free peroxyacetic acid and malic acid were stopped with 1X PBS buffer (pH 7.05 ±

0.01) + 1mM $CaCl_2$. After sanitization all samples were transferred to a sterile sampling bag containing 1X PBS buffer + 1mM $CaCl_2$ and sonicated for 1-minute (25 kHz, 80% power) to remove all viruses attached to the surface. The virions collected were used for plaque forming unit (PFU) assay.

5.3.5 Plaque forming unit (PFU) assay

Trypsin-activated PRV and TV stocks was serially diluted with serum-free MEM. Afterwards, PRV and TV aliquots were applied to MA104 cellular monolayers in a 6-well plate and incubated at 37 °C for 60 minutes in a 5% CO_2 incubator. Following the incubation, an agarose overlay solution containing 1µg/ml of trypsin was added to each well and the plates were incubated at 37 °C for 72 hours in a 5% CO_2 incubator (Thermo Scientific, MA USA) to allow the viruses to replicate. Afterwards, the cells were fixed by adding a solution of 10% (v/v) formaldehyde in 1X PBS buffer for 2 hours. Following that, the medium was removed from each well, and the cells were stained with 0.05% (w/v) crystal violet in 10% ethanol and plaques were counted manually.

5.3.6 Surface hydrophobicity

Surface hydrophobicity was measured as previously described by Lu et al., 2015. Briefly, nine disks (approximate area of each disk 2.01 cm^2) from ‘Outredgeous’ romaine lettuce, ‘Carmel’ spinach, or artificial surfaces with and without epicuticular chemical deposition were excised (for fresh leaves) and taped (3M, Minnesota, USA) to a microscope glass slide exposing the adaxial surface of the leaves. To prevent contamination with debris and dust particles, each microscope slide was covered with aluminum foil. Water contact angle (WCA) of all surfaces was obtained using a goniometer (KSV Instruments, Stockholm, Sweden) model CAM 200. Using a calibrated

pipette 5 μl of deionized water was placed at the center of each disk and within 20 seconds five contact angle readings were taken. The artificial surfaces were considered to be hydrophilic when WCA= 70° and of hydrophobic when WCA= 90°.

5.3.7 Surface roughness

Produce surface roughness was measured as described in Chapter 4 §4.3.5.2. A laser confocal microscope (NanoFocus, μSurf explorer, Oberhausen, DE) was used to determine three-dimensional surface parameters. Area-average root mean square roughness (-Sq bar) was obtained from the average of nine linear root mean square roughness (Sq) measured from the 3-D image reconstructed from 2-D laser confocal images over an area of 0.3 mm × 0.3 mm (0.09 mm^2). Image analysis was done using the software Mountains (Digital Surf, France).

5.3.8 Scanning electron microscope characterization

Produce surface characterization was carried using a Scanning Electron Microscope (SEM). Microimages of the epicuticular surfaces were taken using a FEI Quanta FEG 450 ESEM (Hillsboro, OR, USA). The images were captured under vacuum at 20 kV and at 400×, 800×, and 1200× magnification from at least three different samples.

5.3.9 Statistical analyses

The experiments were performed with a complete randomized design (CRD) with each treatment conducted four times. The PRV and TV counts were subjected to log transformation before statistical analysis. Data were analyzed using a general linear model available in SAS version 9.1 (SAS Institute, Raleigh, NC, USA), and with Origin-Pro 2016 (OriginLab Corporation,

MA, USA). Separation of treatment means was performed using Tukey's test with $\alpha = 0.05$. For the sanitization experiments of PRV and TV adhering to leaves, the significant differences in sanitization efficacies between cultivars and sanitizer treatments were determined using a one-way analysis of variance (ANOVA). The efficacy of ultrasound treatment was analyzed using t-tests, normality of data distribution was determined with Shapiro-Wilk test. Relationships were considered significant when $P < 0.05$.

5.4 RESULTS AND DISCUSSION

5.4.1 Attachment of porcine rotavirus (strain OSU) and tulane virus to 'Outredgeous' romaine lettuce and 'Carmel' spinach fresh and artificial surfaces.

As shown by the PFU assay (Figure 5.1), a higher attachment was observed of PRV to fresh leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach (-0.09 ± 0.05 Log_{10} PFU/PFU_0 and -0.06 ± 0.05 Log_{10} PFU/PFU_0, respectively) than to artificial leaf surfaces. However, the difference in PRV attachment between fresh and artificial surfaces was not significant different ($P > 0.05$). Similarly, a higher attachment TV occurred on fresh leaves of both 'Outredgeous' romaine lettuce and 'Carmel' spinach (-0.09 ± 0.02 Log_{10} PFU/PFU_0, -0.08 ± 0.01 Log_{10} PFU/PFU_0 respectively) compared to artificial leaf surfaces (Figure 5.2), but again the differences were not significantly different ($P > 0.05$). In addition, no significant differences were observed on the attachment of PRV and TV to hydrophobic surfaces (WCA = 110°) of 'Outredgeous' romaine lettuce and 'Carmel' spinach compared to the control (smooth PDMS, WCA= 120°) which had a smooth surface ($P > 0.05$).

The results shown in Figures 5.1 and 5.2 indicate that surface roughness and surface hydrophobicity have no significant effect on the attachment of PRV and TV to fresh produce and

artificial surfaces. This finding can be explained by the difference in the size of viruses and that of the features on produce surfaces. As shown in Table 5.1, the vertical height variations (surface roughness) on the surface of fresh leaves and artificial phylloplanes are in micrometers while the sizes of tulane virus virion (400 Å diameter) and porcine rotavirus virion (70 nm diameter) are in nanometers (Kapikian 1996, Yu, Zhang et al. 2013). (Lu, Ku et al. 2015) also reported that viral attachment was not correlated with surface hydrophobicity of 24 leafy green cultivars but rather with the presence of three-dimensional wax structures on produce leaf surfaces. The fact that no significant differences in viral attachment to fresh and artificial produce surfaces indicated that the artificial produce surfaces developed by our group can be used in produce sanitation studies to provide a surface that mimic both the topological and chemical properties of fresh produce leaves to eliminate the biological variation among produce samples.

From the SEM microimages in Figure 5.3, one can see that the epicuticular wax on fresh and artificial leaf surfaces exhibited different hierarchical structures. The epicuticular wax on the fresh leaves of 'Carmel' spinach (Fig. 5.3a) and romaine lettuce (Fig. 5.3b) was a mixture of monolayer long chain hydrocarbons and three-dimensional crystals, whereas the epicuticular wax on the artificial phylloplanes (Fig. 5.3c-d) was mainly three-dimensional crystals. The smooth PDMS (control surface) (Fig. 5.3e) had no epicuticular wax. Since no significant differences were found among different leaf surfaces with regard to the effect of surface topological and chemical properties in this study, we hypothesized that the high viral counts present in the inoculum utilized in the experiments e.g., PRV= 8.4 ± 0.4 Log_{10} PFU/ml; TV= 7.3 ± 0.3 Log_{10} PFU/ml, may have override the effect of surfaces physiochemical characteristics tested; similar behavior has been observed in the studies of (Kukavica-Ibrulj, Darveau et al. 2004, Kramer, Schwebke et al. 2006, Yang, Chambers et al. 2018) on attachment of viruses to inanimate surfaces.

5.4.2 Removal of porcine rotavirus (strain OSU) and tulane virus with sodium hypochlorite and ultrasound combined treatment

The effects of sodium hypochlorite and ultrasound (25 kHz) combined treatment on removal of PRV and TV on fresh leaves and artificial surfaces of ‘Outredgeous’ romaine lettuce and ‘Carmel’ spinach are shown in Figures 5.4 and 5.5. A significantly higher reduction ($P < 0.05$) of PRV was achieved by the sodium hypochlorite alone wash compared to the control (1X PBS buffer). When ultrasonication was added in the sodium hypochlorite wash, no significant ($P > 0.05$) enhancement in the removal of PRV and TV from the artificial spinach and lettuce leaves was observed (Figures 5.4-5.5). However, compared to the chlorine alone wash, the addition of ultrasonication increased the removal of PRV and TV from fresh leaves of ‘Outredgeous’ romaine lettuce and ‘Carmel’ spinach by up to 2.0 ± 0.5 Log_{10} PFU/ml ($P < 0.05$) (Figure 4).

One issue in fresh produce sanitation with a chlorine-based sanitizer is the depletion of chlorine due to its interaction with organic matter and electrolytes released by cut produce tissues. Thus, the observed enhancement in viral removal by adding ultrasonication to the fresh produce wash may be caused by the physical dislodging effect of ultrasound which is independent of sanitizer concentration. Many studies have reported similar enhancement in microbial inactivation by application of ultrasonication to a produce chlorine wash (Zhou, Feng et al. 2009, Luo, Nou et al. 2012, Palma-Salgado, Pearlstein et al. 2014). We hypothesize that since artificial phylloplanes are silicon based, there is less or no release of organic matter that can cause depletion of the chlorine therefore the effect of adding ultrasonication became less significant.

