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Aqueous Ozone Inactivation of Viruses and Bacteria on Biotic and Abiotic Surfaces

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Aqueous Ozone Inactivation of Viruses and Bacteria on Biotic and Abiotic Surfaces

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Abstract:

Produce is susceptible to contamination by foodborne pathogens. Food service establishments utilize sanitizing agents to reduce microbes on produce surfaces. The research objectives were to evaluate the efficacy of aqueous ozone 1) on the inactivation of viruses and bacteria on produce; 2) on the inactivation of viruses on stainless steel; and 3) against viruses in association with bacteria on produce surfaces. For objective 1, Boston bibb lettuce and cherry tomatoes were spot inoculated with a cocktail of viruses (murine norovirus (MNV) and MS2 bacteriophage) or bacteria (*Enterobacter cloacae* and *Bacillus cereus*) and washed for 40 min with samples taken every 10 min. For objective 2, stainless steel (SS) coupons were spot inoculated with the same cocktail of viruses and washed for 0.5, 3, and 10 min. For objective 3, Boston bibb was spot inoculated with either MNV and *E. cloacae* or MNV and *B. cereus* and washed for 40 min with samples taken every 10 min. Inocula were allowed to dry for $\geq$ 90 min. A batch wash ozone sanitation system (BWOSS) was prepared with ice (3-5°C) and 0.5 ppm ozone or no ozone. Surfaces were treated with either an ozone or water wash with samples taken over time and repeated in at least duplicate. Samples were processed to determine plaque forming units (PFU) and colony forming units (CFU). In objective 1, there were no significant differences in inactivation of MNV, MS2, or bacteria with ozone compared to water only. There was greater variability in viral reduction while bacterial inactivation increased over time. In objective 2, there was no significant difference in inactivation of MNV or MS2 on SS, but the variability was reduced. The log reduction difference between ozone and water for MNV and MS2 after 10 min was 0.25 and 0.51 PFU/ml, respectively. In objective 3, MNV-bacteria association on lettuce did not impact ozone inactivation of MNV. The log reduction differences between ozone and water

for MNV with *B. cereus* and MNV with *E. cloacae* after 40 min were 0.95 and -0.36 PFU/ml, respectively. Further research is needed on how food matrices effects viral inactivation by ozone.

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List of Unpublished Papers

1. Chapter 2: Dawley, Cailin and Gibson, Kristen. (2018) Virus-Bacterial Interactions: Implications for Prevention and Control of Human Enteric Viruses from Environment to Host. *Foodborne Pathogens and Disease.* Submitted for Publication.

Chapter 1: Literature Review

1. Foodborne Illnesses

Of 31 major foodborne pathogens, it is estimated there are 9.4 million cases of foodborne illness in the United States each year (Scallan et al., 2011). Foodborne pathogens can contaminate food anywhere from the farm to the fork, due to the range of environments it incurs before reaching the consumer. These environments are the production field, processing facilities, transportation, handling by food service establishment employees, or in homes during preparation. Foods that are especially susceptible to contamination are produce, dairy, poultry, eggs, and beef (Painter et al., 2013). Major foodborne pathogens are introduced into these environments through a variety of routes. In some cases, animals are hosts to pathogens such as *Salmonella*, *Listeria*, *Escherichia coli*, and *Campylobacter* (Swartz, 2002). Sometimes humans are hosts and can transfer the pathogen (e.g. human norovirus) during handling (Berger et al., 2010). Of the 31 major pathogens, human noroviruses are the primary cause of foodborne illnesses in the United States (Scallan et al., 2011).

a. Outbreaks and Fresh Produce

Fresh produce, specifically leafy greens, have been found to be a common source of foodborne pathogens resulting in 46% of estimated foodborne illnesses (Painter et al., 2013). Norovirus is often associated with leafy greens as well; from 2001-2008, 33% of norovirus outbreaks were associated with leafy greens (Hall et al., 2012). Produce is susceptible to contamination with pathogens for several reasons. It is consumed raw, unlike products that are cooked or pasteurized before eating. Produce is also processed to be ready to eat which can cause tears and breaks to the surface of the produce allowing some microbes to grow on the surface or be internalized (Benson, 2010). Produce can become contaminated prior to retail or during preparation at the food service establishment (FSE). Prior to harvest, contamination can come

from the soil, water, or wild animal excrements carrying pathogens that adhere to produce (Benson, 2010). Then as produce is transferred from the field to the store there are more opportunities for contamination: field workers, packaging, and transportation (Johnston et al., 2005). At the FSE, the contamination more than likely comes from the employees. This could be in the form of poor personal hygiene or from improper hand washing leading to the transmission of viruses such as norovirus and Hepatitis A virus (Strohbehn et al., 2008).

Contamination of fresh produce has led to several outbreaks. For instance, *E. coli* O157:H7 has been associated with contaminated leafy greens resulting in various outbreaks. A multistate outbreak of *E. coli* O157:H7 was linked to bagged spinach that was sold for at home use (Grant et al., 2008). The authors hypothesized that the bacteria were either internalized by the spinach plant in the field or attached to the cut surfaces of the prepackaged spinach. Another outbreak of *E. coli* O157:H7 in Wisconsin was linked to bagged spinach where the pathogen was detected in river water, cattle feces, and pig feces near the field the spinach was grown in (Wendel et al., 2009). Non-typhoidal *Salmonella* has been associated with outbreaks due to contaminated tomatoes. In a multistate outbreak across 26 states, tomatoes were contaminated by *Salmonella* Newport which was found to be present in the irrigation water at the field the tomatoes were grown in (Greene et al., 2008).

2. Strategies to control pathogens in retail food service

The U.S. Food and Drug Administration (FDA) has published recommendations on how to prevent contamination of food products after receiving. It includes separating products, washing hands, and cleaning equipment. There is a specific section on washing produce which is as follows:

> Washing Fruits and Vegetables A) Except as specified in (B) of this section and except for whole, raw fruits and vegetables that are intended for washing by the consumer before consumption, raw fruits and vegetables shall be thoroughly washed in water to remove soil and other contaminants before being cut, combined with other ingredients, cooked, served, or offered for human consumption in ready to eat form. (B) Fruits and vegetables may be washed by using chemicals as specified under 21 CFR 7-204.12 (U.S. Department of Health and Human Services, 2009).

Retail and institutional food service can apply several different strategies to control pathogens and prevent contamination once produce reaches the retailer. Retailers can take steps to prevent improper food handling by workers. These steps can include enforcing good hygiene, hand washing, and sending ill workers home. Retailers can also make sure that suppliers are following good manufacturing practices (GMPs) to prevent contamination before the products reach them. The use of refrigeration when the produce is not being served can keep bacterial pathogens from multiplying (Lynch et al., 2009). Retailers can also wash produce with a sanitizing solution of chlorine, ozone, or lemon juice and vinegar to reduce microbes on the surface of produce (Berger et al., 2010). A study by Allende et al. (2008) investigated commercial sanitizing agents for the inactivation of epiphytic microbes on the surface of leafy greens via submersion in these sanitizers. The sanitizers they used were chlorine (sodium hypochlorite), Sanova (acidified sodium chlorite), Sanoxol 20 (hydrogen peroxide and peroxyacetic acid), Tsunami 100 (peroxyacetic acid and hydrogen peroxide), Purac FCC 80 (lactic acid), Citrox 14W (organic acid and flavonoids) and Catallix (lactoperoxidase, hydrogen peroxide and thiocyanate) under the manufacturer's suggested concentrations and exposure time. The researchers found that chlorine, Sanova, Purac FCC 80, Citrox 14W and Catallix all had a significant impact on the microbial epiphytes present on the leafy greens compared with the water only wash.

3. Ozone as a disinfectant

Ozone (O_3) is a strong oxidant that can be effective against several different microorganisms (Alexandre et al. 2011). Aqueous ozone is being used in the food industry during produce washes, disinfection of processing equipment, and disinfection of the environment (Mahapatra et al., 2005) Ozone is an effective disinfectant against *E. coli* O157:H7, *Salmonella, Listeria monocytogenes, Staphylococcus aureus,* foodborne viruses, and protozoa (Achen et al., 2001; Cursons et al., 1980; Lim et al., 2010; Restaino et al., 1995). When considering ozone as a disinfectant, it has some positive characteristics that aid in its ability to disinfect and some drawbacks that need to be considered. Ozone is soluble in water which creates a solution, but it is not stable long term; it will decompose into oxygen over time. Due to the short half-life of ozone, there is no residue left on the produce after being washed unlike chlorine that leaves residues on produce and surfaces that can be toxic in high concentrations (Karaca and Velioglu, 2007).

Ozone is also sensitive to pH as it is more stable in acidic conditions than alkaline (Khadre et al., 2001). Additionally, the half-life of ozone is increased at colder temperatures thus enhancing its stability (Batakliev et al., 2014). Ozone effectiveness can be impacted by the presence of organic matter by changing the pathway of ozone. Some organic matter converts the hydroxyl radical into a superoxide radical which slows the effectiveness of ozone while other organic compounds can stabilize ozone (Kim et al., 1999).

a. Ozone inactivation of bacteria

Ozone has been shown to be effective in inactivating a range of bacteria. Ozone oxidizes lipids present in the cell membrane of the bacteria, intracellular enzymes, as well as the bacterial genome (Guzel-Seydim et al., 2004)**.** The effectiveness is in relation to the type of bacteria: gram

positive or gram negative. Gram negative bacteria are reported to be less resistant to ozone while gram positive bacteria have greater resistance due to the cellular structure of the outer membrane. Gram positive bacteria contains a higher concentration of peptidoglycan which is more resistant to ozone than the lipoproteins present in gram negative bacteria (Khadre et al., 2001). Ozone also has the potential to inactivate bacterial spores such as *Bacillus cereus* by degrading the outer spore coat and exposing the core to disinfection (Khadre et al., 2001).

Ozone has been used with bacteria in suspension and bacteria inoculated on food products. Table 1 highlights studies that have used ozone as a sanitizing agent for bacteria. Kim and Yousef (2000) investigated the inactivation of bacteria in suspension when exposed to ozone. The researchers used a low concentration of ozone (0.2-0.3ppm) and observed at least a one log reduction after 30s for all of the microbes tested. Ozone is highly effective against bacteria in suspension; however, adding in a food matrix requires higher concentrations of ozone and a longer contact time to experience the same reduction. This can be seen in the study by Bialka and Demirci (2007). Here, the authors inoculated blueberries with *E. coli* and *Salmonella* and required over 2 minutes to observe at least a log reduction with a higher concentration of ozone (1.7-8.9ppm) compared to the previously mentioned study by Kim and Yousef (2000). The main limitation in comparing these studies is the difference between microbes in suspension and microbes on the surface of produce which effects the results. Besides the presence of a biotic surface, there are two different ways of generating aqueous ozone: ozonated water which was utilized in the study by Kim and Yousef (2000) and bubbling ozone which was utilized by Bialka and Demirci (2007). Ozonated water is water that ozone has been generated in to reach a certain concentration and then the generator of ozone is removed whereas bubbling ozone has a continuous supply of ozone added to the water (Achen and Yousef, 2001). Bubbling ozone

generates a greater log reduction than ozonated water due to the ozone always being replenished (Achen and Yousef, 2001) (Table 1).

b. Ozone inactivation of viruses

Viruses are also reportedly susceptible to ozone. This is primarily due to the composition of a virus particle. It is genetic material surrounded by a capsid made of proteins that can be denatured by ozone thus releasing the genetic material which leads to inactivation (Khadre et al., 2001). It could also be hypothesized that ozone could damage the viral capsid and lead to an inability to bind to receptors which has been documented in thermal treatments (D. Li et al., 2012). Ozone inactivation of viruses has been investigated with virus in suspension. Ozone has been found to be more effective in deactivating viruses at lower pH's and with lower temperatures. Table 2 highlights studies that have documented the impact of ozone on the inactivation of viruses. Lim et al. (2010) investigated the inactivation of murine norovirus (MNV) at 5°C and 20°C and at pH 5.6 and 7. The authors observed a more rapid inactivation of MNV at 5°C than at 20°C, but this was not significantly different. The pH did have a significant impact on the inactivation of MNV, with a more rapid inactivation of the viral surrogate at pH 5.6 than at pH 7, regardless of temperature. Studies have also been conducted on biotic and abiotic surfaces such as green onions and stainless-steel utensils inoculated with viruses. Green onions were inoculated with adenovirus, MNV, and Hepatitis A virus, and log reductions of 4.3, 2.5, and 2.9 were achieved, respectively, in 6.25 ppm ozone after 10 minutes (Hirneisen and Kniel, 2013). Gibson and Almeida (2016) reported dipping a stainless-steel utensil in a virus solution containing PRD1 bacteriophage then placing the inoculated utensil in a circulating ozone washer at 0.5ppm. The authors observed a 6.44 log reduction at 30 seconds (Almeida and Gibson, 2016) (Table 2).

4. Microbe-Microbe Interactions

Microorganisms do not exist in monoculture but rather in a diverse community of microbes. A community that is often discussed in the food industry are biofilms. Biofilms are microorganisms that attach to and multiply on a surface while attracting nutrients (Kumar and Anand, 1998). These communities may allow interactions to form which could create stronger resistances to being inactivated. Research has begun to investigate these interactions but several questions still remain.

a. Virus and Bacteria

As previously stated, microbes do not exist in isolation. Microbial communities such as biofilms may harbor bacteria including pathogens, but can also protect viruses from desiccation and other environmental stressors (Lacroix-Gueu et al., 2005). Similar studies that occurred *in vivo* showed that in the presence of bacteria (gram positive and gram negative) or in the presence of bacterial cell components viruses had increased infectivity (Kuss et al., 2011).

When it comes to foodborne pathogens, human noroviruses are a primary concern due to the volume of illnesses. A majority of human noroviruses bind to histo-blood group antigens (HBGA) (Huang et al., 2005). Some bacteria, such as enteric bacteria, have been known to possess HBGA-like structures that viruses are able to bind to as well (Miura et al., 2013). Jones et al. (2014) took this knowledge and showed that enteric bacteria can act as co-factors to aid in viral attachment to B-cells. These studies show that viral interactions with bacteria have the potential to exist and increase viability and virulence of the virus.

i. Disinfection

Understanding the impact of virus-bacteria interactions on the efficacy of a disinfection method is an important research area. If these interactions occur, how do the microbes respond to

current disinfection processes? Heat treatment is the only disinfectant that has been applied to these interactions thus far. Li et al. (2015) utilized several genera of norovirus allowing the virus to interact with various bacteria. Three different treatment groups were evaluated: virus like particles (VLPs) of human norovirus GI.1; VLPs of human norovirus GI.1 and bacteria with HBGA-like substances; and VLPs of human norovirus GI.1 and bacteria with no HBGA-like substances. Results indicated that when viruses interacted with bacteria with HBGA-like structures, the viruses had a higher immunoreactivity after applying heat (Li et al., 2015). On the other hand, Li et al. (2017) found that when Tulane virus (TV) was in association with bacteria with HBGA-like structures the interaction did not protect the virus against heat inactivation. The authors hypothesized that the difference in results was due to the different viruses used: VLPs of human norovirus versus a viral surrogate (TV).