5.4.3 Removal of porcine rotavirus (strain OSU) and tulane virus with peroxyacetic acid and ultrasound combined treatment.

The results of the peroxyacetic acid (80 mg/L) and ultrasound (25 kHz) combined treatment on removal of PRV and TV on the surface of fresh leaves and artificial phylloplanes of 'Outredgeous' romaine lettuce and 'Carmel' spinach are shown in Figures 5.6 and 5.7. A significantly higher reduction of PRV and TV was achieved by the peroxyacetic acid alone wash compared to the control (water wash) ($P < 0.05$).

A higher removal of PRV was obtained from the spinach artificial phylloplanes compared to the fresh leaves (Figure 5.6). As shown in Figure 5.3, the artificial phylloplanes of spinach had a mixture of monolayer and three-dimensional epicuticular wax structure. Even though the rotavirus *spp*. outer viral capsid is hydrophilic (Farkas, Varsani et al. 2015, Fuzawa, Ku et al. 2016), due to the presence of three-dimensional epicuticular wax, the interfacial interaction virus/solid might not be strong enough to provide shelter to rotavirus viral particles. These structures might become the place that harbor PRV on the artificial phylloplane surfaces thus becoming readily available to be inactivated by peroxyacetic acid.

However, as can be seen in Figure 5.7, no clear trend was observed for the removal of tulane virus from fresh leaves of spinach and lettuce as well as from the artificial phylloplanes. This may be caused by the small size of TV and the strong oxidizing capabilities of peroxyacetic acid (pH of washing solution 2.6 ± 0.01) that suppressed the differences among treatments.

5.4.4 Removal of porcine rotavirus (strain OSU) and tulane virus with 0.5% Malic acid + 0.05% TDS and ultrasound combined treatment.

The effects of 0.5% Malic acid + 0.05% TDS in combination with ultrasound (25 kHz) for removal of PRV and TV on the fresh leaves and artificial phylloplanes of 'Outredgeous' romaine lettuce and 'Carmel' spinach was examined. Figures 5.8 and 5.9 show that similar to the wash with an oxidant-base sanitizer, the addition of surfactant-base sanitizer yielded significantly higher reductions of PRV and TV compared to the control (water wash) ($P < 0.05$).

Although less removal of both PRV and TV was observed in samples treated with the surfactant-base sanitizer compared to the oxidant-base sanitizers (Figures 5.6-5.7), the addition of ultrasound to the malic acid + TDS wash resulted in a significantly higher reduction of PRV on fresh leaves of 'Outredgeous' romaine lettuce (3.4 ± 0.5 Log_{10} PFU/ml vs. 1.4 ± 0.1 Log_{10} PFU/ml) and 'Carmel' spinach (4.1 ± 0.1 Log_{10} PFU/ml vs. 2.6 ± 0.4 Log_{10} PFU/ml). The surfactant-base sanitizer was effective in removing both PRV and TV attached to fresh and artificial leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach. The addition of surfactants to sanitizing solutions is a common practice in the food industry; the purpose is to reduce the surface tension between microorganism and solid and that between aqueous solution and microorganism, thus allowing the sanitizer to reach crevices on fresh produce surfaces (Zhao, Zhao et al. 2009, Salgado, Pearlstein et al. 2014). This may be the reason for the enhanced removal by the 0.5% Malic acid + 0.05% TDS treatment.

Similarly, a significantly ($P < 0.05$) higher reduction of TV on both the fresh leaves of 'Carmel' spinach (3.6 ± 0.5 Log_{10} PFU/ml vs. 1.9 ± 1.1 Log_{10} PFU/ml) and spinach artificial phylloplane (2.5 ± 0.6 Log_{10} PFU/ml vs. 1.1 ± 0.3 Log_{10} PFU/ml) was observed when ultrasound was added in the wash. This might also be caused by the presence of three-dimensional

epicuticular wax on artificial phylloplanes as discussed above which reduced the interaction between the virus and solid interface which was further weakened by the action of the surfactant.

5.4.5 Mean comparison of virus, produce type, surface type and sanitizer type by T-test

The contribution of virus and produce type, produce surface type (fresh vs. artificial), sanitizer type (oxidant- or surfactant-based), or addition of ultrasound to viral removal from produce surfaces is presented in Figure 5.10. The comparisons in Figure 5.10 were made with a t-test by pooling all the target viral removal data together. For instance, when comparing the effect of virus type on viral removal, all the viral removal data of the PRV, regardless of produce, sanitizer type, and with or without sonication, were compared to all the removal data from the TV. It can be seen from Fig 5.10A that the removal of PRV from produce leaves treated by different sanitizers is significantly higher than that of the TV. Noticeably, there is no difference in viral removal from both the fresh and artificial produce surfaces (Fig. 5.10B), confirming that the later can be used as a replica of true produce leaf surface for sanitation studies. The viral removal is also produce-type dependent as shown by a higher (but not significant) removal of viruses from spinach surfaces than that from the lettuce surfaces (Fig. 5.10C). The oxidant-base sanitizers were generally more effective than the surfactant-based sanitizer in removal of virus on both fresh and artificial surfaces (Fig. 5.10D) The effect of ultrasound is evidenced by a significantly higher viral removal when ultrasound was added to a sanitizer wash (Fig. 5.10E).

5.5 CONCLUSIONS

No significant differences were observed in the attachment of PRV and TV inoculated to fresh leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach and their artificial counterparts. In sanitation tests, the removal of virus attached to fresh and artificial surfaces was virus type, sanitizer type, and produce cultivar dependent. For the same sanitation treatment on the same produce (fresh or artificial) surface, more PRV can be remove than TV. The oxidant-base sanitizers were generally more effective than the surfactant-based sanitizer in removal of virus on both fresh and artificial surfaces. In most treatments except the fresh lettuce wash with peroxyacetic acid, more viral removal was observed on spinach surfaces than on lettuce. Introduction of ultrasound into a sanitization treatment enhanced the disinfection efficacy in the surfactant-based sanitizer treatment. The design of an effective viral disinfection procedure needs to consider the effect of sanitizer and produce type in order to maximize the removal of a target virus.

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5.7 FIGURES AND TABLES

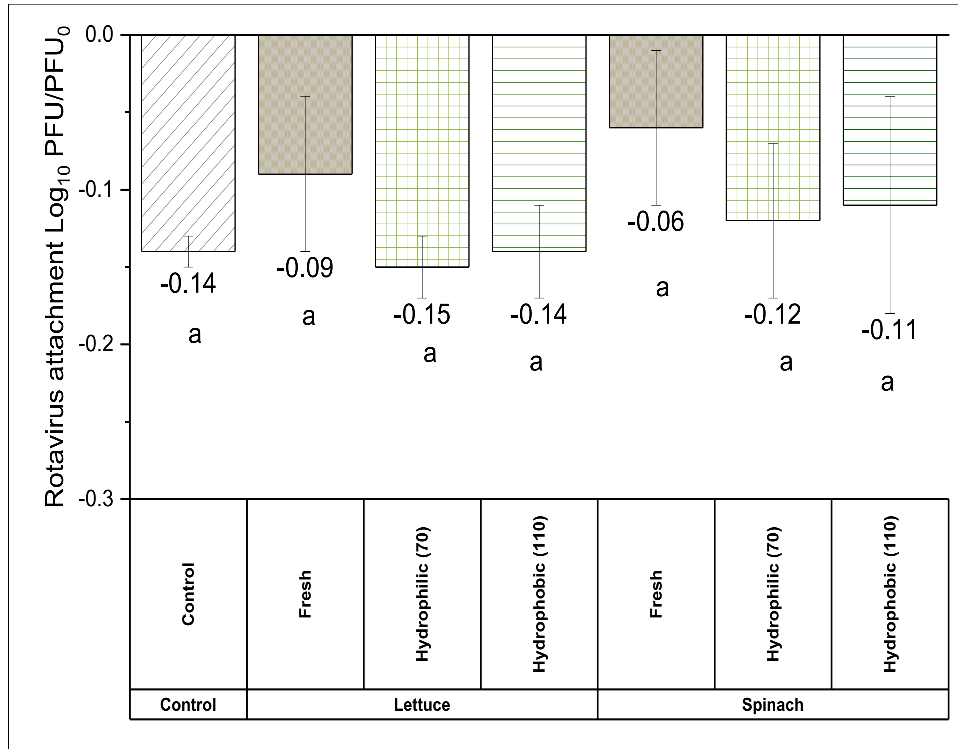

Figure 5.1. Attachment of **porcine rotavirus** (strain OSU), to ‘Outredgeous’ romaine lettuce and ‘Carmel’ spinach fresh and artificial phylloplanes.

a: Means with same letter within each produce type together with the Control are no significantly different by Tukey's test (α=0.05)

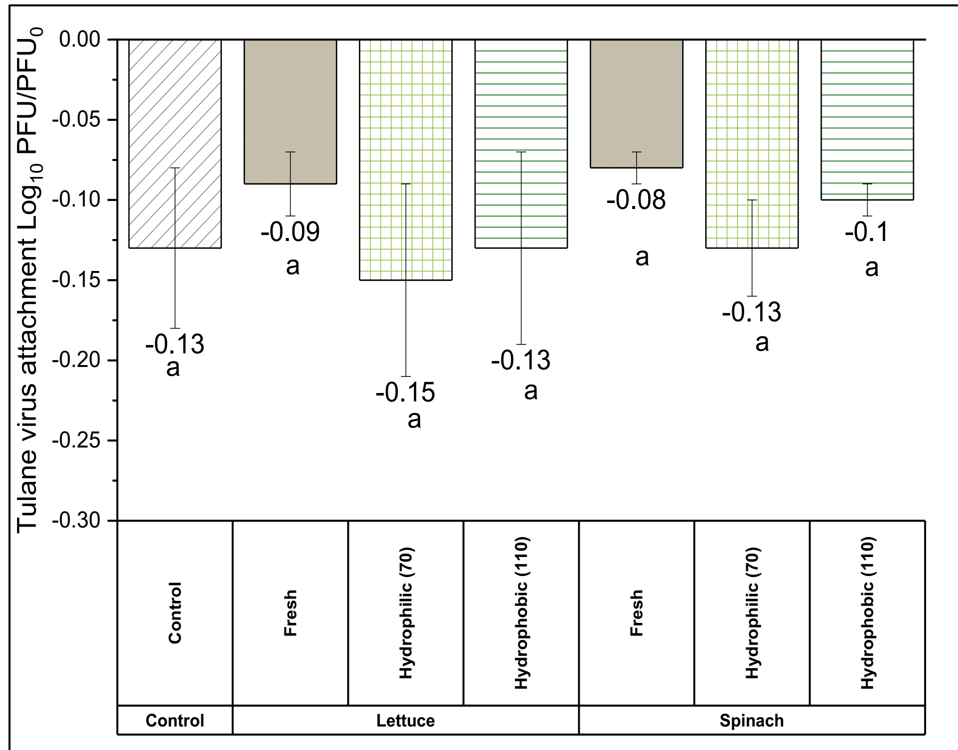

Figure 5.2. Attachment of **tulane virus**, to 'Outredgeous' romaine lettuce and 'Carmel' spinach fresh and artificial phylloplanes.

a: Means with same letter within each produce type together with the Control are no significantly different by Tukey's test (α=0.05

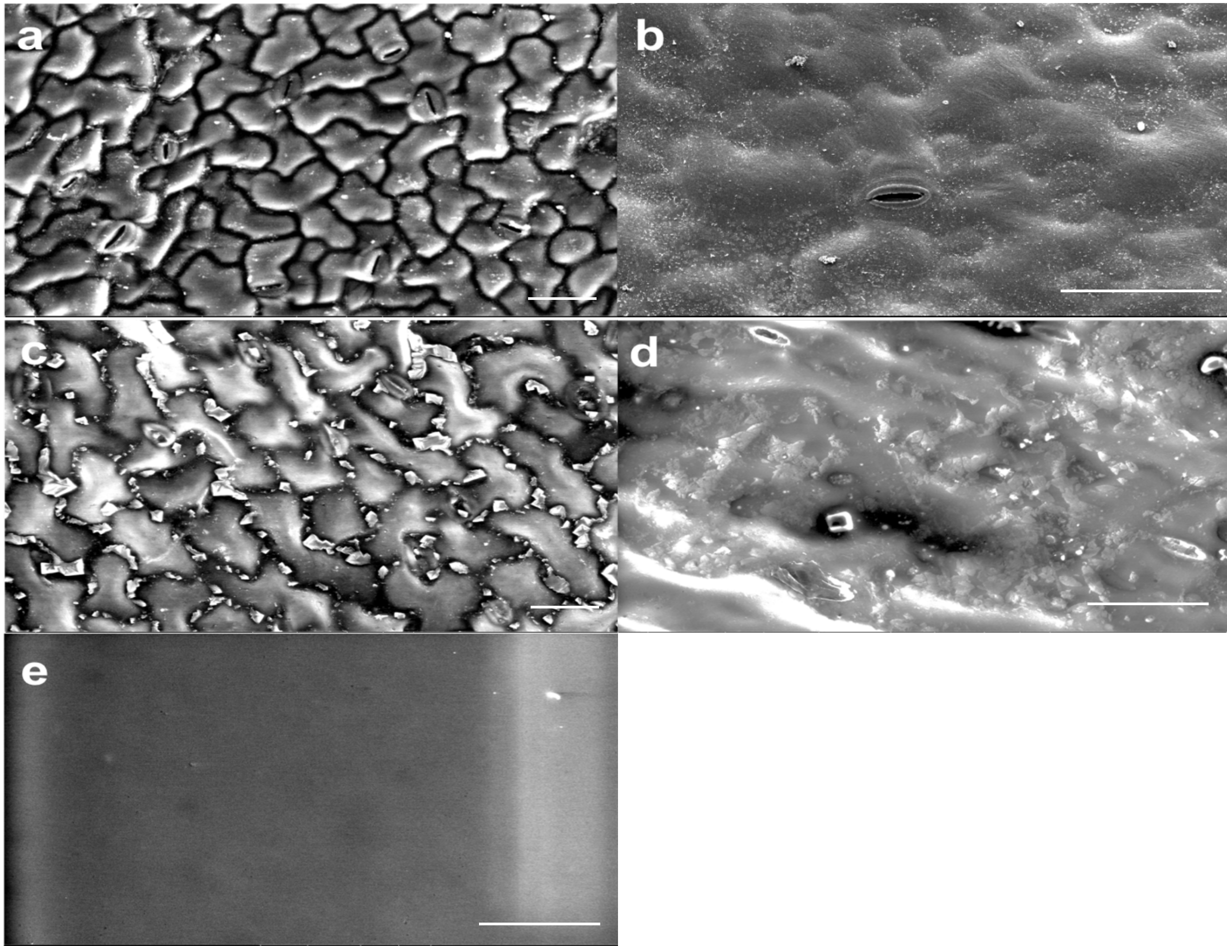

Figure 5.3. Epicuticular characterization of fresh leaves and artificial phylloplanes used for adherence assay. (a) 'Carmel' spinach fresh leaf (b) 'Outredgeous' romaine lettuce fresh leaf (c) spinach artificial phylloplane (d) lettuce artificial phylloplane (e) smooth PDMS (control surface). White scale bar in each figure indicate 50 µm.

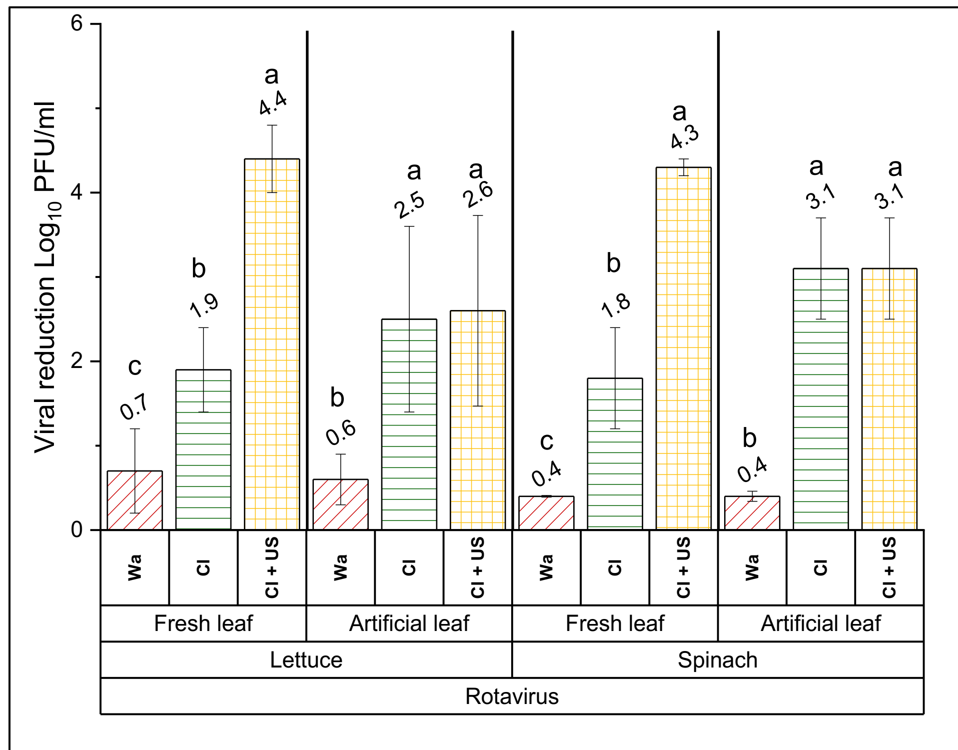

Figure 5.4. Removal of **porcine rotavirus** (strain OSU), using the oxidant-base sanitizer, **sodium hypochlorite (20 mg/L)** in combination with ultrasound (25 kHz)**.**