5. Future Research and Objectives

Due to heat stress being the only form of inactivation that has been applied to virus-bacteria interactions, there is a range of possible research in this area of microbiology, such as investigating common disinfectants including chlorine, bleach, peroxide, or ozone and their impact on viruses in association with bacteria. The disinfectant of interest in this specific study is ozone. This is due to ozone being effective against several microbes individually. This could mean that ozone might be an effective disinfectant for microbe-microbe interactions that have very little published research.

Ozone washers have been proposed for the retail setting especially for fresh produce. Produce is a possible environment that microbe-microbe interactions could occur. Since leafy greens have been linked to several outbreaks of foodborne illnesses especially norovirus

outbreaks, this commodity is a possible environment that could highlight microbe-microbe interactions.

A batch wash ozone sanitation system (BWOSS) will be used to evaluate the efficacy of an ozone washer to inactivate viruses with different attachment times. This research will further elucidate the susceptibility of viruses when adhered to different surfaces: Boston bib lettuce, cherry tomatoes, and stainless steel. The second area of research focus is to investigate the ability of the BWOSS to inactivate viruses associated with bacteria when in combination on the fresh produce surface. These interactions could possibly prevent current control strategies from working at the optimal level which is important for food safety.

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Appendix

Table 1. Previous studies on bacterial survival while using ozone

Bacteria	Method of Ozone (Aqueous)	Ozone Concentration	Microbe Concentration	Results	Reference
P. fluorescens	Lettuce inoculated and washed in water with bubbling ozone	3-10ppm	10^4 cfu/mL	1.5-1.9 log reduction in 3min 3.9-4.6 log reduction in 5min	(Kim et al., 1999)
E. coli O157:H7 *L. monocytogenes* *L. mesenteroides* *P. fluorescens*	Cell suspension was placed in ozonated water and samples taken at 30s	Varied by batch	EC:1-3x10^9cfu/mL LMo:1-2x10^8cfu/mL LMe: 10^7cfu/ml PM: 1-3x10^9cfu/mL	EC: 0.3ppm-1.3 log red. LMo: 0.4ppm-4.6 log red LMe: 0.3ppm-1.3 log red PM: 0.2ppm-0.9 log red.	(Kim and Yosef, 2000)
E. coli O157:H7	Inoculated apples dipped in ozonated water and immersed in water with bubbling ozone	Ozonated Water: 22-24ppm Bubbling Ozone: 25ppm	10^9 cfu/mL	Maximum decreases: OW: 3min immersion 2.6 log red BO: 3min washing 3.7 log red	(Achen and Yousef, 2001)
E. coli O157:H7 *Listeria monocytogenes*	Inoculated lettuce and strawberries in an aqueous ozone till there is a one log reduction	3 ppm	10^6 cfu/g	EC: 1 log reduction in shredded lettuce 92s and strawberries in 20s LMo: 1 log reduction in shredded lettuce 96s in strawberries 20s	(Rodgers et al., 2004)

EC *Escherichia coli* O157:H7, LMo *Listeria monocytogenes*, LMe *Leuconostoc mesenteroides*, PM *Pseudomonas fluorescens*, S *Salmonella enterica*, OW ozonated water, BO bubbling ozone

Table 1. (Continued) Previous studies on bacterial survival while using ozone

Bacteria	Method of Ozone (Aqueous)	Ozone Concentration	Microbe Concentration	Results	Reference
E. coli O157:H7	Lettuce inoculated immersed in ozone solution in a bag with agitation for different time	5.2ppm, 9.7ppm, 16.5ppm	8.10$\log_{10}$ cfu/g	5.2 ppm no significant reduction 9.7 ppm (10min):1.41$\log_{10}$ (15min):1.42$\log_{10}$ reduction 16.5ppm (10min):1.68$\log_{10}$ reduction (15min):1.8$\log_{10}$ reduction	(Koseki and Isobe, 2006)
Yersinia enterocolitica	Inoculated surfaces of potatoes were immersed in aqueous ozone for one minute	5 ppm	10^9cfu/ml	1.6 log reduction in 1 min	(Selma et al., 2006)
E. coli O157:H7 *Salmonella enterica*	Inoculated blueberries were immersed in aqueous ozone solution log reductions recorded over time	Varied (1.7-8.9ppm)	10^6cfu/g	EC: 2min-1.7ppm-1.3 log red. 16 min-7.6ppm-2.5 log red. 64min-8.9ppm-4.9 log red. S: 2min-1.7ppm-0.7log red. 16min-7.6ppm-3.5log red. 64min-8.9ppm-4.7log red.	(Bialka and Demirci, 2007)
Shigella sonnei	Shredded lettuce was inoculated then submerged into different ozonate water concentrations for 5 min	1ppm 2ppm 5ppm	10^9cfu/ml	1ppm: 0.7 log red. 2ppm: 1.4 log red. 5ppm: 1.8 log red.	(Selma et al., 2007)

EC *Escherichia coli* O157:H7, LMo *Listeria monocytogenes*, LMe *Leuconostoc mesenteroides*, PM *Pseudomonas fluorescens,* S *Salmonella enterica*, OW ozonated water, BO bubbling ozone

Table 2. Previous studies on virus survival while using ozone

Virus	Aqueous Ozone Method	Ozone Concentration	Virus Concentration	Results	Reference
Poliovirus 2 Echovirus 1 Poliovirus 1 Coxsackievirus B5 Echovirus 5 Coxsackievirus A9	Viral suspension was added with aqueous ozone in a beaker and samples were collected at 2 min, 20°C, and pH 7.2	0.15ppm	10^6 PFU/mL	PV2: 1.4 log reduction EV1: 2.6 log reduction PV1: 2.8 log reduction CXB5: 2.9 log reduction E5: 3.4 log reduction CXA9: 3.9 log reduction	(Roy et al., 1982)
Simian Rotavirus SA-11 Human Rotavirus type 2	Virus in suspension was added to ozonated water and gently mixed with a stirrer with samples taken over time at pH 6 and 9 over time	Varied	10^7 PFU/mL	SA-11 (pH6): (0.10ppm) 5 log reduction in 30s (0.25ppm) 5 log reduction in 10s pH 9 (0.15ppm) 3.4 log reduction in 60s (0.30ppm) 5 log reduction in 10s HRV (pH 6): (0.05ppm) 5 log reduction in 10s pH 9 (0.10ppm) 5 log reduction in 10s	(Vaughn et al., 1987)
Murine Norovirus	Virus was suspended in a solution and ozonated water was then added at two pH and temps. Then measured by plaque assay	1ppm	10^4 PFU/mL	20° (pH 7): 2 log reduction 20° (pH 5.6): 2.4 log reduction 5° (pH 7): 2.8 log reduction 5° (pH 5.6): 3 log reduction	(Lim et al., 2010)

V2 Poliovirus 2, EV1 Echovirus 1, PV1 Poliovirus 1, CXB5 coxsackievirus B5, E5 Echovirus 5, CXA9 Coxsackievirus A9, SA-11 Simian Rotavirus SA-11, HRV Human Rotavirus type 2, AD41 Adenovirus 41, MNV Human Murine Norovirus, HAV

Table 2. (Continued) Previous studies on virus survival while using ozone

Virus	Aqueous Ozone Method	Ozone Concentration	Virus Concentration	Results	Reference
Adenovirus 41 Human Murine Norovirus Hepatitis A	Virus inoculated on the surface of onions and then placed in water with bubbling ozone at 20°C for 10min	6.25ppm	AD41: 10^5PFU/mL MNV: 10^6 PFU/mL HAV: 10^6 PFU/mL	AD41: 4.3 log reduction MNV: 2.5 log reduction HAV: 2.9 log reduction	(Hirneisen and Kniel, 2013)
PRD1 bacteriophage	Virus particles were added to a solution then a stainless-steel scoop was dipped in it. Then placed in a continuous ozone washer	0.5ppm	10^5-10^6 PFU/mL	30s: 6.44 log reduction 180s: 6.44 log reduction	(Almeida and Gibson, 2016)

V2 Poliovirus 2, EV1 Echovirus 1, PV1 Poliovirus 1, CXB5 coxsackievirus B5, E5 Echovirus 5, CXA9 Coxsackievirus A9, SA-11 Simian Rotavirus SA-11, HRV Human Rotavirus type 2, AD41 Adenovirus 41, MNV Human Murine Norovirus, HAV Hepatitis A

Chapter 2: Virus-Bacterial Interactions: Implications for Prevention and Control of Human Enteric Viruses from Environment to Host

Abstract

Human enteric viruses, specifically human noroviruses (hNoV), are the most common cause of foodborne illness boasting a wide range of transmission routes. These include person-to-person, contact with contaminated fomites as well as ingestion of contaminated water and food. Because of this, the control and prevention of enteric viruses in food and other relevant environments has been a research focus over the past few decades. Interestingly, viruses as well as many other pathogens are often studied in isolation even though it is known that microorganisms do not occur in isolation but rather as part of complex microbial communities—both external from the host as well as within the host. Therefore, the overall goal of this review is to present the current evidence on virus-microbe interactions as these relate to the infectivity as well as the control and prevention of epidemiologically relevant foodborne viruses (such as hNoV) within our food systems. Therefore, this review is divided into *in vivo*, *in situ*, and *in vitro* implications of virus-microbe interactions through discussion of studies investigating the complex relationships between human enteric viruses and microbial co-habitants, specifically hNoV and bacteria.

1. Introduction

The U.S. Centers for Disease Control and Prevention (CDC) estimates that there are 9.4 million foodborne illnesses caused by 31 major pathogens each year in the United States, of which 5.5 million of those illnesses are associated with foodborne viruses (Scallan *et al.*, 2011). Due to the high prevalence of virus related foodborne illnesses, it is important to characterize how viruses behave in food systems in order to implement prevention and control strategies. The most common foodborne viruses are human norovirus (hNoV) and Hepatitis A virus (HAV) (Hall, 2016). These viruses have various, well-documented modes of transmission including from person-to-person, contact with contaminated fomites as well as ingestion of contaminated water and food. One of the most common modes of transmission is via infected food handlers with inadequate hygiene while in contact with food (Koopmans *et al.*, 2002). In addition, food may become contaminated prior to preparation such as during production which is often the case with shellfish (e.g., bivalve mollusks) as well as for leafy greens and berries—the most common commodities implicated in viral foodborne disease outbreaks (Marsh *et al.*, 2018). With respect to shellfish and fresh produce, these commodities are susceptible to contaminants from the surrounding production environment, especially from water sources utilized during production and processing (Greening and Cannon, 2016).

While oftentimes pathogens are studied in isolation, it is known that viruses—and microorganisms in general—do not occur in isolation but are rather a part of complex microbial communities. The most well-known example of a complex microbial community are biofilms that form on various types of surfaces. Biofilms are composed of microorganisms that attach to surfaces and multiply, and as the microbes multiply the colonies attract nutrients, organic compounds, and other microbes thus creating a biofilm (Kumar and Anand, 1998). These

microbial communities have had an impact on the food industry for years. Pathogenic microbes can be attracted to biofilms that develop on food contact surfaces. Due to this, there is an increased chance of contamination in food processing when biofilms are present (Shi and Zhu, 2009). Biofilms also reside in the natural environment – aquatic and sediment – in which food production occurs (Winkelströter *et al.*, 2013). Besides microbial communities in the form of biofilms, there are also communities on the surfaces of foods as well as within the human gastrointestinal system. These communities are being investigated in order to understand how they affect the infectivity and pathogenicity of viruses (Jones *et al.*, 2014; Kuss *et al.*, 2011; Monedero *et al.*, 2018).

The past ten years has seen an explosion of studies attempting to better understand complex microbial communities including the interactions of viruses with other microbes (e.g., free-living protozoa and bacteria) as well as specific bacterial cell components (Atanasova *et al.*, 2018; Hsueh and Gibson, 2015; Moore and Jaykus, 2018). The overall goal of this review is to present the current evidence on virus-microbe interactions as it may relate to the infectivity as well as the control and prevention of epidemiologically relevant foodborne viruses within our food systems. Therefore, this review is divided into *in vivo*, *in situ*, and *in vitro* implications of virus-microbe interactions through discussion of studies investigating the complex relationships between human enteric viruses and microbial co-habitants, specifically hNoV and bacteria.

2. In Vivo Implications of Virus-Bacteria Interactions

It is established that viruses exist in diverse microbial communities, and it is important to understand how these ecosystems impact infectivity and pathogenesis of the viruses within the host. Several studies utilizing cell culture model systems over the past decade have investigated these interactions, and these data are used here to consider the potential implications to the virus

host. Moreover, Berger and Mainou (2018) recently provided an in-depth review on the interaction of enteric bacteria with eukaryotic viruses—such as those discussed herein—and the impact on the viral infection process.

a. Infectivity and pathogenesis

One such study by Kuss *et al.* (2011) explored interactions of poliovirus (PV; serotype 1, Mahoney) with bacteria and bacterial cell components. The authors found that when PV is incubated in the presence of gram-negative and gram-positive bacteria, the virus had increased viability as determined by plaque assays. The largest increase in viability was seen with the gram-positive bacterium *Bacillus cereus.* Further investigation revealed that *B. cereus* increased adherence of PV to HeLa cells thus aiding the infection process. In addition, Kuss *et al.* (2011) reported an increased yield of plaque forming units (PFU) of polioviruses in the presence of bacterial components, such as lipopolysaccharide (LPS) and peptidoglycan (PG). Further research was conducted based on that of Kuss *et al.* (2011) findings. Robinson *et al.* (2014) investigated the mechanisms leading to the increase in yield of poliovirus. More specifically, following incubation of PV with LPS, the authors observed that LPS associated with PV binds directly to the PV receptor. As a result, PV associated with bacterial cell components had an increase in attachment to the host cells. It was also discovered that only a few sites on the viral capsid—specifically, the lysine amino acid at position 99 located in the surface exposed BC loop region of viral protein 1 (VP1)—had to bind with LPS to lead to an increase in attachment.

Another study examined murine norovirus (MNV)—a hNoV surrogate—strain types 1 and 3, and the ability of MNV to infect B cells in the presence of enteric bacteria (Jones *et al.,* 2014). To begin, the authors investigated whether MNV infects B-cells which then led the authors to determine whether hNoV (GII.4 Sydney) also infected B-cells. Once it was established that

hNoV also infected B-cells, further investigation examined what occurs with the addition of the enteric bacteria, *Enterobacter cloacae*. It has been shown that hNoV can bind to histo-blood group antigen (HBGA) like structures (Harrington *et al.* 2004), and *E. cloacae* is documented to possess the H-type HGBA that allows hNoV to bind (Miura *et al.*, 2013). The results of the study by Jones *et al.* (2014) revealed that enteric bacteria, such as *E. cloacae*, can act as co-factors to aid in the virus's attachment to and infection in B-cells. These two key studies demonstrate that the interactions between viruses and specific bacterial strains have the potential to increase infectivity during *in vitro* studies. However, do these observed interactions and enhanced infectivity translate to the infection process in the actual host? The authors of the two cell culture studies above did in fact use the mouse host to provide further evidence. Both treated one group of mice with antibiotics to deplete the natural microbiota in the gut and then challenged the mice with poliovirus or MNV. In both studies, the group of mice treated with antibiotics had a reduction in viral replication (Jones *et al.*, 2014; Kuss *et al.*, 2011). These results further support the idea that virus-bacteria interactions can potentially impact the infectivity of viruses in a host.

b. Protection and competitive exclusion

A further review of gut microbiota and viruses indicates that these principles can translate into other hosts including humans. In a study investigating the effectiveness of a vaccine against porcine rotavirus (pRV)—an enteric virus that infects swine—the researchers first inoculated gnotobiotic pigs with either healthy or diseased children's feces and then administered the pRV vaccine (Twitchell *et al.*, 2016). The diseased feces were from children in Nicaragua that demonstrated a high enteropathy score (i.e., an indication of intestinal inflammation and poor gastrointestinal health) and had previously received the human rotavirus vaccine. Next, the pigs were challenged with infectious pRV particles, and the pigs inoculated with healthy feces

demonstrated a lower incidence of infection and a stronger adaptive immunity to the pRV vaccine than compared to the pigs inoculated with diseased feces. These results indicate that the intestinal microbiota affects the infection process of the virus for better (e.g., protective) or for worse (e.g., increased susceptibility) (Twitchell *et al.*, 2016). In an editorial by Iturriza-Gómara and Cunliffe (2017), the authors discuss the link between the gut microbiome and the efficacy of enteric virus vaccines used in areas with elevated morbidity due to infectious disease. Iturriza-Gómara and Cunliffe highlight the findings of Harris *et al.* (2017) who reported significant differences in the gut microbiota of infants who responded positively versus that of infants that responded poorly to an administered RV vaccine. While the difference could be strictly due to a decrease in the immune-modulating capacity of the LPS of the more abundant bacteria in the infants with low response to the RV vaccine, another theory is possible. More specifically, because the RV vaccine contains live attenuated virus, the gut bacteria in the positive response group could be expressing HBGA or other relevant glycans that are necessary for RV cell entry and replication; thus, these bacteria are helping to elicit an immune response and future protection from infection with wild-type RV (Harris *et al.*, 2017).

Investigations have also explored the role probiotics may play in host protection from and/or during viral infection as observed in the Twitchell *et al.* (2016) study discussed previously. More specifically, Rubio-del-Campo *et al.* (2014) explored the interaction of hNoV (GI.1 and GII.4) P-particles—the protruding domain of the viral protein 1 [VP1] capsid protein—with lactic acid bacteria including *Lactococcus lactis* and nine types of *Lactobacillus* sp. along with *Escherichia coli* strain Nissle 1917. The authors observed varying degrees of ability to bind hNoV P-particles among all eleven bacteria assayed with the best and worst binding observed for *Lactobacillus casei* BL23 and the gram-negative *E. coli* Nissle 1917, respectively. Following confirmation of

bacterial cell binding, the investigators explored the effects of bacteria on the binding of hNoV GI.1 P-particles to HT-29 enterocyte cultures. These studies revealed that total inhibition of P-particle binding to HT-29 cells was achieved in the presence of high concentrations ($OD_{550} \geq$ 0.5) of *E. coli* and less so with *L. casei* BL23. Of more interest, however, is the observation of this inhibitory effect only via competitive exclusion (i.e., simultaneous inoculation of bacteria and P-particles) and not when HT-29 cells were pre-treated with bacteria or when P-particles were already attached to the cells. In the latter scenario, the addition of bacteria to the cells with P-particles already attached actually enhanced P-particle retention on the enterocytes by up to 4-fold depending on the bacteria type and density. Rubio-del-Campo and co-authors hypothesized that during competitive exclusion hNoV GI.1 P-particle association with bacterial cells may limit binding to HT-29 cells; however, this simple association may not fully explain the inhibitory mechanism, especially in the case of *E. coli* Nissle 1917. It is plausible that this probiotic strain of *E. coli* could prevent hNoV GI.1 P-particle binding to enterocytes via a non-microbicidal substance as was previously shown for preventing invasion of intestinal cells by bacterial pathogen (Altenhoefer *et al.*, 2004).

Along with Rubio-del-Campo *et al.* (2014), additional studies on the role of probiotics in both hNoV and its surrogates binding to host cells have also been published. Li *et al.* (2016) investigated the effect of *Bifidobacterium adolescentis* against both MNV and hNoV virus-like particles (VLP). The authors determined that *B. adolescentis* primarily decreased MNV replication in the murine macrophage cell line (RAW 264.7 cells) as opposed to denaturing the MNV protein capsid via lactic acid production or inhibition of host cell binding. With respect to hNoV VLPs, the authors observed that *B. adolescentis* actually did impact the binding of VLPs to the cells. More specifically, hNoV GI.1 VLP binding to Caco-2 cells was decreased

significantly whereas binding to HT-29 cells was marginally impacted. Interestingly, hNoV GII.4 VLP binding to Caco-2 cells was not impacted by the presence of *B. adolescentis*. Additional investigations by Shearer *et al.* (2014) and Aboubkar *et al.* (2014) also explored probiotic interactions with hNoV surrogates—specifically Tulane virus and MNV as well as feline calicivirus, respectively. However, these studies primarily consider cell-free spent media from probiotic culture for the purpose of viral inactivation.

Based on this evidence, researchers have recently considered the role gut microbiota may play in hNoV infection. Prior to 2016, the hNoV research community relied on surrogates and limited human volunteer studies to understand the mechanisms behind hNoV infection processes; however, Ettayebi *et al.* (2016) published the first evidence of reproducible hNoV replication using an *ex vivo* human intestinal enteroid (HIE) model. Following the lead of Jones *et al.* (2014) who reported MNV as well as hNoV infection of B cells in the presence of enteric bacteria, Ettayebi and others claimed that hNoV did not require bacterial co-factors for infection nor did LPS promote replication. Although, the investigators acknowledge that hNoV replication within the HIEs varied greatly by strain type as well as HIE origin (i.e. FUT2 secretor status of the patient from which the biopsy was taken). In the area of hNoV-bacteria interactions within this new culture model, more work is needed to characterize the individual requirements for infection of each hNoV genotype. This is especially apropos given the nearly parallel (in time) publication on the role *E. cloacae* plays in the shedding of hNoV by gnotobiotic pigs inoculated with the hNoV GII.4/200b variant (Lei *et al.*, 2016). The authors support the conclusions of Ettayebi *et al.* (2016) with respect to enterocytes specifically being the site of infection. However, Lei *et al.* (2016) also reported that pigs colonized with *E. cloacae* inhibited hNoV infection by reducing both the concentration of hNoV in the feces and the duration of shedding

compared to the control group. Similarly, Rodríguez-Díaz (2017) reported that individuals with a greater abundance of certain bacterial families—for example, *Ruminococcaceae* bacteria—might have lower susceptibility to infections with RV and hNoV. However, limitations linked to the sample population and the interdependency of gut microbiota composition and secretor status are not conducive to generalizability of results to the greater population.

c. Role in recombination

Besides directly impacting virus infectivity, virus-bacteria interactions *in vivo* may also indirectly play a role in recombination events that viruses can undergo. Recombination happens as viruses interact with other viruses during the replication process within the host, and this allows the virus to acquire new genes (Worobey and Holmes, 1999). These newly acquired genes can lead to viral evolution and a potential increase in virulence (Bull *et al.*, 2007). These recombination events can happen in a variety of ways and settings including during animal production. For instance, Mattison *et al.* (2007) examined swine and cattle fecal samples and retail meat (raw chicken, beef, and pork) for the presence of noroviruses—both animal and human. It has been established that swine and bovine-specific NoV strains are present in these animals and can infect their respective hosts (Scipioni *et al.*, 2008). For this reason, the authors were interested in whether hNoV strains could simultaneously be present in livestock, and thus possibly cause indirect zoonotic transmission through fecal contamination of retail meat products. The authors reported the detection of human-like GII.4 (genogroup II, cluster 4) NoV in cattle and swine fecal samples alongside GIII (bovine) and GII.18 (swine). In addition, one raw pork meat sample tested positive for a hNoV in the GII.4 cluster. Since hNoV strains were found to be in the presence of NoV strains infectious to cattle and swine, Mattison *et al.* (2007) suggested the opportunity for recombination of the virus along with its new virulence factors.

More recently, Sisay *et al.* (2016) confirmed the presence of hNoV GII.1 in collected swine fecal samples—demonstrating both zoonotic as well as viral evolutionary potential.

Other studies have investigated the whole virome—a collection of viruses that make up a viral community within a given ecosystem. A study by Shan *et al.* (2011) looked specifically at the virome of food production animals. Here, the authors explored the virome associated with the feces of healthy and diarrheic piglets on high density farms. The majority (68%) of classified sequences in the piglets' intestines were viruses with 99% of those being mammalian RNA viruses from the families *Picornaviridae* (kobuviruses, enteroviruses), *Astroviridae*, *Coronaviridae,* and *Caliciviridae* (sapoviruses). Shan and co-authors posit that the level of presumed co-infection of diverse viruses observed in their study presents favorable conditions for viral recombination and viral evolution. In the context of virus-bacteria interactions, what role do these interactions play in co-infections and the possibility of allowing accelerated viral evolution? Fortunately, an *in vitro* study by Erickson *et al.* (2018) took this step forward. The authors investigated the bacterial strains that aid in co-infection of cells and found that, when co-infection occurs, the bacteria 1) aids in recombination events and 2) prevents deleterious mutations from occurring, ultimately causing an advantageous impact on the fitness of the virus and viral population diversity. The authors established this using poliovirus (serotype 1 Mahoney) and forty-one bacterial isolates recovered from the feces of healthy mice. Through experimental procedures, Erickson and co-authors were able to observe a 4.6-fold increase in recombination in the presence of co-infection aiding bacteria over the control group that contained no bacteria.

The research presented above provides evidence that virus-bacteria interactions can increase viability and virulence by allowing co-infection and recombination of viruses. There are still

many questions on how these interactions affect human viruses since most research is completed using virus surrogates. These surrogates represent the human enteric virus well, but do not behave completely like the human strains.

3. *In Situ* Implications of Virus-Bacteria Interactions

Besides the interactions within the body, viruses can also form interactions with bacteria in the environment, whether on surfaces, conduits, or food. Biofilms are an example where microbial communities aid in persistence of microbes which in this case could include viruses. Biofilms are composed of microbes that adhere to a surface and include bacteria that secrete extracellular polymeric substances (EPS). The EPS act as a binding agent and allows the microbes to stay attached and attract other microbes to the biofilm creating a community of diverse microbes. Similar to bacteria, viruses can imbed themselves into bacterial biofilms to gain protection from desiccation and other environmental stressors (Lacroix-Gueu *et al.*, 2005). While previous work is related to bacteriophage in biofilms, it gives merit to the hypothesis that these microbial communities present *in situ* have an impact on foodborne viruses as a result of the interactions between human enteric viruses and bacteria.

a. Food contact surfaces

Food products have natural microflora—some may be pathogenic, while others are naturally occurring (Wang *et al.*, 2017). As food is processed, the ingredients and products encounter non-porous surfaces where microorganisms can be transferred resulting in adherence to the surface and possibly biofilm formation. There have been several studies that look at the development of biofilms with bacterial foodborne pathogens such as *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 (da Silva Meira *et al.*, 2012; Di Bonaventura *et al.*, 2008; Dourou *et al.*, 2011; Yang *et al.*, 2009). Because viruses do not propagate outside of a

host, studies on viruses and fomite surfaces have been limited to investigating their persistence under varying conditions. For instance, Escudero *et al.* (2012) examined viral persistence on food contact surfaces (stainless steel, ceramic, and formica) and reported that hNoV GI.1 (Norwalk strain), GII.2 (Snow Mountain strain), and MNV (type 1) were able to survive on surfaces for 42 days. These results have been substantiated by other researchers as reviewed by Kotwal and Cannon (2014). Unfortunately, most published studies investigate viruses in isolation as opposed to in complex microbial systems, such as biofilms, that are present in the real-world. This paucity of published data related to interactions between viruses and bacteria on surfaces was also previously noted by Vasickova *et al.* (2010).

Recently, Schumacher *et al.* (2016) investigated the spread of porcine epidemic diarrhea virus (PEDV)—an animal coronavirus—within an animal food manufacturing facility. The authors reported that one batch of feed contaminated with PEDV distributed the virus to both animal and non-animal food contact surfaces throughout the facility. Moreover, the control measures typically employed for the prevention of cross-contamination of bacterial contaminants were not adequate for the control of PEDV. While the authors did not specifically look at the interaction of PEDV with bacteria, research has shown that—once diffusion through the biofilm occurs—viruses can utilize the protective aspects of the biofilm in order to avoid environmental stressors (Bridier *et al.*, 2015; Habimana *et al.*, 2011). It can be speculated that specific associations of viruses with bacteria may allow for easier entry of virus particles into the biofilm resulting in a reservoir of viruses that are as difficult to remove and inactivate as their bacterial counterparts (Belessi *et al.*, 2011; Corcoran *et al.*, 2014; Coughlan *et al.*, 2016). However, specific studies on virus-bacteria interactions on food contact surfaces are nearly nonexistent and is an area that needs to be further explored.

b. Water resources – Biofilms

Biofilms in our water conveyance systems are not a novel occurrence and have been investigated for years. A review by Skraber *et al.* (2005) examined how viruses in water distribution systems can cause health concerns. Another review by Wingender and Flemming (2011) discussed research on the ability of pathogenic bacteria to persist in drinking water biofilms and act as reservoirs for a variety of pathogenic microorganisms. These reviews point to similar references, such as Quignon *et al.* (1997). The authors of this seminal study demonstrated that viruses can incorporate into biofilms within water distribution systems. The researchers evaluated how poliovirus-1 (Sabin strain) behaved in a water distribution system and found that the virus was always recovered at a higher percentage from the biofilms than from the water alone. The main concern within the water industry is that sloughing off of the biofilm can occur and result in pathogenic microorganisms reaching the consumer (Ashbolt, 2015). This transmission of pathogens via water to the consumer could occur either directly or indirectly. Directly from drinking the contaminated water while indirectly via consumption of contaminated food products that have come in contact with the water through irrigation or processing (Lynch *et al.*, 2009).