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

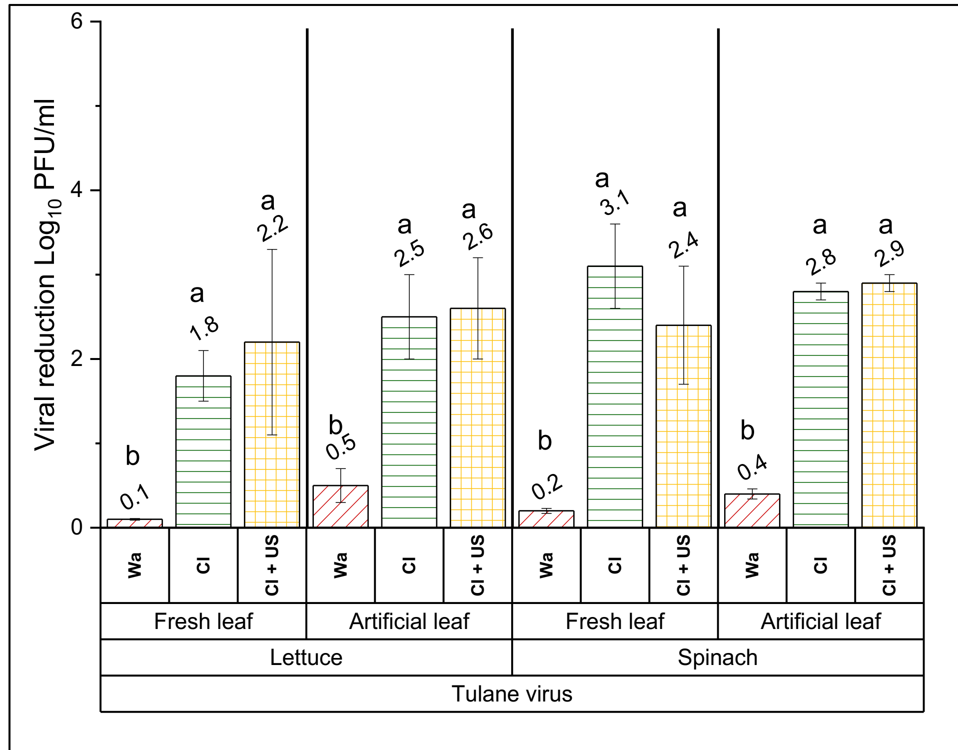

Figure 5.5. Removal of **tulane virus,** using the oxidant-base sanitizer, **sodium hypochlorite (20 mg/L)** in combination with ultrasound (25 kHz).

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

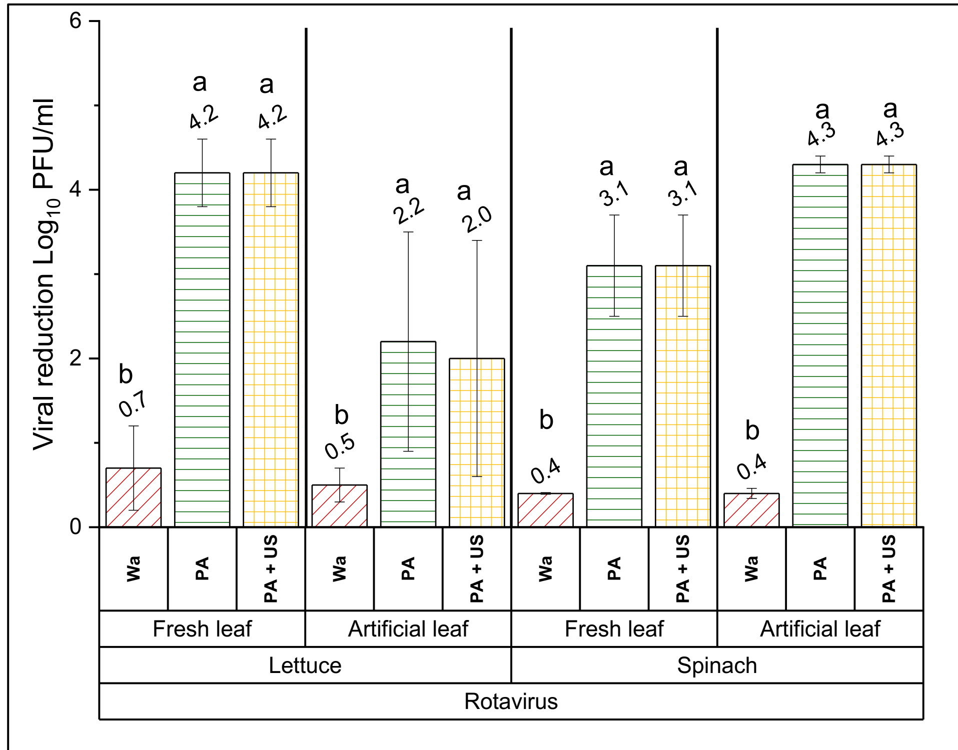

Figure 5.6. Removal of **porcine rotavirus** (strain OSU), using the oxidant-base sanitizer, **peroxyacetic acid (80 mg/L)** in combination with ultrasound (25 kHz).

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

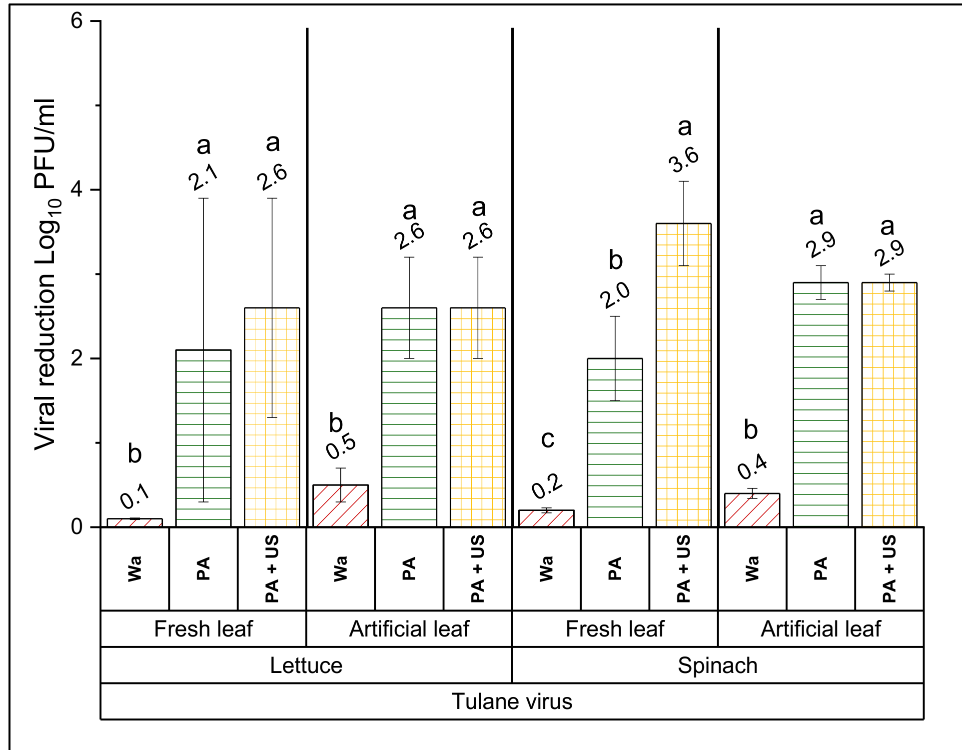

Figure 5.7. Removal of **tulane virus**, using the oxidant-base sanitizer, **peroxyacetic acid (80 mg/L)** in combination with ultrasound (25 kHz).