There are several types of microbes that have been detected in irrigation waters, and Uyttendaele *et al.* (2015) recently published a thorough review of irrigation water quality in the fresh produce industry. A study conducted in Belgium monitored microbes not only on the surface of the produce but also in the irrigation water of several farms (Holvoet *et al.*, 2014). The authors found that within the irrigation water *E. coli* were a regular occurrence with positive detection in 75% of the samples and that *Campylobacter* spp. was occasionally detected with a 30.9% presence in samples. As indicated by Holvoet and co-authors, the prevalence of both

Campylobacter and *E. coli* was quite high and comparable to previous reports. Of the farms that were sampled, six used open wells and two used bore hole water for irrigation, and the samples were collected either from the water source or if able, from the outlet of irrigation. While this study targeted only bacteria, it provides evidence of the susceptibility of irrigation water sources to human pathogens including viruses as reported by Kokkinos *et al.* (2017).

Regarding viruses, Kokkinos *et al.* (2017) investigated the presence of enteric viruses in irrigation waters within leafy green and berry production chains in multiple countries. The researchers reported Hepatitis E virus and hNoV GII in 1 of 20 and 4 of 28 samples within leafy green production, respectively. In berry production, norovirus GII was detected in 2 of 56 samples. Here, the water samples were collected from a variety of systems in which water was most often pumped directly to the produce while some production water sources were stored in open basins. In these instances, the contamination could be introduced via direct fecal contamination or even association with and detachment from biofilms within the water pipes. For instance, Pachepsky *et al.* (2012) focused on the effect of biofilms in aluminum irrigation pipes and observed that the concentration of *E. coli* was always greater in the biofilm rather than the water. Moreover, *E. coli* concentrations were higher in the sprinkler water, or irrigation output, than the intake creek water—indicating the release of microbes from the biofilms. Given that viruses can associate with *E. coli* along with other bacteria within biofilms, one can speculate that viruses could enter the irrigation water just as easily as bacteria, especially if physically associated with bacteria during biofilm detachment.

Another less obvious reservoir of human pathogens in water resources used in food production are those found in fresh water sediments. Interestingly, sediments contain their own biological compartments (i.e., biofilms) and if disturbed via heavy rains, increased flow, or

activities occurring within the waterbody, these sediments can significantly contribute to the microbial population of the water column (Pachepsky and Shelton, 2011). A study by Yakirevich *et al.* (2013) observed the prolonged release of *E. coli* after artificial high-water flow events even when water levels returned to base flow indicating continued detachment from sediments. Unfortunately, this study did not measure levels of pathogens. For some perspective on the potential contributions of the sediments to microbial load, Pachepsky and Shelton (2011) described sediment densities of *E. coli* ranging from 1 to 500,000 colony forming units (CFU) or most probable number (MPN) per gram of dry weight sediment from an analysis of over 20 published studies. It is also well-known that viruses associate with particulates in the environment including aquatic environments (Gerba, 1984). Although specific to coastal and estuarine sediments, Hassard *et al.* (2016) reviewed the reported abundance of enteric viruses in these sediments and listed levels ranging from non-detect to >6,000,000 viruses per 100 grams of weight wet sediment. Research in this area has also revealed that protection from degradation is conferred to viruses when associated with sediments (Hassard *et al.* 2016). Therefore, it is conceivable that microbial settling and resuspension—including bacteria-associated viruses—are essential processes driving microbial contamination of freshwater including water sources used for irrigation purposes.

c. Specialty crops

As with other natural environments, specialty crops such as fresh produce have their own unique microflora. Several studies have investigated the microbial diversity present on the phyllosphere—the total above-ground portions of plants—of a variety of fresh produce. Leff *et al.* (2013) observed that, while each produce type has a distinct microbial community, the majority of the microorganisms belonged to the family *Enterobacteriaceae* in the case of sprouts,

spinach, lettuce, tomatoes, peppers, and strawberries. Meanwhile, Jackson *et al.* (2013) reported that *Pseudomonas* spp. were ubiquitous in leafy greens by both culture-dependent and culture-independent analyses. As reviewed by Deng and Gibson (2017), numerous types of microorganisms inhabit leafy green phyllospheres including viruses, some of which may be pathogenic to humans. Baert *et al.* (2011) investigated the prevalence of hNoV on a variety of fresh produce: leafy greens, red fruits, cucumbers, and tomatoes. Out of 850 samples, 216 (25.4%) tested positive for hNoV (GI or GII) by real time, reverse transcription PCR (RT-PCR); however, these presumptive positives could not be confirmed via sequencing. Similarly, Stals *et al.* (2011) reported that 18 of 75 (24%) fruit samples tested positive for hNoV (GI and/or GII) and also could not confirm their results.

Looking beyond hNoV, Aw *et al.* (2016) were the first to characterize the virome of lettuce. The researchers collected samples of romaine and iceberg head lettuce from a produce distribution center and then conducted viral metagenomic analysis. The authors observed that human and animal viruses—rotavirus and picobirnavirus, respectively—were present on the samples prior to retail distribution. Aw and co-authors also confirmed presents of numerous viruses that require other hosts such as plants, bacteria, invertebrate, amoeba, fungi, and alga. Along these lines, the interactions of viruses with fresh produce, specifically leafy greens, in the presence of both biotic and abiotic (i.e. flooding, heat stress, mechanical stress) factors have been investigated (Esseili *et al.*, 2015; Gao *et al*. 2016). Deng and Gibson (2017) described various interactions that may be occurring including specific binding, non-specific binding, internalization, and microbial-assisted binding.

As discussed in the previous section *"Water resources – biofilms"*, irrigation water can transport and harbor microorganisms and deliver them to crops; thus, irrigation waters also effect

the microbiome on the surface of fresh produce. Jongman *et al.* (2017) applied next generation sequencing to characterize the bacterial composition of both irrigation waters and leafy greens in South Africa by targeting the V1-V3 hypervariable region of the 16S ribosomal RNA (rRNA) gene found in prokaryotes. The authors examined several variables and determined that the bacterial microbiome of the fresh produce sample in their study was influenced by water quality, similar to the findings of Kokkinos *et al.* (2012). With both bacteria and viruses being present on fresh produce, this could result in interactions as previously speculated (Deng and Gibson, 2017).

4. In Vitro Implications of Virus-Bacteria Interactions

Interactions have been demonstrated *in vivo* and *in situ*, and the observations by Miura *et al.* (2013) set the stage for investigating these interactions. As mentioned previously, Miura *et al.* (2013) revealed that hNoV virus-like particles (VLPs) bound to the EPS of *Enterobacter* sp. SENG-6 which contained HBGA-like structures similar to A type. The knowledge that bacteria have HBGA-like structures that viruses are able to bind to has led to additional investigations on how these interactions impact persistence and survival of enteric viruses relevant to human health. Li *et al.* (2015) examined how thermal treatment would affect hNoV VLPs (GI.1 and GII.4 strains) associated with bacteria. The authors revealed that, when VLPs were in association with bacteria expressing HBGA-like structures, there was a higher antigen integrity versus interactions with bacteria not expressing similar structures. This means that hNoV VLPs could be detected via antibody-based assays such as direct ELISA and porcine gastric mucin-binding assays at greater frequency with associated with an HBGA expressing *E. coli* than with non-HBGA expressing *E. coli*. Conversely, Li *et al.* (2017) investigated viral infectivity of thermally-treated Tulane virus (TV)—a hNoV surrogate—in association with the HBGA type B expressing bacterium, *E. coli* O86:H2. Here, the authors revealed that the association of TV with *E. coli*

O86:H2 did not confer any heat resistance to TV compared to a TV+PBS control. Moreover, the infectivity of TV due to heat denaturation was reduced in a dose-dependent manner. The divergent results of these two similar studies could be due to the use of TV as opposed to hNoV VLPs, different bacterial strains, and differences in methods of evaluation (plaque assay for infectivity versus antibody-antigen based detection).

Yet another investigation explored the association of enteroviruses (coxsackievirus A21, coxsackievirus B5, echovirus 30, poliovirus) with bacterial cell components (LPS and PG), and the impact this association had on common drinking water disinfection strategies (chlorine, ultraviolet radiation) as well as thermal treatment. Waldman *et al.* (2017) demonstrated that LPS and PG of bacterial origin could stabilize the capsid of echovirus 30 resulting in enhanced thermal protection at 50°C for at least 1 h. Based on previous data by Robinson *et al.* (2014), with regard to the specific capsid binding protein for LPS, the authors hypothesized that differences would exist amongst the enterovirus serotypes selected for the study based on polymorphisms in the BC loop protein sequence across the enterovirus genus within the *Picornaviridae* family. As such there was variability in protection across enterovirus serotypes as well as across the LPS and PG of different bacterial origins. Similar to heat treatment, protective effects were demonstrated when echovirus 30 was exposed to 0.2 ppm free chlorine over a period of 3 to 12 minutes. Meanwhile, these bacterial components did not exert any protective effects on echovirus 30 during exposure to UV radiation. Based on these data, Waldman and co-authors suggest that when inactivation steps target the viral genome (e.g. UV radiation), bacterial cell components do not provide a protective effect and that these virus-bacteria interactions may only be beneficial when the mechanism of action is for destabilization of viral capsid. Overall,

the limited published studies on the protective effect of virus-microbe interactions regarding persistence and resistance to common control measure still leave many questions unanswered.

5. Conclusion

It has been established that virus-bacteria interactions can happen in a variety of settings: *in vivo, in situ,* and *in vitro.* Therefore, a key question is how do these interactions impact virus prevention and control? There are scientifically-validated strategies available to prevent enteric virus transmission, but most of these validated approaches are based on lab-controlled studies using viruses within single organism environments. Based on the evidence presented, whole cell bacteria as well as their cell components can impact the efficacy of the strategies aimed at the prevention and control of viruses. Moreover, virus selection in studies related to bacterial interactions – or microbial interactions in general – will be critical as previous work has already demonstrated variability between virus types within a given virus family as well as at the genus level. These differences are effectively demonstrated by the differing observations of thermal stability of viruses in the presence of bacteria reported in Li *et al. (*2015) and Li *et al.* (2017) where one used hNoV VLPs and the other used Tulane virus—a virus within the same family as hNoV—respectively. Another aspect to consider is the role virus-microbe interactions may play in downstream virus detection. Can these interactions be capitalized on to aid in virus recovery and detection, or do they provide another source of interference and complexity to the already tedious methods utilized in foodborne virus research? In conclusion, while human enteric virus research is moving into a new frontier, there is clearly more work to be done to further elucidate the significance of microbe-microbe interactions within the host as well as the environment.

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Chapter 3: Inactivation of microorganisms on Boston bibb lettuce and cherry tomatoes by aqueous ozone

Abstract

Produce is susceptible to contamination throughout the supply chain. To further protect consumers, retail foodservice establishments may treat produce with sanitizing agents, such as aqueous ozone, to reduce microorganisms that may be present on the surface of produce. The primary objective of this study was to investigate the efficacy of aqueous ozone against viruses and bacteria on fresh produce. Boston bibb lettuce and cherry tomatoes were inoculated with either viruses (murine norovirus [MNV-1] and MS2 bacteriophage) or bacteria (*Enterobacter cloacae* and *Bacillus cereus)* and then treated with aqueous ozone or water only in a batch wash ozone sanitation system (BWOSS). Virus experiments also compared 90 min and 24 h virus attachment times before ozone treatment. Each wash consisted of 40 min with 25 g samples taken every 10 min. Ozone concentrations ranged from 0.5 ppm to 1 ppm with water temperatures of 3 to 5°C achieved by ice prior to wash. After treatment, samples were processed for recovery of plaque forming units (PFU) or colony forming units (CFU), and log reductions in PFU/ml or CFU/ml were calculated. The log reduction difference between ozone and water at the 24 h attachment time for MS2 and MNV-1 on Boston bibb lettuce after a 40 min wash was 0.33 and -0.16 log10 PFU/ml, respectively. The difference between ozone and water only wash for cherry tomatoes under the same conditions for MS2 and MNV-1 was -0.99 and 0.31 log10 PFU/ml, respectively. Ozone achieved a 0.55 and 0.93 log10 CFU/ml greater reduction over water alone of *E. cloacae* and *B. cereus*, respectively, on Boston bibb after a 40 min wash. For tomatoes, the difference between ozone treatment and water only for *E. cloacae* and *B. cereus* was 0.12 and 0.92 log10 CFU/ml, respectively. Overall, there were no significant differences (p-value > 0.05) between ozone and water only washes for any experimental combinations. Future

studies should focus on continued optimization of aqueous ozone treatment for enhanced inactivation of microorganisms on fresh produce.

1. Introduction

It is estimated that there are over 9 million illnesses that are due to foodborne pathogens every year (Painter et al., 2013). Of the 9 million illnesses, contaminated produce contributed to 46% of illnesses with leafy greens associated with 22% of the illnesses alone (Painter et al., 2013). Produce is at risk for foodborne illness because it is consumed raw unlike other food products that have a pathogen kill step in place: pasteurization, cooking, or sterilization (Benson, 2010). Pathogens that are often associated with produce include: *Escherichia coli* O157:H7, *Salmonella*, and human norovirus (Grant et al., 2008; Greene et al., 2008; Hall et al., 2012).

Produce contamination can come from anywhere between farm and fork. Pathogens can be transmitted to produce from water, soil, animal excretions, or during preparation at retail (Benson, 2010). Retail settings present several opportunities for cross-contamination of fresh produce to occur: from mishandling of the product, poor handwashing, or poor hygiene by employees. Therefore, it is also important to prevent and control these pathogens at retail prior to reaching the consumer. Wash treatments are the primary prevention method considered for fresh produce; however, issues arise when wash treatments do not inactivate all of the microbes on the produce allowing any remaining viable microorganisms to potentially cross-contaminate a subsequent batch of fresh produce (Olaimat and Holley, 2012). Due to this, there is an opportunity to implement new processes for inactivation of pathogens on fresh produce in retail foodservice settings. One such mitigation strategy includes the addition of ozone—a strong oxidant and sanitizer—to wash water used for fresh produce prior to preparation.