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

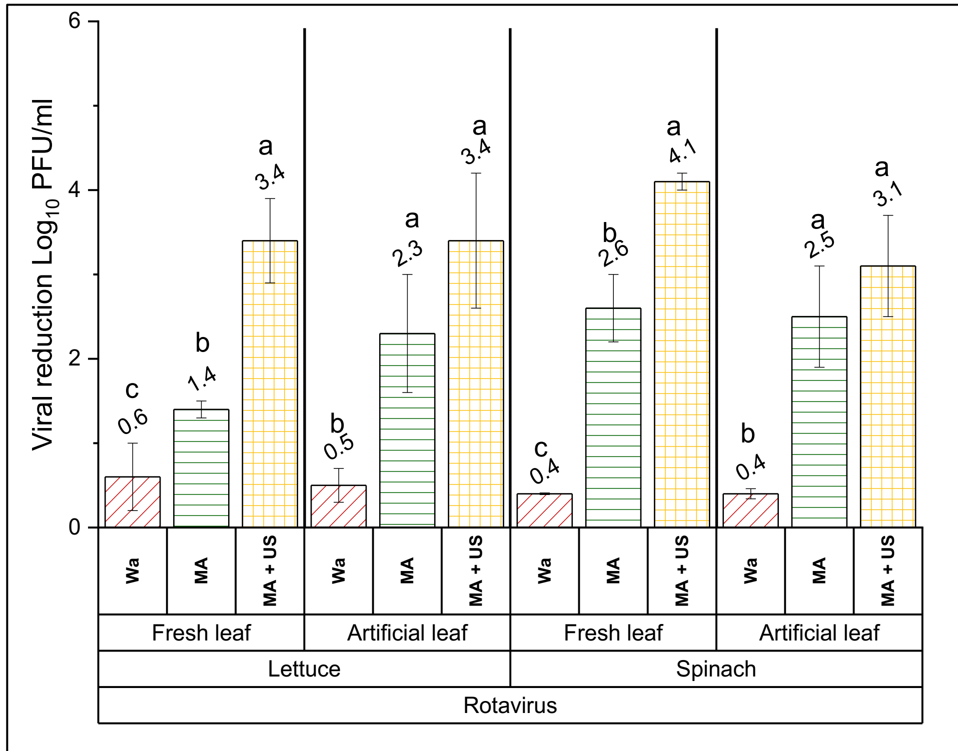

Figure 5.8. Removal of **porcine rotavirus** (strain OSU), using the surfactant-base sanitizer, **0.5% Malic acid + 0.05% TDS** in combination with ultrasound (25 kHz).

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

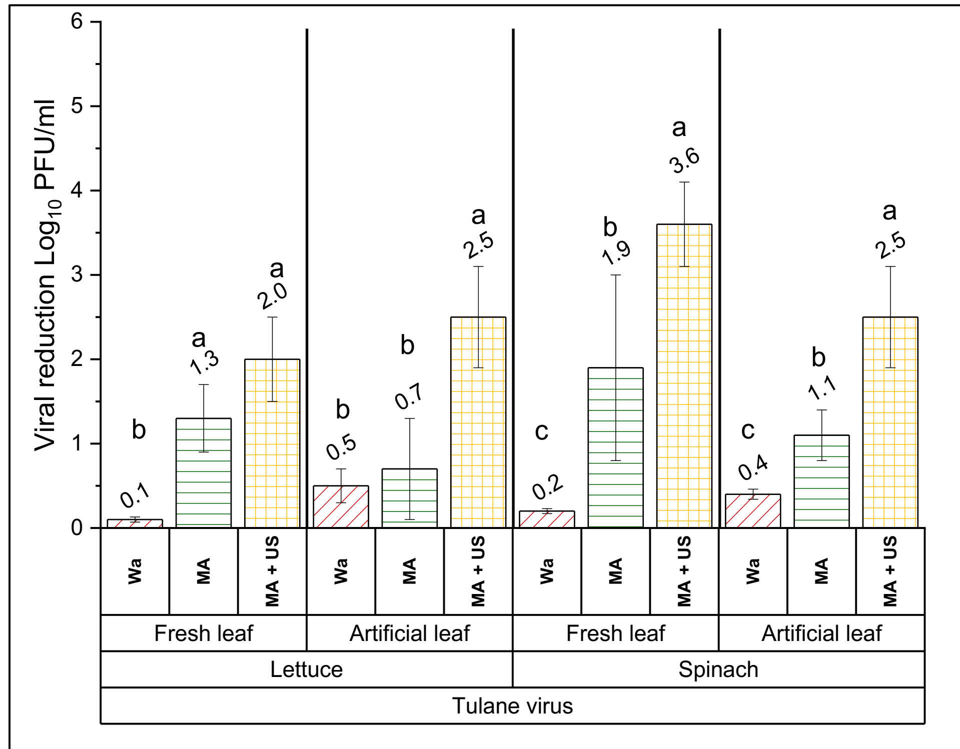

Figure 5.9. Removal of **tulane virus**, using the surfactant-base sanitizer, **0.5% Malic acid + 0.05% TDS** in combination with ultrasound (25 kHz).

a-c: means (bars) within each leaf type (fresh or artificial) with different letters are significantly different by Tukey's test (α=0.05)

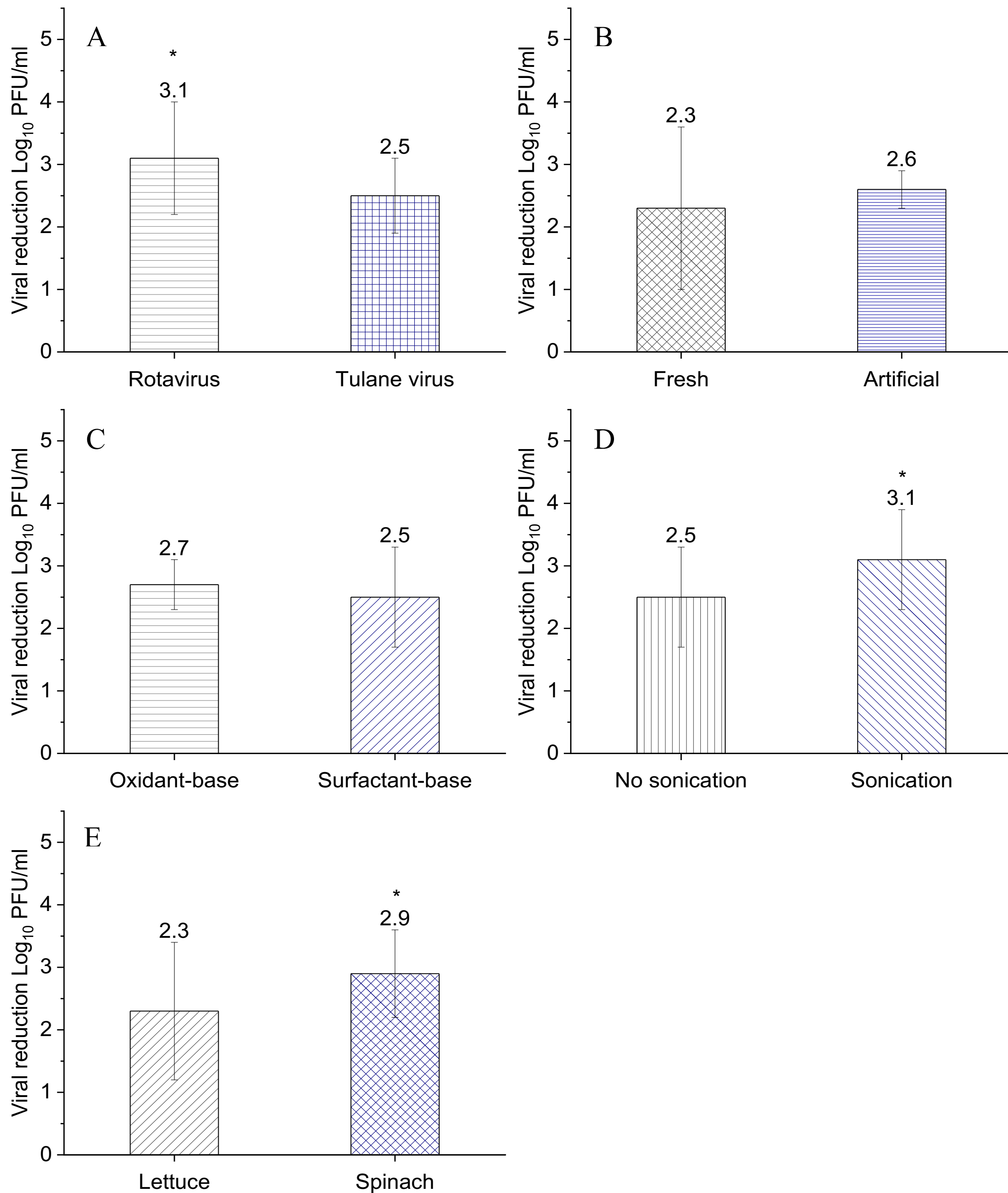

Figure 5.10. Mean comparison by T-test. (A) comparison by type of virus -Porcine rotavirus vs Tulane virus- (B) comparison by type of leaf -Fresh vs Artificial- (C) comparison by type of cultivar -lettuce vs spinach-(D) comparison by type of sanitizer (E) comparison by use of sonication.