By implementing an ozone wash into the retail setting, this additional control strategy could inactivate pathogens potentially present on the produce and ultimately reduce the number of foodborne illnesses. When using ozone as a sanitizer, there are several variables that impact

efficacy. There are extrinsic factors, such as water temperature, free chlorine, and pH, that effect the stability of the ozone and ultimately the efficacy. Besides extrinsic factors, there are intrinsic factors that need to considered: concentration and form (gaseous or aqueous) (Jin-Gab Kim, Yousef, & Khadre, 2003). When it comes to disinfection, ozone has been shown to reduce viruses including murine norovirus, poliovirus, and human rotavirus while in suspension (Lim et al., 2010; Roy et al., 1982; Vaughn et al., 1987). Unfortunately, there is very limited research on the reduction of viruses in association with food matrices. On the other hand, aqueous ozone has been shown to be effective for several different types of bacteria on produce. Examples include *E. coli* on the surface of apples (3.7 log reduction in 3 min); 1 log reduction in 96 s of *Listeria monocytogenes* on the surface of lettuce; and 3.5 log reduction in 16 min of a cocktail of *Salmonella enterica*—including the serovars Agona, Baildon, Gaminara, Michigan, and Montevideo—on the surface of blueberries (Achen and Yousef, 2001; Bialka and Demirci, 2007; Rodgers et al., 2004).

Inactivation of various spoilage and opportunistic microbes by ozone has also been previously reported. However, there are still some bacterial strains of interest that have not been investigated for ozone inactivation *Enterobacter cloacae* and *Bacillus cereus*. *B. cereus* is a gram positive bacterium that can cause illness when the toxins are ingested; it is found in the soil and thus has also been found on produce (Stenfors Arnesen et al., 2008). *E. cloacae* is a gram negative rod that is found in the soil and the intestinal tract of humans, and often related to nosocomial infections (Harbarth et al., 1999). Similar to *B. cereus*, *E. cloacae* has been found on the surface of produce (Al-Kharousi et al., 2016). Since these bacteria may naturally occur on the surface, they could possibly interact with viruses (Moore and Jaykus, 2018). This interaction could result in a change in inactivation of viruses due to the presence of the bacteria (Li et al.,

2015; Li et al., 2017). In chapter 5, these bacteria will be investigated in association with viruses, but prior to investigating the association it was important to establish the impact of ozone on the bacteria alone.

Viral surrogates such as murine norovirus (MNV-1) are often used in place of human noroviruses because they are easier to cultivate and may behave similarly to the human strain (Wobus et al., 2006). Therefore, the purpose of this research is 1) to evaluate the inactivation of viruses and bacteria by aqueous ozone when they are associated with different produce types (leafy greens and tomatoes) and 2) to investigate attachment time of viruses to fresh produce and its impact on ozone inactivation.

2. Materials and Methods

a. Microbe cultivation

i. Viruses

1. MS2 Bacteriophage

MS2 stock was generated, as described previously by Gibson, Crandall, and Ricke (2012), and aliquoted in small volumes. MS2 was kindly provided by Dr. Stephanie Friedman from the United States Environmental Protection Agency (EPA) Gulf Ecology Division in Gulf Breeze, FL. Stock was generated by scraping the soft agar layer containing bacteriophage lysed *E. coli* C3000 (ATCC 15597) into centrifuge tubes. The cell lysate was then suspended with 23 ml of 1 × phosphate buffered saline (PBS), vortexed, and centrifuged at 185 × *g* for 25 min. The supernatant was collected and filtered through a 0.22 μm filter (Millipore Corporation, Billerica, MA) and then aliquoted and stored at -80°C. The MS2 stock concentration was determined based on titration by double agar layer (DAL) method with bacterial host *E. coli* C3000. DAL assay involves adding 100 μl of sample plus 100 μl of log phase bacterial host (*E. coli* C3000) to 5 ml

of soft agar (0.7% tryptic soy agar (TSA)) and then poured on the top of TSA plates. The soft agar was allowed to solidify and then the plates were incubated for 24 h at 37°C. The plaques were counted and PFU/ml was then calculated.

2. Murine norovirus (MNV-1)

Murine norovirus type 1 (MNV-1) was prepared as described previously by Bae and Schwab (2008) with modifications. MNV-1 was kindly provided by Dr. Kellogg Schwab at Johns Hopkins Bloomberg School of Public Health in Baltimore, MD. MNV-1 was propagated in monolayers of RAW 264.7 (mouse leukameic monocyte macrophage, ATCC TIB-71) cells. Cells were cultured in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing less than 10% low endotoxin, fetal bovine serum (FBS: GibcoLife Technology, Gaithersburg, MD), 1% 100 × penicillin-streptomycin solution (GibcoLife Technology), 1% HEPES (Sigma-Aldrich), 1% glutamine (Hyclone, Logan, UT), and 1% non-essential amino acids (Corning, New York, NY). After reaching 90% confluence, cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.05 for virus stock production. The virus was extracted from cell lysate after complete cytopathic effect as described by Hsueh and Gibson (2015) with modifications. Briefly, culture flasks containing infected cells were subjected to three freeze-thaw cycles at -80°C and 37°C respectively, followed by centrifugation at 5000 × *g* for 20 min at 4°C then filtration with 0.1 μm filter and stored at -80°C. To determine infectious titer, the plaque assay as reported by Hsueh and Gibson (2015) was used with modifications. Briefly, six-well plates were seeded with $2x10^6$ RAW cells per well and grown to 90% confluence in 2 ml of complete growth medium. Cell monolayers were inoculated with virus stocks for 1 h at 37°C with rocking followed by removal of the inocula. Cells were covered with 2 ml of prepared overlay medium containing: 25% of 6% low melting point agarose, 50% 2 × minimum essential

medium eagle (MEM) (Corning) (100 ml 10× MEM, 10 ml glutamine, 29.3 ml sodium bicarbonate, and 360.7 ml sterile distilled water filtered through 0.22 μm filter (Millipore Corporation, Billerica, MA)), 10% low endotoxin FBS, 1% 100 × penicillin-streptomycin solution, 1% glutamine, 0.5% HEPES, and 12.5% sterile distilled water. The plates were incubated for 72 h. Next, 2 ml of 0.01% neutral red (Sigma-Aldrich) prepared in 1 × phosphate buffered saline (PBS) was added to each well to visualize plaques. Plaques were counted after 1h to determine virus titer (PFU/ml).

ii. Bacteria

Enterobacter cloacae (ATCC 39979) with rifampicin resistance was streaked from a frozen 50% glycerol stock onto Luria Bertani (LB) agar (Alfa Aesar, Tewksbury, MA) with 100 μg/ml of rifampicin (Alfa Aesar) using a sterile inoculation loop and incubated overnight at 37°C. A single CFU was selected from the plate, placed in 5 ml of LB broth with 100 μg/ml rifampicin in a 50 ml centrifuge tube, and incubated overnight at 37°C with shaking at 150 rpm. *Bacillus cereus* (ATCC 14579) was also streaked from a frozen, 50% glycerol stock onto a nutrient agar (NA) plate (Becton, Dickson, and Company, Franklin Lakes, NJ) using a sterile inoculation loop and incubated overnight at 30°C. A single CFU was selected from the plate, placed in 5 ml of nutrient broth (NB) (Becton, Dickson, and Company) in a 50 ml centrifuge tube, and incubated overnight at 30°C with shaking at 150 rpm. Following overnight growth of both bacteria, the culture tubes were centrifuged at 5,000 × *g* for 10 min to pellet the bacterial cells. The supernatant was decanted, and the pellet was resuspended in 5 ml of 1 × PBS, vortexed, and centrifuged again. This washing step was repeated two more times. After the final wash, the bacterial pellet was resuspended in 5 ml of buffered phosphate water (BPW), and the concentration of each bacterial culture was determined by spread plate enumeration of

microorganisms. LB agar with 100 µg/ml of rifampicin and *B. cereus* agar (Oxoid, Altrincham, Cheshire, England) supplemented with selective supplement (Oxoid) and egg yolk emulsion (Dalynn, Calgary, Canada) were used for *E. cloacae* and *B. cereus*, respectively.

b. Produce

Boston bibb loose leaf lettuce and cherry tomatoes were used. The Boston bibb was grown hydroponically and procured from two separate places: 1) grown in a greenhouse at Ozark All Seasons in Windsor, AR and purchased from a local grocer and 2) grown in a freight farm—a hydroponic farming system that is built into a shipping box—on the University of Arkansas Fayetteville campus and donated. In both instances, Boston bibb leaves were placed in a sterilized plastic container with a lid and stored at 4°C until use. Cherry tomatoes were received from a local produce distributor or from a local grocery store and transported and stored at 4°C until use.

c. Inoculation of Produce

For virus inoculation, 25 g of Boston bibb leaves were measured out for each sampling time point (n=5) for a total of 125 g for each experiment. Each 25 g was then spot inoculated with 100 µl each of 10^8 PFU/ml MS2 and 10^6 PFU/ml MNV-1. For cherry tomatoes, 2 tomatoes (20-25 g) were measured out for each sampling point (n=5), and inoculated similarly. For inoculation with bacteria, the same amount of produce and similar inoculation methods were used as described for viruses. The Boston bibb was inoculated with 100 µl each of 5×10^7 CFU/ml *E. cloacae* and 6×10^6 CFU/ml *B. cereus*. Due to a low recovery of bacteria based on preliminary work, tomatoes were inoculated with 100 µl each of 7×10^8 CFU/ml *E. cloacae* and 3×10^8 CFU/ml *B. cereus*.

Following inoculation, the produce was allowed to dry until the surface was visibly dry, approximately 1 h. Following the initial 1 h drying period, for virus experiments, the produce

was stored overnight and then exposed to the ozone wash or to the water only wash. An additional short attachment time of 90 min followed by an ozone wash was also conducted for viruses. For bacteria experiments, the produce was stored overnight prior to the ozone wash or to the water only wash.

d. Treatments

The batch wash ozone sanitation system (BWOSS) utilized in the present study was developed by Recycled Hydro Solutions (Rogers, AR). The BWOSS contains a one compartment sink measuring 43 cm^2 with a depth of 30 cm and fabricated using 16-gauge stainless steel. During operation, the sink is filled with water, and once filled, water from the sink is passed through a Venturi injector, which creates aqueous ozone continuously. The ozonated water is then passed back into the sink basin. The BWOSS has a dissolved ozone meter (Model Q46, ATI, Collegeview, PA) which was corroborated by the indigo trisulfonate method (SM 4500-OS3 B) using a Hach Pocket Colorimeter II (Hach Company, Loveland, CO) and Ozone AccuVac Ampules (Hach) (American Public Health Association, American Water Works Association, and Water and Environment Federation, 2012).

The sink holds approximately 34.07 L (9 gallons) of water. Ice was added to the sink to aid in stabilization of ozone until the water temperature reached 4°C. The produce was placed in the BWOSS once ozone reached a concentration of 0.5 ppm—as indicated by the dissolved ozone meter—and the water temperature in the sink was stable at 4°C. The produce samples were submersed in the sink for a total of 40 min with subsamples taken every ten minutes.

e. Microbial analysis

Following treatment, produce samples were placed in Whirl-pak bags with 75 ml of BPW. The bags containing lettuce were then placed in a stomacher (Seward Stomacher 400 Circulator,

West Sussex, United Kingdom) for one minute at 260 rpm. The eluate was then serially diluted. Based on preliminary data, the cherry tomatoes were hand massaged for 1 min in order to prevent the tomatoes from breaking. We speculate that the pH of the eluate dropped when the tomatoes broke resulting in interference with the plaque assay leading to inconclusive results due to cytopathic effects. The resulting eluate from the hand massaged tomatoes was then serially diluted in BPW.

For MS2, 100 µl of each dilution was plated in duplicate using DAL method. Then the plaque forming units (PFU) were counted and PFU/ml was calculated. For MNV-1, plaque assay was performed as previously described (Section MNV-1) and PFU were counted in order to calculate PFU/ml. Viral experiments were conducted at least in duplicate.

For bacteria, the eluate was ten-fold serially diluted, and 100 µl of each dilution was plated on LB agar with 100 µg/ml of rifampicin and *B. cereus* agar for detection of *E. cloacae* and *B. cereus,* respectively. The plates were incubated overnight at 37°C and 35°C, respectively. Colonies were counted to determine CFU/ml. All bacterial experiments were conducted in duplicate.

f. Statistical analysis

Statistical analyses were performed using JMP Pro 13 software (SAS institute, Inc., Cary, NC). Significance was determined by a critical p-value 0.05 by one-way analysis of variance (ANOVA) for all scenarios. Based on the results, a Tukey-Kramer honestly significant difference (HSD) test was conducted to confirm differences between groups.

3. Results

a. Viral Inactivation

A baseline recovery was used to calculate the amount of virus that can be recovered from each produce without any treatment. The average recovery was 10^7 PFU/ml for MS2 and 10^5 PFU/ml for MNV-1 for both Boston bibb and cherry tomatoes. The samples were then compared to the baseline recovery to give the log reductions over time for the various treatments.

MS2 inactivation on Boston bibb did not reveal a significant difference between the ozone wash and the water only wash or between the two attachment times (Table 1). There was a small observed difference between attachment times where a 90 min attachment resulted in a lower reduction in MS2 on Boston bibb compared with a 24 h attachment at all sampling points except at 40 min. When comparing ozone washes to water only washes there was a greater reduction based on PFU/ml values with ozone achieving a greater reduction of MS2 at both attachment times; however, it was not a significant difference.

MNV-1 inactivation on Boston bibb also did not display a significant difference between the ozone wash and the water only wash or between the two attachment times (Table 2). There was a small observed difference between attachment times. However, differing from MS2, there was a greater reduction in MNV-1 after a 90 min attachment compared to a 24 h attachment at all sampling points. When comparing ozone washes to water only washes, there was a greater reduction with ozone than water, except at time 40 min with 24 h attachment. Again, this observed trend was not statistically significant.

In Tables 3 and 4, MS2 and MNV-1 inactivation on cherry tomatoes is reported, respectively. Besides at time 10 min, a 90 min attachment time has a higher reduction than a 24 h attachment time on cherry tomatoes, which was also seen with Boston Bibb. When comparing the results

between the treatments for cherry tomatoes, there is not a trend that can be observed given the variation within the data. Again, there is not a significant difference between the treatments. For MNV-1, unlike the Boston bibb results, the 24 h attachment on tomatoes had a greater reduction than the 90 min attachment when treated with aqueous ozone. There was variability in the trend in log reduction of MNV-1 over time when comparing ozone and water only treatments. Specifically, water only had a greater log reduction than ozone at 10 and 20 min, but at 30 and 40 min, ozone washes had a greater log reduction than water.

b. Bacterial Inactivation

A baseline recovery was also used for bacteria in order to calculate the log reduction over time. The average recovery from both produce types was 10^6 CFU/ml for *E. cloacae* and 10^5 *B. cereus.* Bacterial inactivation on Boston bibb is shown in Table 5. When comparing ozone and water only treatments of lettuce inoculated with both *E. cloacae* and *B. cereus,* initially water had a greater reduction than ozone at 10 min. However, in general, ozone achieved greater reduction than water for the remaining time points. Despite ozone having a greater reduction than water, there were no statistically significant differences. With cherry tomatoes, the bacterial inactivation is shown in Table 6. Ozone treatment had a higher observed log reduction than water only for both bacteria. When comparing the two bacteria on Boston bibb, *E. cloacae* experienced a greater log reduction of 3.33 log CFU/ml after a 40 min exposure compared to *B. cereus* which had a 2.83 log CFU/ml. Although for tomatoes, *B. cereus* experienced a greater log reduction of 2.82 log CFU/ml over the 2.58 log CFU/ml for *E. cloacae*.

c. Ozone Concentration

The average ozone concentration for each treatment is recorded in Table 7. The ozone concentration ranges from 0.48-0.99 ppm. The treatments all have an initial concentration of at

least 0.48 ppm of ozone. As time increases, the concentration of ozone also increases reaching at least 0.90 ppm in all treatments except viruses on tomatoes with a 24 h attachment which reached a concentration of 0.69 ppm.

4. Discussion

One of the major conclusions from this study was that ozone did not provide a significant impact on virus reduction on Boston bibb lettuce or cherry tomatoes when compared to the water only treatments. When examining the effects of aqueous ozone on viral inactivation, there are very few studies that have investigated the inactivation on fresh produce. Hirneisen et al. (2011) reported a more than three-log reduction of MNV-1 after 10 min on lettuce and on green onions. Moreover, the authors observed a five-log reduction of MNV-1 inoculated in water after a 10 min exposure to ozone and concluded that the food matrices played a role in viral inactivation. The authors reported that there was a greater log reduction of the viruses in suspension than on onions or lettuce. However, Hirneisen and co-authors did not publish the results of a water wash only, but rather referenced that a water wash was performed as a control. Through comparison of ozone and water only treatments in the present study, it seems these results contradict those reported by Hirneisen et al. (2011). More specifically, the present study showed that ozone did not have a significant impact on log reduction of viruses. In fact, it may be hypothesized that reduction in viruses on produce is primarily due to physical removal followed by actual inactivation of viruses in the wash water by aqueous ozone. However, these discrepancies could be due to the differing sample sizes, the volume of water the samples were treated with, or the concentration of the ozone applied. More specifically, Hirneisen et al. (2011) exposed 5 g of produce to an ozone wash in a beaker at a concentration of 6.25 ppm whereas, in the current study, 25 g of produce was submerged in a sink of aqueous ozone at a concentration between 0.5

ppm and 1 ppm. Even though there are conflicting results, both studies indicate that food matrices play a key role in protecting viruses from ozone inactivation.

It was also shown in this study that attachment time for viruses does not affect the efficacy of ozone. To our knowledge, no other published studies that have investigated the attachment time of viruses to fresh produce and the impact on sanitizer efficacy. The results reported here show that the attachment time of viruses has no significant effect with respect to ozone inactivation. This may indicate that no matter how long the virus has been present, whether that means contamination in the retail setting or contamination in the field, aqueous ozone has a similar level of efficacy.

Even though the conclusions are the same for both Boston bibb and cherry tomatoes, there was a visible difference in the reductions of microorganisms between the two produce types. This could be due to the surface topography of the produce. Lu et al. (2015) looked at the influence of the epicuticular surfaces of lettuce and tomatoes as it related to the adsorption of rotavirus. The authors found that these surfaces play a role in the effectiveness of sanitation treatments and could be the cause of the differences in log reductions of viruses between produce types. Interactions of the microbe with the produce may also play a role in the inactivation difference between produce types. A majority of human noroviruses are known to bind to histo-blood group antigens (HBGA) on cells lining the gastrointestinal tract which can lead to infections in human. It has been previously observed that lettuce possess HBGA-like carbohydrates and other carbohydrate moieties that norovirus particles were able to attach to and thus could not be removed by simple washing (Gao et al., 2016). The viral surrogate used in the present study, MNV-1, binds to sialic acid, another type of carbohydrate moiety (Taube et al.,

2009). These types of interactions could be an explanation as to why there was a lower reduction of viruses on the lettuce than tomatoes.

When it comes to bacteria, Takeuchi et al. (2000) observed that species of bacteria (*E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium, and *Pseudomonas fluorescens*) attach to lettuce differently. The authors found that *E. coli* and *L. monocytogenes* attached to cut edges while *P. fluorescens* preferred the surface of the lettuce; meanwhile, *Salmonella* did not have a preference in attachment site. They further explained that these differences are due to the ability of the bacteria to bind to the hydrophobic cuticle layer. This could be an explanation as to why log reductions are different between the two bacteria for the produce analyzed. In general, the present study demonstrates that ozone does not have a significant impact on the removal and/or inactivation of *E. cloacae* and *B. cereus* on either produce type. Previous studies on ozone inactivation of bacteria on fresh produce have reported findings that conflict with those reported here. Kim et al. (1999) investigated the inactivation of *P. fluorescens* on the surface of shredded lettuce. The authors found that bubbling ozone into the wash water was significantly different from the water only wash for inactivation of *P. fluorescens*. However, their samples were placed in a beaker with 500 ml of water and stirred while the ozone is generated which may have impacted the result. The stirring could have increased the physical removal of the bacteria from the surface. Similarly, Selma et al. (2007) concluded that ozone significantly reduced *Shigella sonnei* when inoculated on shredded lettuce. This experiment took place in a 50-L tank and the sample size was 30 g with the longest exposure time being 5 min at various ozone concentrations (1, 2, and 5 ppm). In the present study, the sample size was 25g which is similar to Selma et al. (2007) but the studies differ in contact times (5 min compared to 40 min in the current study) and in the concentrations of ozone applied (1, 2, and 5 ppm compared to 0.5-1 ppm in the current

study). These differences could be a possible explanation for the discrepancies between the present study and other published studies.

The bacteria investigated in the present study where chosen because both bacteria are present in the phyllosphere of leafy greens and fresh produce in general (Al-Kharousi et al., 2016; Stenfors Arnesen et al., 2008). In addition, these bacteria also represent both gram positive and gram negative species. Gram negative bacteria are reportedly more sensitive to ozone due to the composition of the cell membrane not containing as much peptidoglycan that is present in gram positive bacteria (Kim et al., 1999). Although that was true for gram negative bacteria on Boston bibb in the present study, tomatoes had a greater reduction of gram positive bacteria. This could mean that produce type may be a key aspect in the inactivation of the microbes. It is also important to note that unpublished data has shown that when the sink is drained, microbes were left on the surface of the sink with water only washes whereas there were no microbes left on the surface of the sink when ozone was used. This indicates that adding ozone to wash water could prevent cross contamination during produce washing.

5. Conclusion

In conclusion, an ozone wash does not increase inactivation of viruses on fresh produce compared to the use of water alone. Additionally, the two different attachment times had no significant difference in viral inactivation by ozone. Ozone does, however, have the potential to make an impact on bacterial inactivation when compared to water only. The surface of the produce could play a key role in the extent of inactivation of microbes and needs to be considered when evaluating sanitizers.

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Appendix

Table 1. Log reduction of MS2 on Boston bibb lettuce after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in PFU (±SD) by Treatment[1]		
	Ozone (90 min)	Ozone (24 h)	Water (24 h)
10	2.14 (±0.30)	3.85* (±1.13)	2.02 (±0.42)
20	2.99 (±0.93)	3.49 (±0.27)	2.64 (±0.55)
30	2.78 (±0.70)	2.92 (±1.20)	2.14 (±0.09)
40	2.95 (±0.73)	2.46 (±0.51)	2.13 (±0.47)

SD = one standard deviation from the mean
[1]Ozone (90 min) = exposure to ozone after 90 min drying time for inoculant; Ozone (24 h) = exposure to ozone after 24 h drying time for inoculant; Water (24 h) = no ozone exposure after 24 h drying time for inoculant
*A sample reached the limit of detection (10 PFU/ml in 75ml sample)

Table 2. Log reduction of MNV-1 on Boston bibb lettuce after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in PFU (±SD) by Treatment[1]		
	Ozone (90 min)	Ozone (24 h)	Water (24 h)
10	2.54* (±1.16)	1.12* (±1.08)	0.91 (±1.33)
20	2.51* (±1.21)	1.50* (±0.90)	1.47 (±0.93)
30	1.69 (±0.53)	1.53 (±0.52)	1.22 (±0.51)
40	1.63 (±0.17)	1.47 (±0.31)	1.63 (±0.70)

SD = one standard deviation from the mean
[1]Ozone (90 min) = exposure to ozone after 90 min drying time for inoculant; Ozone (24 h) = exposure to ozone after 24 h drying time for inoculant; Water (24 h) = no ozone exposure after 24 h drying time for inoculant
*A sample reached the limit of detection (10 PFU/ml in 75ml sample)

Table 3. Log reduction of MS2 on cherry tomatoes after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in PFU (±SD) by Treatment[1]		
	Ozone (90 min)	Ozone (24 h)	Water (24 h)
10	3.32* (±1.02)	3.64 (±0.70)	2.42 (±0.92)
20	3.91 (±0.49)	3.36 (±0.33)	3.74 (±0.10)
30	3.89* (±0.85)	3.54* (±0.84)	3.32 (±0.05)
40	3.30* (±1.10)	2.71* (±1.33)	3.70 (±0.23)

SD = one standard deviation from the mean

[1]Ozone (90 min) = exposure to ozone after 90 min drying time for inoculant; Ozone (24 h) = exposure to ozone after 24 h drying time for inoculant; Water (24 h) = no ozone exposure after 24 h drying time for inoculant

*A sample reached the limit of detection (10 PFU/ml in 75ml sample)

Table 4. Log reduction of MNV-1 on cherry tomatoes after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in PFU (±SD) by Treatment[1]		
	Ozone (90 min)	Ozone (24 h)	Water (24 h)
10	0.99 (±0.52)	1.60 (±0.39)	2.08 (±0.56)
20	1.85 (±0.65)	2.12 (±0.28)	2.62 (±0.19)
30	1.87 (±0.08)	2.34 (±0.49)	1.96 (±0.71)
40	1.74 (±0.36)	2.60 (±0.01)	2.29 (±0.92)

SD = one standard deviation from the mean

[1]Ozone (90 min) = exposure to ozone after 90 min drying time for inoculant; Ozone (24 h) = exposure to ozone after 24 h drying time for inoculant; Water (24 h) = no ozone exposure after 24 h drying time for inoculant

*A sample reached the limit of detection (10 PFU/ml in 75ml sample)

Table 5. Log reduction of *Enterobacter cloacae* and *Bacillus cereus* on Boston bibb lettuce after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in CFU (±SD) by Treatment			
	E. cloacae		*B. cereus*	
	Ozone	Water	Ozone	Water
10	1.81 (±0.05)	2.80 (±0.35)	1.61 (±0.32)	2.13 (±0.42)
20	2.13 (±1.57)	1.67 (±0.02)	2.45 (±0.06)	2.11 (±0.01)
30	2.41 (±0.60)	1.48 (±0.09)	2.13 (±0.32)	2.15 (±0.28)
40	3.33* (±0.95)	2.78* (±1.73)	2.83* (±1.08)	1.90 (±0.44)

SD = one standard deviation from the mean
*= A sample reached the limit of detection (10 CFU/ml in 75ml sample)

Table 6. Log reduction of *Enterobacter cloacae* and *Bacillus cereus* on cherry tomatoes after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in CFU (±SD) by Treatment			
	E. cloacae		*B. cereus*	
	Ozone	Water	Ozone	Water
10	1.58 (±0.33)	1.39 (±0.53)	1.62 (±0.47)	0.36 (±0.23)
20	1.44 (±0.66)	1.35 (±0.14)	2.06 (±0.22)	1.51 (±0.10)
30	2.46 (±0.14)	2.01 (±0.76)	2.71 (±1.07)	1.59 (±0.86)
40	2.58 (±1.11)	2.46 (±0.21)	2.82* (±1.38)	1.90 (±0.21)

SD = one standard deviation from the mean
*A sample reached the limit of detection (10 CFU/ml in 75ml sample)

Table 7. Mean ozone concentration (ppm) for ozone treatments over time.

Time (min)	Mean Ozone Concentration in ppm (±SD) by Treatment[1]					
	Boston Bibb-Viruses		Cherry Tomatoes-Viruses		Boston Bibb-Bacteria	Cherry Tomatoes-Bacteria
	90 min	24 h	90 min	24 h		
0	0.51 (±0.02)	0.65 (±0.15)	0.51 (±0.04)	0.48 (±0.05)	0.54 (±0.06)	0.51 (±0.01)
10	0.78 (±0.01)	0.69 (±0.05)	0.78 (±0.06)	0.76 (±0.03)	0.73 (±0.01)	0.72 (±0.02)
20	0.91 (±0.02)	0.74 (±0.01)	0.90 (±0.08)	0.89 (±0.03)	0.84 (±0.01)	0.85 (±0.00)
30	0.94 (±0.04)	0.74 (±0.5)	0.96 (±0.11)	0.96 (±0.06)	0.92 (±0.01)	0.93 (±0.03)
40	0.96 (±0.02)	0.69 (±0.12)	0.98 (±0.10)	0.99 (±0.07)	0.94 (±0.00)	0.97 (±0.28)

SD = one standard deviation from the mean

Chapter 4: Aqueous ozone inactivation of viruses on stainless steel surfaces

Abstract

Norovirus is a common foodborne illness, most often related to an infected food handler transferring the virus directly to food or indirectly to food contact surfaces. Implementing risk management strategies, such as aqueous ozone, to reduce viral contamination is important for the retail industry. Stainless steel coupons (10 cm^2) were spot inoculated with 100 µl of both MNV-1 and MS2 at 10^6 and 10^8 PFU/ml, respectively. The viruses were allowed to dry and attach to the coupons for 90 min and then the coupons were placed in a batch wash ozone sanitation system (BWOSS) with either an ozone wash or a water only wash. The water was iced to achieve a temperature between 3-5°C with an ozone concentration of 0.5 ppm to 1 ppm. The coupons were exposed to either treatment for times of 0.5, 3, and 10 min. The coupons were removed from the sink and flooded with buffered phosphate water in a sterile petri dish in order to recover the viruses. The eluate was serially diluted and processed by double agar layer method and plaque assay for MS2 and MNV-1, respectively, to determine PFU/ml. The log reduction difference between ozone and water for MNV-1 and MS2 after 10 min was 0.25 and 0.51 PFU/ml. Overall, there was no significant difference between an ozone wash and a water only wash for the inactivation of MNV-1and MS2 on stainless steel surfaces.

1. Introduction

In Chapter 3, ozone was investigated for its inactivation of viruses and bacteria on fresh produce, and ozone was not found to significantly contribute to the inactivation of viruses. Based on those results, it was hypothesized that the food matrix may contain structures and other compounds that aid in viral attachment and prevent ozone inactivation. For instance, Gao et al. (2016) showed that virus like particles (VLPs) of human norovirus GII.4 were able to bind to surface carbohydrates in the cell wall of lettuce.