* Significant differences at α=0.05

Table 5.1 Physiochemical characteristics of the samples used.

SAMPLE	SURFACE ROUGHNESS (μm)	CONTACT ANGLE (Degrees)
Smooth PDMS (control surface)	1.1 ± 0.01 [b,y]	120.0 ± 1.0 [a,x]
'Carmel' spinach - fresh	8.0 ± 1.0 [a]	74.0 ± 5.0 [c]
Spinach –artificial, hydrophilic	12.0 ± 3.8 [a]	70.0 ± 2.0 [c]
Spinach – artificial, hydrophobic	12.0 ± 3.0 [a]	110.0 ± 2.0 [b]
Romaine lettuce - fresh	8.5 ± 4.0 [x]	71.0 ± 7.0 [z]
Lettuce –artificial, hydrophilic	9.0 ± 1.5 [x]	75.0 ± 5.0 [z]
Lettuce – artificial, hydrophobic	9.0 ± 0.3 [x]	110.0 ± 3.0 [y]

a-c: Means with different letter (column) for spinach sample and control (surface roughness and contact angle) are significantly different by Tukey's test (α=0.05).

x-z: Means with different letter (column) for lettuce sample and control (surface roughness and contact angle) are significantly different by Tukey's test (α=0.05).

CHAPTER 6

CONCLUSIONS AND SUGESTIONS FOR FUTURE RESEARCH

The overall goal of this project was to conduct a comprehensive investigation to develop a new method for the fabrication of artificial phylloplanes that mimic the three-dimensional topological features of natural produce leaves, and have surface hydrophobicity, roughness values and epicuticular composition resembling those of the most commonly consumed fresh produce. Specifically, the adhesion and removal of *Escherichia coli* K12 as affected by the epicuticular wax composition, surface roughness, and hydrophobicity features of fresh produce, and the hydrophobicity of the bacterial surface was investigated. Then, a rapid-replication "double-casting" method to produce these artificial phylloplanes that mimic the physicochemical characteristics of selected fresh produce and examine their use in bacterial attachment/removal studies was developed. Finally, the attachment and removal of porcine rotavirus (OSU) and tulane virus from fresh and artificial leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach as affected by ultrasonication in combination with oxidant- or surfactant-based sanitizers was investigated.

In the first stage of this research, the outcome indicated that: (1) factors such as produce genotype, produce surface roughness, and wax composition affected the attachment and removal of *Escherichia coli* K12 to/from five leafy green cultivars; (2) Surface roughness was positively correlated to attachment of *Escherichia coli* K12 and negatively correlated to its removal; and (3) vegetables with higher surface wax content resulted in less rough surface and more bacterial removal than the produce with lower wax content.

In the second stage of this research, the outcomes showed that: (1) polydimethylsiloxane (PDMS) can be used as a substrate to create artificial phylloplanes that mimic surface

characteristics of fresh produce; (2) mixing a non-ionic surfactant with different HLB values resulted in modification of surface hydrophobicity of PDMS to match the hydrophobicity of produce leaves; and (3) similarities in bacterial attachment patterns between the fresh produce leaves and artificial phylloplanes were observed; (4)The PDMS leaf replicas are reusable, economical, and recyclable.

In the third stage of this research the findings demonstrated that: (1) artificial phylloplanes can be used as a tool in produce sanitation studies since no significant differences were observed in the attachment of PRV and TV inoculated to fresh leaves of 'Outredgeous' romaine lettuce and 'Carmel' spinach and their artificial counterparts; (2) In sanitation tests, the removal of virus attached to fresh and artificial surfaces was virus type, sanitizer type, and produce cultivar dependent; (3) ultrasonication enhanced the efficacy of disinfection in the surfactant-based sanitizer treatment.

In summary, the newly developed artificial phylloplanes mimic fresh produce surface physiochemical characteristics have a similar surface hydrophobicity, similar epicuticular wax composition, produce similar bacterial and viral attachment and are reproducible and reusable, including autoclave-able and compatible with stomacher. Thus, they are a perfect tool to use during fresh produce sanitation studies and to screen chemical and physical sanitizers.

For future studies, modifications to the artificial phylloplanes can be made so that abaxial surface is also utilized during attachment and removal studies. In addition, the attachment pattern of porcine rotavirus and tulane virus in artificial phylloplanes using Transmission Electron Microscope can be investigated. Finally, the method to create PDMS-based artificial phylloplane can be modified to recreate artificial phylloplanes of other leafy greens such as iceberg lettuce, kale, cilantro leaves and microgreens for future sanitation studies.

APPENDIX A.

PHYSICAL PROPERTIES OF EPICUTICULAR LAYER OF 24 VEGETABLE LEAVES AND TOMATO FRUIT.

Table A1 Surface hydrophobicity and surface roughness of 24 vegetable leaves and tomato fruit

Sample name	Genus	Species	Contact angle-ad (˚)	Contact angle-ab (˚)	Surface roughness-ad (µm)	Surface roughness-ab (µm)
Tokyo bekana	*Brassica*	*rapa*	95.6 ± 7.1	95.6 ± 21.0	2.7 ± 0.9	7.4 ± 3.4
‘Perseo’ radicchio	*Cichorium*	*intybus*	53.1 ± 15.0	55.6 ± 2.6	4.1 ± 2.0	2.9 ± 0.8
‘Rhodos’ endive	*Cichorium*	*endivia*	52.7 ± 8.5	44.6 ± 3.5	5.4 ± 4.0	6.0 ± 2.7
‘Southern Giant Curled’ mustard	*Brassica*	*juncea*	100.2 ± 4.6	116.9 ± 11.2	8.0 ± 0.6	8.9 ± 2.2
Mizuna	*Brassica*	*rapa*	93.1 ± 3.9	96.4 ± 0.7	2.5 ± 0.2	5.0 ± 0.9
‘Tyee’ spinach	*Spinacia*	*oleracea*	99.3 ± 5.8	99.5 ± 13.9	3.5 ± 1.9	2.9 ± 0.2
‘Racoon’ spinach	*Spinacia*	*oleracea*	104.2 ± 3.0	110.0 ± 2.4	2.8 ± 0.2	3.9 ± 0.8
‘Carmel’ spinach	*Spinacia*	*oleracea*	87.7 ± 7.0	99.8 ± 2.7	3.0 ± 0.7	4.1 ± 1.4

Continuation of APPENDIX A

Sample name	Genus	Species	Contact angle-ad (˚)	Contact angle-ab (˚)	Surface roughness-ad (µm)	Surface roughness-ab (µm)
Tatsoi	*Brassica*	*rapa*	73.8 ± 16.2	85.2 ± 4.3	6.7 ± 0.9	3.1 ± 0.8
‘Top Bunch’ collards	*Brassica*	*oleracea*	115.1 ± 4.5	127.6 ± 11.3	1.4 ± 0.5	1.9 ± 0.9
‘Starbor’ kale	*Brassica*	*oleracea*	128.9 ± 9.8	126.3 ± 3.7	1.4 ± 0.1	1.6 ± 0.4
‘Red Russian’ kale	*Brassica*	*napus*	125.1 ± 4.9	130.4 ± 7.5	2.1 ± 0.3	3.6 ± 1.3
Arugula	*Eruca*	*sativa*	92.4 ± 8.3	96.3 ± 3.3	5.3 ± 1.2	7.4 ± 1.8
‘Totem’ Belgian Endive	*Cichorium*	*intybus*	56.5 ± 1.6	43.9 ± 2.9	4.0 ± 0.9	3.3 ± 0.9
‘Two Star’ lettuce	*Lactuca*	*sativa*	49.1 ± 9.5	53.4 ± 2.5	2.6 ± 0.6	5.0 ± 1.6
‘Tropicana’ lettuce	*Lactuca*	*sativa*	53.5 ± 10.3	67.4 ± 6.6	2.9 ± 1.2	7.1 ± 0.1
‘Outredgeous’ romaine lettuce	*Lactuca*	*sativa*	60.2 ± 8.6	59.4 ± 6.5	2.5 ± 0.5	2.5 ± 0.8
‘Super Red’ cabbage	*Brassica*	*oleracea*	77.9 ± 2.5	103.4 ± 4.6	1.3 ± 0.6	1.7 ± 0.9
‘Gonzales’ cabbage	*Brassica*	*oleracea*	115.5 ± 1.9	107.3 ± 2.4	1.6 ± 0.2	1.9 ± 0.5