Viral inactivation on food matrices has been shown to be variable. Hirneisen et al., (2010) indicates that the efficacy of ozone on food matrices is greatly affected by variables such as the surface topography of the food, the type of microorganism contaminating the food, and the strength of association of the microbe to the produce. There are only a few studies that have researched viral inactivation on produce by ozone. Hirneisen et al. (2011), researched the inactivation of murine norovirus (MNV) and feline calcivirus (FCV) on green onions and lettuce by ozone. The researchers did not always have viral inactivation over time there were a few cases of variability recorded. However, the researchers recorded at least a 2 log reduction of FCV on both produce and over a 3 log reduction of MNV. They concluded that the main contributor to variability was due to produce type. The authors believe that the difference in organic composition of the produce could be the reason for the variability. Similarly, Chapter 3 had variability in inactivation of MNV and MS2 bacteriophage on both Boston bibb lettuce and cherry tomatoes.

Due to the variability in virus inactivation observed in Chapter 3, the use of an abiotic surface such as stainless steel for the evaluation of ozone efficacy against viruses was considered here in order to eliminate the impact of the specific interactions occurring on biotic surfaces.

2. Materials and Methods

a. Microbe cultivation

i. MS2 Bacteriophage

MS2 stock was generated, as described previously by Gibson, Crandall, and Ricke (2012), and aliquoted in small volumes. MS2 was kindly provided by Dr. Stephanie Friedman from the United States Environmental Protection Agency (EPA) Gulf Ecology Division in Gulf Breeze, FL. Stock was generated by scraping the soft agar layer containing bacteriophage lysed *E. coli* C3000 (ATCC 15597) into centrifuge tubes. The cell lysate was then suspended with 23 ml of 1 × phosphate buffered saline (PBS), vortexed, and centrifuged at 185 × *g* for 25 min. The supernatant was collected and filtered through a 0.22 μm filter (Millipore Corporation, Billerica, MA) and then aliquoted and stored at -80°C. The MS2 stock concentration was determined based on titration by double agar layer (DAL) method with bacterial host *E. coli* C3000. DAL assay involves adding 100 μl of sample plus 100 μl of log phase bacterial host (*E. coli* C3000) to 5 ml of soft agar (0.7% tryptic soy agar (TSA)) and then poured on the top of TSA plates. The soft agar was allowed to solidify and then the plates were incubated for 24 h at 37°C. The plaques were counted and PFU/ml was then calculated.

ii. Murine Norovirus (MNV-1)

Murine norovirus type 1 (MNV-1) was prepared as described previously by Bae and Schwab (2008) with modifications. MNV-1 was kindly provided by Dr. Kellogg Schwab at Johns Hopkins Bloomberg School of Public Health in Baltimore, MD. MNV-1 was propagated in monolayers of RAW 264.7 (mouse leukameic monocyte macrophage, ATCC TIB-71) cells. Cells were cultured in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing less than 10% low endotoxin, fetal bovine serum (FBS: GibcoLife Technology,

Gaithersburg, MD), 1% 100 × penicillin-streptomycin solution (GibcoLife Technology), 1% HEPES (Sigma-Aldrich), 1% glutamine (Hyclone, Logan, UT), and 1% non-essential amino acids (Corning, New York, NY). After reaching 90% confluence, cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.05 for virus stock production. The virus was extracted from cell lysate after complete cytopathic effect as described by Hsueh and Gibson (2015) with modifications. Briefly, culture flasks containing infected cells were subjected to three freeze-thaw cycles at -80°C and 37°C respectively, followed by centrifugation at 5000 × *g* for 20 min at 4°C then filtration with 0.1 μm filter and stored at -80°C. To determine infectious titer, the plaque assay as reported by Hsueh and Gibson (2015) was used with modifications. Briefly, six-well plates were seeded with $2x10^6$ RAW cells per well and grown to 90% confluence in 2 ml of complete growth medium. Cell monolayers were inoculated with virus stocks for 1 h at 37°C with rocking followed by removal of the inocula. Cells were covered with 2 ml of prepared overlay medium containing: 25% of 6% low melting point agarose, 50% 2 × minimum essential medium eagle (MEM) (Corning) (100 ml 10× MEM, 10 ml glutamine, 29.3 ml sodium bicarbonate, and 360.7 ml sterile distilled water filtered through 0.22 μm filter (Millipore Corporation, Billerica, MA)), 10% low endotoxin FBS, 1% 100 × penicillin-streptomycin solution, 1% glutamine, 0.5% HEPES, and 12.5% sterile distilled water. The plates were incubated for 72 h. Next, 2 ml of 0.01% neutral red (Sigma-Aldrich) prepared in 1 × phosphate buffered saline (PBS) was added to each well to visualize plaques. Plaques were counted after 1h to determine virus titer (PFU/ml).

b. Stainless Steel

Stainless steel sheets were (type 304/16 gauge, unpolished; Rose Metal Products, Springfield, Missouri, USA) cut into 10 cm^2 coupons. The stainless steel was first autoclaved then 100μl of

MS2 at 10^8 PFU/ml and 100µl of MNV at 10^6 PFU/ml were spotted on the surface of each coupon in 10 µl drops and allowed to dry for 90 min in a biological safety cabinet.

c. Treatments

The stainless steel coupons were placed in a batch wash ozone sanitation system (BWOSS). The BWOSS utilized in the present study was developed by Recycled Hydro Solutions (Rogers, AR). The BWOSS contains a one compartment sink measuring 43 cm^2 with a depth of 30 cm and fabricated using 16-gauge stainless steel. During operation, the sink is filled with water, and once filled, water from the sink is passed through a Venturi injector, which creates aqueous ozone continuously. The ozonated water is then passed back into the sink basin. The BWOSS has a dissolved ozone meter (Model Q46, ATI, Collegeview, PA) which was corroborated by the indigo trisulfonate method (SM 4500-OS3 B) using a Hach Pocket Colorimeter II (Hach Company, Loveland, CO) and Ozone AccuVac Ampules (Hach) (American Public Health Association, American Water Works Association, and Water and Environment Federation, 2012).

The sink holds approximately 34.07 L (9 gallons) of water. Ice was added to the sink to aid in stabilization of ozone until the water temperature reached 4°C. The coupons were placed in the BWOSS once it reached 0.5 ppm as indicated by the dissolved ozone meter and the water temperature in the sink was stable at 4°C. The coupons were in the sink for a total of 10 minutes with two samples (n=6) taken at 30 s, 3 min, and 10 min. A water only wash was also conducted in the same sink as a control. Experiments were conducted in duplicate.

d. Microbial analysis

The coupons were placed in sterile Petri dishes and flooded with 2 ml of buffered phosphate water (BPW), and the coupons were then scraped with a cell scraper. The eluate was serially diluted and analyzed.

For MS2, 100 µl of each dilution was plated in duplicate using DAL method. Then the plaque forming units (PFU) were counted, and PFU/ml was calculated. For MNV-1, plaque assay was performed as previously described (Section MNV-1), and PFU were counted in order to calculate PFU/ml.

e. Statistical analysis

Statistical analyses were performed using JMP Pro 13 software (SAS institute, Inc., Cary, NC). Significance was determined by a critical p-value 0.05 by one-way analysis of variance (ANOVA) for all scenarios. Based on the results, a Tukey-Kramer honestly significant difference (HSD) test was conducted to confirm differences between groups.

4. Results

A baseline recovery was used to calculate the amount of the viruses that can be recovered from stainless steel without any treatment. The average baseline recovery for MNV-1 was 6.4×10^4 PFU/ml and for MS2 1.2×10^7 PFU/ml. The treatment results are the difference between the baseline values and the experimental sample values. The average ozone concentration for 0, 0.5, 3, and 10 min are as follows: 0.52, 0.56, 0.66, 0.80 ppm.

Both treatments applied to stainless steel coupons resulted in log reductions over time (Table 1). An observed increase in log reduction of 0.83 to 3.53 PFU/ml over time with ozone was seen for MNV-1. Similarly, with a water only wash, the log reduction was 0.57 to 3.28 PFU/ml over time for MNV-1. There was a visible difference between the ozone wash and water only wash

for MNV-1, though there was not a significant difference. An increase in log reduction from 0.56 to 4.56 PFU/ml over time with ozone was also observed for MS2. Again, the water wash also showed an increase in log reduction over time of 0.34 to 4.05 PFU/ml for MS2. Similar to MNV-1, MS2 did not have a significant difference between the treatments.

5. Discussion

Even though there is not a significant difference between ozone and water wash, it was hypothesized that log reductions would increase over time as viruses are inactivated and/or physically removed. This was seen with the stainless steel results and not in the produce results (See Chapter 3). This is related to the limitations in recovery. Produce was washed in a larger volume (75 ml) than the stainless steel (2 ml) which dictates the limit of detection. Recovery from produce is also more difficult because of the surface topography hindering the removal of the virus in comparison to the smooth surface of the stainless steel (K A Hirneisen et al., 2011). Viruses have also been known to form interactions with the surface of lettuce due to surface glycans (Gao et al., 2016). However, there are no known studies that have looked at the inactivation of viruses by ozone and compared biotic and abiotic surfaces.

Previous research has reported similar log reductions on abiotic surfaces as are reported here. A study showed that a 6-log reduction in the bacteriophage, PRD1, was achieved on the surface of a stainless steel ice cream dipper in an aqueous ozone dipper well (Almeida and Gibson, 2016). The results in the current study are not near a 6-log reduction, but there is still an overall log reduction of 3.5 and 4.5 for MNV-1 and MS2, respectively, after a 10 min exposure. There are differences between the current study and the study by Almeida and Gibson (2016). The first notable difference is the use of a different bacteriophage. PRD1 is a double stranded DNA bacteriophage while MS2 is a single stranded RNA bacteriophage (Anders and Chrysikopoulos,

2006). DNA and RNA phage are reported to behave differently. When exposed to sanitizing practices such as peracetic acid and UV irradiation, DNA phage are more sensitive to inactivation than RNA phage (Rajala-Mustonen et al., 1997). Peracetic acid and ozone are both strong oxidizers which could indicate that similar tendencies for phages could be seen with ozone; conversely, UV irradiation damages the genetic material which could have been the reason Rajala-Mustonen et al. (1997) saw that DNA phage were more sensitive than RNA phage. Besides the bacteriophage used, the method of inoculation between the two studies was different. Almeida and Gibson (2016) dipped the ice cream dipper in the inoculum whereas the current study spot inoculated. Moreover, the ice cream dipper was then immediately submerged in the ozone wash as opposed to allowing for a drying period. The dip inoculation method is limited due to the application of an unknown microbial concentration thus leading to consistency issues between inoculation events; meanwhile, a spot inoculation of a known concentration eliminates this issue (Beuchat et al., 2001). Last, the ice cream dipper was then swabbed for the presence of microorganisms whereas the stainless steel coupons used in the present study were flooded in a buffer solution. This difference in recovery methods affects the limit of detection since the eluate volumes also differe. The differences discussed above could certainly account for the opposing results.

6. Conclusion

By exploring the effect of aqueous ozone on viruses on an abiotic surface, we were able to show ozone efficacy without confounding variables that are present on biotic surfaces such as on produce. By eliminating confounding variables, the variation in the data was reduced in comparison to the produce results in Chapter 3. Surface results show an increase in log reduction over time which contrasts what was shown on produce in Chapter 3. Even though there was not a

significant difference between the ozone and water washes on the surface of the stainless steel, there was a visible difference which indicates that the food matrix plays a critical role in a virus's ability to evade inactivation via ozone.

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Appendix

Table 1. Log reduction of MNV-1 and MS2 on stainless steel after exposure to aqueous ozone or water over time.

Time (min)	Mean Log Reduction in CFU (±SD) by Treatment			
	MNV-1		MS2	
	Ozone	Water	Ozone	Water
0.5	0.83 (±0.09)	0.57 (±0.01)	0.56(±0.14)	0.34 (±0.34)
3	2.39 (±0.50)	1.89 (±0.15)	1.82 (±1.40)	0.72 (±0.29)
10	3.53* (±0.03)	3.28* (±0.64)	4.56* (±0.99)	4.05 (±0.50)

SD = one standard deviation from the mean

*= A sample reached the limit of detection (10 CFU/ml in a 2ml sample)

Chapter 5: Aqueous ozone inactivation of viruses in association with bacteria on Boston bibb lettuce

Abstract

Viruses do not exist in monocultures in the environment; they instead exist in microbial communities. The extent to which these communities effect virus inactivation is not well known. These communities may exist on the surface of fresh produce, and the interaction of human enteric viruses with their microbial co-habitants is an under explored area of research. A key question is how do these virus-bacteria interactions impact the efficacy of control strategies? Therefore, aqueous ozone was applied as a sanitizing agent on Boston bibb lettuce to investigate the impact of virus-bacteria interactions on the inactivation of viruses on fresh produce. Samples of Boston bibb (25 g) were spot inoculated with 200 µl of a mixture of 100 µl of murine norovirus (MNV-1; 10^6 PFU/ml) and 100 µl of either *Bacillus cereus* or *Enterobacter cloacae* (10^6 CFU/ml) that had been allowed to incubate for 1 h at room temperature prior to inoculation. The mixture was allowed to dry and attach to the lettuce (90 min) and then the lettuce was placed in the batch wash ozone sanitation system (BWOSS) with either an ozone wash or a water only wash. The water was iced to achieve a temperature between 3-5°C and the ozone concentration was 0.5 ppm to 1 ppm. The lettuce was exposed to either treatment for 10, 20, 30, and 40 min. The lettuce was removed from the sink and washed with an antibiotic solution. The eluate was serially diluted and processed by plaque assay to determine the concentration of MNV-1 in PFU/ml. The log reduction differences between ozone and water for MNV-1 only, MNV-1 with *B. cereus*, and MNV-1 with *E. cloacae* after 40 min were 0, 0.95, and -0.36 PFU/ml, respectively. Overall, there was no significant difference between virus alone and virus associated with either bacterium. However, the results from MNV-1 with *B. cereus* indicate that association with bacteria of different gram types (i.e. gram positive versus gram negative) could impact viral inactivation by aqueous ozone.

1. Introduction

Throughout the supply chain there are several opportunities for microorganisms to interact with microbial co-habitants on the surface of produce. These interactions could possibly create stronger resistance to current microbial control strategies thus placing the consumer at an increased risk for foodborne illness. Most studies focus on microbes in isolation; however, that is not the case as these microbes exist in microbial communities and interact with one another. Currently, there is very little research on how existing control strategies respond to these interactions, especially viral interactions with bacteria. Enteric viruses, for example human norovirus, are often of concern when it comes to food safety. Norovirus and leafy greens have the highest number of illnesses when comparing pathogen-commodity pairs (Gould et al., 2013).