Continuation of APPENDIX A

Sample name	Genus	Species	Contact angle-ad (˚)	Contact angle-ab (˚)	Surface roughness-ad (µm)	Surface roughness-ab (µm)
‘Ruby Perfection’ cabbage	*Brassica*	*oleracea*	115.3 ± 3.0	119.1 ± 2.0	2.4 ± 0.3	1.4 ± 0.3
‘Alcosa’ cabbage	*Brassica*	*oleracea*	110.8 ± 5.4	115.0 ± 5.1	6.0 ± 2.6	4.6 ± 0.9
‘Sun Gold’ cherry tomatoes	*Solanum*	*lycopersicum*	85.4 ± 4.4	-	1.1 ± 0.5	-
‘Indigo Rose’ tomatoes	*Solanum*	*lycopersicum*	97.9 ± 10.6	-	7.1 ± 1.8	-
‘Rose’ tomatoes	*Solanum*	*lycopersicum*	110.7 ± 11.0	-	2.9 ± 0.4	-
LSD			14.9	13.6	2.2	2.9

(Contact angle is presented in °, and roughness is in µm.). Ad and ab indicate adaxial and abaxial leaf, respectively.

APPENDIX B

FIRST PROTOTYPES OF ARTIFICIAL SURFACES USING SOFT LITHOGRAPHY

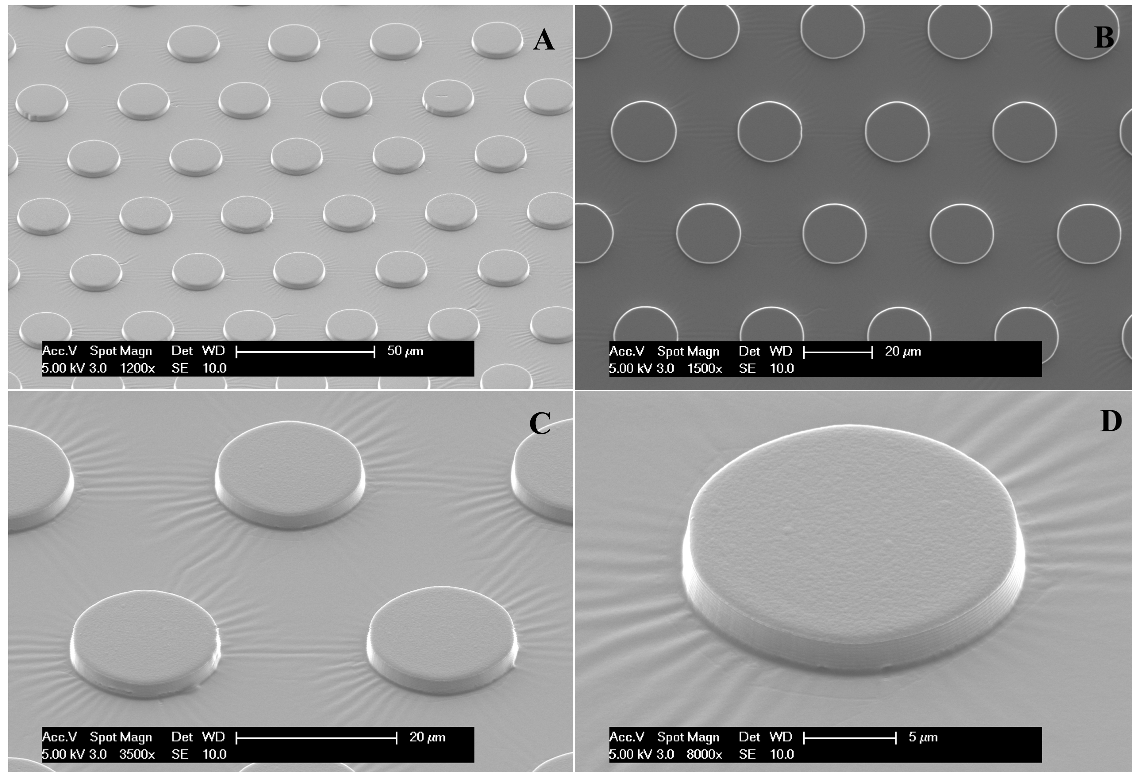

Figure B1. First prototype of artificial leaf designed with periodic array of identical circles spatial distribution every 20 μm (A) sample at 1200×, (B) aerial view at 1500×, (C) magnification 3500×, (D) magnification 8000×.

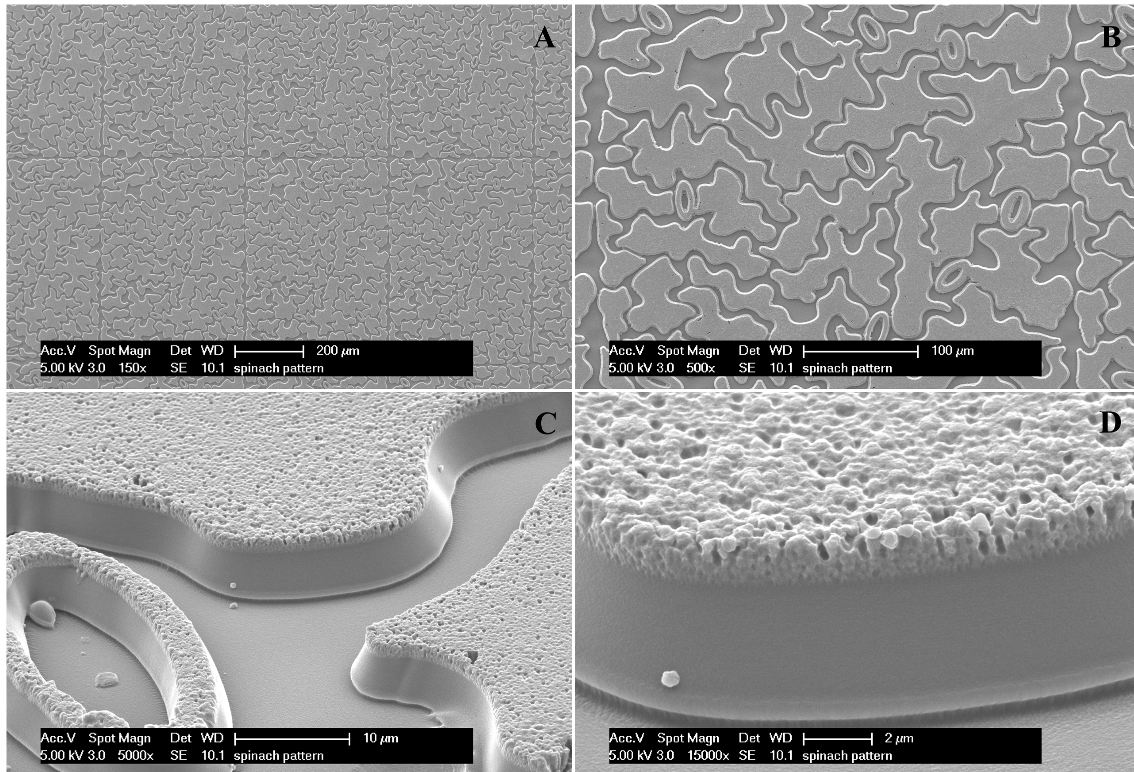

Figure B2. Second prototype of artificial leaf designed with periodic array of identical shapes that mimic adaxial surface of spinach leaf (A) aerial view at 150×, (B) aerial view at 500×, (C) magnification 5000×, (D) magnification 15000×

APPENDIX C

ATTACHMENT OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA TO NEW NANOSTRUCTURED-POLYSTYRENE COATING AND PLANAR-POLYSTYRENE COATING ON GLASS

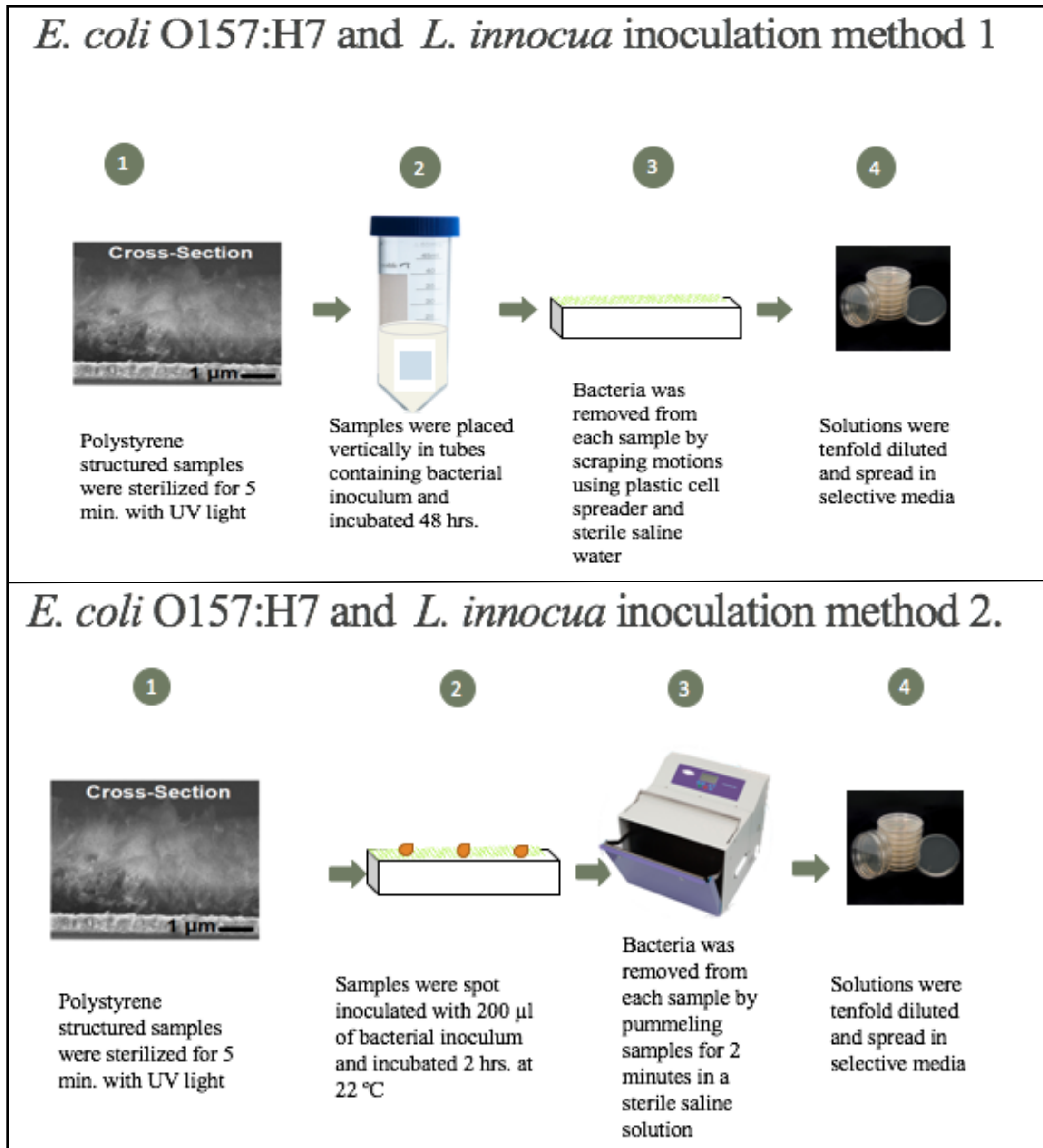

Figure C1. Graphical abstract of adhesion studies of bacteria in nanostructured-polystyrene coating and planar-polystyrene coating on glass

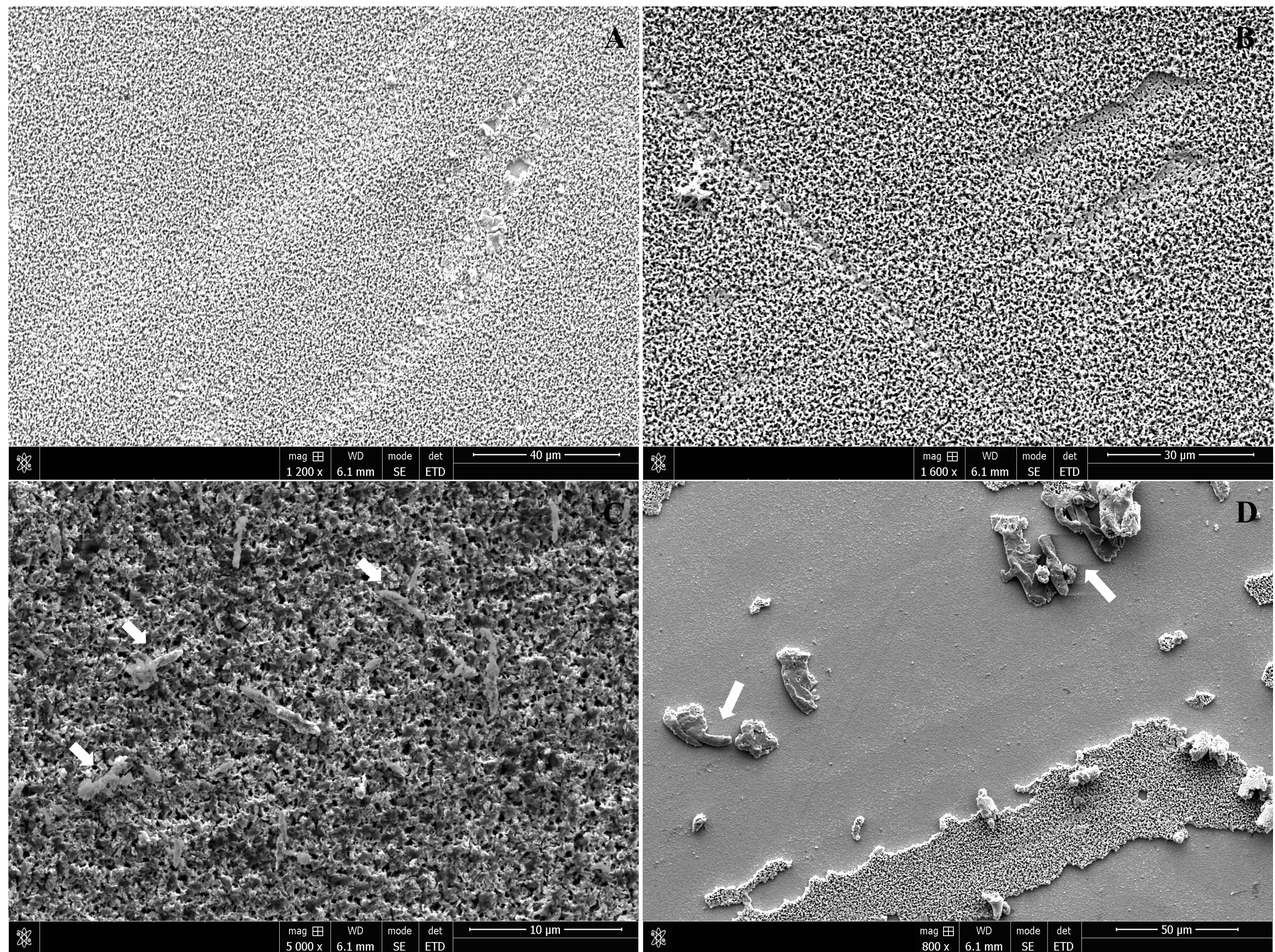

Figure C2. Attachment of *Escherichia coli* O157:H7 on nanostructured-polystyrene coating. (A) uninoculated surface, (B) sample inoculated with method 2, rinsed twice to remove loosely attached bacteria, (C) sample inoculated with method 2, rinsed once to remove loosely attached bacteria, (D) deterioration of polystyrene structure during attachment studies.

Continuation of APPENDIX C

Table C1. Relative adhesion of gram-negative (*Escherichia coli* O157:H7) and gram-positive (*Listeria innocua*) bacteria on bare glass control surfaces and nanostructured PS surfaces

Sample	*E. coli* O157: H7 (Log_{10} CFU/ml)		*Listeria innocua* (Log_{10} CFU/ml)	
	Vertical biofilm formation	Horizontal spot-inoculation	Vertical biofilm formation	Horizontal spot-inoculation
Bare glass	6.0 ± 0.05 (ay)	8.0 ± 0.1 (ax)	7.0 ± 0.05 (ax)	7.0 ± 0.01 (ax)
Nanostructured PS	5.5 ± 0.1 (by)	6.2 ± 0.1 (bx)	5.0 ± 0.1 (bx)	5.0 ± 0.4 (bx)
Planar PS	1.0 ± 0.01 (cy)	2.4 ± 0.1 (cx)	4.3 ± 0.1 (cx)	2.2 ± 0.2 (cy)

a-c: Means for inoculation method (columns) with different letter are significantly different (α=0.05).

x-y: Means within inoculation method (row) with different letter are significantly different (α=0.05).