Produce can be contaminated via various routes before reaching the consumer: soil, water, animal excrements, and mishandling by workers (Benson, 2010; Strohbehn et al, 2008). Also, the produce itself has naturally occurring microbes on its surface (Whipps et al., 2008). Each of these could contribute to microbes possibly interacting on the surface of produce. A recent review by Deng and Gibson (2017), discuss more specifically the interactions that may occur between human noroviruses and the microbial community of leafy greens. Interestingly, Almand et al. (2017) reported on the specificity of interactions between noroviruses and bacteria stated that these were specific to norovirus strains since other viruses tested did not readily interact with bacteria. It has also been reported that noroviruses are able to bind to carbohydrate moieties present on the surface of romaine lettuce (Esseili et al., 2015; Gao et al., 2016).

Of the limited research, it has been shown that when viruses interact with bacteria expressing specific surface antigens, histo-blood group antigens (HBGA's), viruses are more resistant to disinfectant processes. For example, when heat is applied, human norovirus particles in

association with bacteria had a higher immunoreactivity than human norovirus particles alone (Li et al. 2015). Conversely, Li et al. (2017) published results indicating that Tulane virus—a cultivable human norovirus surrogate—was not protected from heat while interacting with bacteria expressing HBGA-like molecules. These conflicting results raise several questions when it comes to the interactions of viruses and bacteria and these may impact virus inactivation under specific sanitation practices.

Murine norovirus and poliovirus (serotype 1, Mahoney) have been shown to interact with gram positive and gram negative bacteria including *Bacillus cereus*, *Enterococcus faecalis, Escherichia coli,* and *Enterobacter cloacae* (Jones et al., 2014; Kuss et al., 2011). Therefore, this study aimed to evaluate the efficacy of aqueous ozone on the inactivation of viruses while in association with bacteria often found on the phyllosphere of lettuce.

2. Materials and Methods

a. Microbe Cultivation

i. Virus

Murine norovirus type 1 (MNV-1) was prepared as described previously by Bae and Schwab (2008) with modifications. MNV-1 was kindly provided by Dr. Kellogg Schwab at Johns Hopkins Bloomberg School of Public Health in Baltimore, MD. MNV-1 was propagated in monolayers of RAW 264.7 (mouse leukameic monocyte macrophage, ATCC TIB-71) cells. Cells were cultured in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing less than 10% low endotoxin, fetal bovine serum (FBS: GibcoLife Technology, Gaithersburg, MD), 1% 100 × penicillin-streptomycin solution (GibcoLife Technology), 1% HEPES (Sigma-Aldrich), 1% glutamine (Hyclone, Logan, UT), and 1% non-essential amino acids (Corning, New York, NY). After reaching 90% confluence, cells were infected with MNV-

1 at a multiplicity of infection (MOI) of 0.05 for virus stock production. The virus was extracted from cell lysate after complete cytopathic effect as described by Hsueh and Gibson (2015) with modifications. Briefly, culture flasks containing infected cells were subjected to three freeze-thaw cycles at -80°C and 37°C respectively, followed by centrifugation at 5000 × *g* for 20 min at 4°C then filtration with 0.1 µm filter and stored at -80°C. To determine infectious titer, the plaque assay as reported by Hsueh and Gibson (2015) was used with modifications. Briefly, six-well plates were seeded with $2x10^6$ RAW cells per well and grown to 90% confluence in 2 ml of complete growth medium. Cell monolayers were inoculated with virus stocks for 1 h at 37°C with rocking followed by removal of the inocula. Cells were covered with 2 ml of prepared overlay medium containing: 25% of 6% low melting point agarose, 50% 2 × minimum essential medium eagle (MEM) (Corning) (100 ml 10× MEM, 10 ml glutamine, 29.3 ml sodium bicarbonate, and 360.7 ml sterile distilled water filtered through 0.22 µm filter (Millipore Corporation, Billerica, MA)), 10% low endotoxin FBS, 1% 100 × penicillin-streptomycin solution, 1% glutamine, 0.5% HEPES, and 12.5% sterile distilled water. The plates were incubated for 72 h. Next, 2 ml of 0.01% neutral red (Sigma-Aldrich) prepared in 1 × phosphate buffered saline (PBS) was added to each well to visualize plaques. Plaques were counted after 1h to determine virus titer (PFU/ml).

ii. Bacteria

Enterobacter cloacae (ATCC 39979) with rifampicin resistance was streaked from a frozen 50% glycerol stock onto Luria Bertani (LB) agar (Alfa Aesar, Tewksbury, MA) with 100 µg/ml of rifampicin (Alfa Aesar) using a sterile inoculation loop and incubated overnight at 37°C. A single CFU was selected from the plate, placed in 5 ml of LB broth with 100 µg/ml rifampicin in a 50 ml centrifuge tube, and incubated overnight at 37°C with shaking at 150 rpm. *Bacillus*

cereus (ATCC 14579) was also streaked from a frozen, 50% glycerol stock onto a nutrient agar (NA) plate (Becton, Dickson, and Company, Franklin Lakes, NJ) using a sterile inoculation loop and incubated overnight at 30°C. A single CFU was selected from the plate, placed in 5 ml of nutrient broth (NB) (Becton, Dickson, and Company) in a 50 ml centrifuge tube, and incubated overnight at 30°C with shaking at 150 rpm. Following overnight growth of both bacteria, the culture tubes were centrifuged at 5,000 × *g* for 10 min to pellet the bacterial cells. The supernatant was decanted, and the pellet was resuspended in 5 ml of 1 × PBS, vortexed, and centrifuged again. The bacterial pellet was resuspended in 5 ml of 1 × PBS and the concentration of each bacterial culture was determined by spread plate enumeration of microorganisms. LB agar with 100 µg/ml of rifampicin and *B. cereus* agar (Oxoid, Altrincham, Cheshire, England) supplemented with selective supplement (Oxoid) and egg yolk emulsion (Dalynn, Calgary, Canada) were used for *E. cloacae* and *B. cereus,* respectively.

b. Microbe-Microbe Interaction

Enterobacter cloacae and *B. cereus* were serially diluted to 10^6 CFU/ml in 1×PBS. For interactions, 100 µl of bacteria was mixed with 100 µl of MNV (10^6 PFU/ml) at room temperature in an Eppendorf tube and allowed to associate for 1 h. An abstract presented at the International Association of Food Protection (IAFP) 2017 Annual Meeting in Tampa, FL, confirms association of MNV and *E. cloacae* or *B. cereus* in the time specified (Almeida and Gibson, 2017).

c. Produce

Boston bibb loose leaf lettuce was used. The Boston bibb was grown hydroponically by Ozark All Seasons in Windsor, AR and purchased from a local grocer. The Boston bibb leaves were removed and placed in a sterilized plastic container with a lid and stored at 4°C until use.

d. Inoculation of Produce

For inoculation, 25 g of Boston bibb leaves were measured out for each sampling time point (n = 5) for a total of 125 g for each experiment. Each 25 g was then spot inoculated with the 200 µl of the mixture containing viruses and bacteria. Following inoculation, the produce was allowed to dry for 90 min before being treated.

e. Treatments

The batch wash ozone sanitation system (BWOSS) utilized in the present study was developed by Recycled Hydro Solutions (Rogers, AR). The BWOSS contains a one compartment sink measuring 43 cm^2 with a depth of 30 cm and fabricated using 16-gauge stainless steel. During operation, the sink is filled with water, and once filled, water from the sink is passed through a Venturi injector, which creates aqueous ozone continuously. The ozonated water is then passed back into the sink basin. The BWOSS has a dissolved ozone meter (Model Q46, ATI, Collegeview, PA) which was corroborated by the indigo trisulfonate method (SM 4500-OS3 B) using a Hach Pocket Colorimeter II (Hach Company, Loveland, CO) and Ozone AccuVac Ampules (Hach) (American Public Health Association, American Water Works Association, and Water and Environment Federation, 2012).

The sink holds approximately 34.07 L (9 gallons) of water. Ice was added to the sink to aid in stabilization of ozone until the water temperature reached 4°C. The produce was placed in the BWOSS once ozone reached a concentration of 0.5 ppm—as indicated by the dissolved ozone meter—and the water temperature in the sink was stable at 4°C. The produce samples were submersed in the sink for a total of 40 min with subsamples taken every ten minutes.

f. Microbial Analysis

Produce samples were placed in Whirl-pak bags with 75 ml of buffered phosphate water (BPW) containing 40 μg/ml of penicillin/streptomycin. The bags were then placed in the stomacher (Seward Stomacher 400 Circulator, West Sussex, United Kingdom) for 1 min at 260 rpm. A plaque assay was performed as previously described (Virus Section). PFU were counted and then PFU/ml was calculated.

g. Statistical Analysis

Statistical analyses were performed using JMP Pro 13 software (SAS institute, Inc., Cary, NC). Significance was determined by a critical p-value 0.05 by one-way analysis of variance (ANOVA) for all scenarios. Based on the results, a Tukey-Kramer honestly significant difference (HSD) test was conducted to confirm differences between groups.

3. Results

A baseline recovery was used to calculate the amount of virus that can be recovered from the surface of Boston bibb. This baseline recovery was then used to calculate the log reduction for all time points. In Table 1, the average ozone concentrations are recorded for the treatments. The initial concentration of ozone is at least 0.5 ppm and reached a final concentration of at least 0.85 ppm for all treatments.

In Table 2, the results from the water wash treatments are displayed. Results from Chapter 3 are included for comparison. There was no significant difference between MNV only and any of the virus-bacteria interactions. If log reduction at 40 min is examined separately, there is no visible difference between MNV-1 only (1.63 PFU/ml) and MNV-1 with *B. cereus* (1.65 PFU/ml); however, MNV-1 with *E. cloacae* had a higher log reduction at this time point (2.20 PFU/ml).

The results from the ozone treatment are displayed in Table 3. There was no significant difference between MNV-1 only and any of the virus-bacteria interactions with the ozone wash; however, there is a visible difference. After 40 min, the log reduction with an ozone wash for MNV-1, MNV-1 with *B. cereus*, and MNV-1 with *E. cloacae* was 1.63, 2.60, 1.83 PFU/ml, respectively. The interaction of MNV-1 and *B. cereus* had consistently greater log reductions over time (30 and 40 min) in comparison to the MNV-1 only log reductions.

In comparing the results from the interactions separately, MNV-1 with *B. cereus*, there was a significant difference between the ozone and water treatments. This, however, was not the case for MNV-1 with *E. cloacae* which had no significant difference between the treatments.

4. Discussion

This is the first study that has investigated the effects of aqueous ozone on the inactivation of a norovirus surrogate while in combination with bacteria. As indicated previously, there have been studies that have investigated how these interactions impact norovirus and norovirus surrogate response to heat stress. Li et al. (2015) published that when human norovirus virus like particles (VLP) were in association with HBGA-like expressing bacteria, the VLP's had a higher antigen integrity than in association with bacteria without the antigen. The researchers indicate that this interaction could protect the virus from heat stress. Then Li et al. (2017) conducted a similar study and found that when Tulane virus (TV) was bound to HBGA-like expressing bacteria that this interaction did not protect it from heat stress. The conflicting results in these two studies can be contributed to the different viruses used, human norovirus VLP's versus TV. The study that utilized VLP's had the limitation of a binding assay which does not indicate infectivity whereas the TV study could measure the virus infectivity to demonstrate if the virus

was denatured or not. The discrepancy between the studies testifies to how complex these interactions may be.

The results in this study indicate there is not a significant difference in viral inactivation by aqueous ozone while interacting with bacteria; however, there were visible differences noted. When MNV-1 was associated with *B. cereus* and then exposed to the ozone wash, MNV-1 experienced a greater log reduction than when associated with *E. cloacae*. This could indicate that, when viruses associate with gram positive bacteria, the virus is more susceptible to inactivation due to ozone, or that when in association with gram negative bacteria, it neither aids or enhances the inactivation. This could be due to the difference in the composition of the cell membrane between gram positive and gram negative. Another possibility could be that the interaction between MNV-1 and *B. cereus* has a weaker association than MNV-1 and *E. cloacae* which could lead to the greater reduction that was recorded. Further research is needed in order to further understand these interactions.

5. Conclusion

Viruses do not exist in a monoculture but rather in microbial communities. The interaction between viruses and bacteria had no significant impact on viral inactivation when compared to the virus only results. There is a possibility that viruses in association with gram positive bacteria may be more susceptible to inactivation by ozone, but this is only speculation and further investigation is needed.

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Appendix

Table 1. Mean ozone concentration (ppm) for ozone treatments over time

Time (min)	Mean Ozone Concentration in ppm (±SD) by Treatment		
	MNV only[1]	MNV + *B. cereus*	MNV+ *E. cloacae*
0	0.51 (±0.02)	0.52 (±0.02)	0.54 (±0.06)
10	0.78 (±0.01)	0.69 (±0.01)	0.70 (±0.02)
20	0.91 (±0.02)	0.81 (±0.07)	0.81 (±0.01)
30	0.94 (±0.04)	0.85 (±0.08)	0.82 (±0.03)
40	0.96 (±0.02)	0.86 (±0.09)	0.86 (±0.02)

SD=one standard deviation from the mean
[1]=Results from Chapter 3

Table 2. Log reduction of MNV-1 on Boston bibb lettuce with only a water wash over time.

Time (min)	Mean Log Reduction in PFU (±SD) by water wash		
	MNV-1 alone[1]	MNV-1 and *B. cereus*	MNV-1 and *E. cloacae*
10	0.91 (±1.33)	1.18 (±0.15)	1.41 (±0.01)
20	1.47 (±0.93)	1.95 (±0.34)	1.64 (±0.08)
30	1.22 (±0.51)	1.70 (±0.23)	1.67 (±0.27)
40	1.63 (±0.70)	1.65 (±0.24)	2.20 (±0.51)

SD = one standard deviation from the mean
[1]= Results from Chapter 3
*= A sample reached the limit of detection (10 CFU/ml in 75ml sample)

Table 3. Log reduction of MNV-1 on Boston bibb lettuce by an aqueous ozone wash over time.

Time (min)	Mean Log Reduction in PFU (±SD) by ozone		
	MNV-1 alone[1]	**MNV-1 and *B. cereus***	**MNV-1 and *E. cloacae***
10	2.54* (±1.16)	2.04 (±0.07)	1.42 (±0.16)
20	2.51* (±1.21)	2.38 (±0.07)	2.04 (±0.75)
30	1.69 (±0.53)	2.77* (±0.04)	2.21 (±0.51)
40	1.63 (±0.17)	2.60* (±0.13)	1.84 (±0.25)

SD = one standard deviation from the mean
[1]= Results from Chapter 3
*= A sample reached the limit of detection (10 CFU/ml in 75ml sample)

Chapter 6: Overall Conclusion

Contaminated produce is responsible for a large portion of foodborne illnesses (Painter et al., 2013). Produce is susceptible to contamination because it is a ready to eat product that is consumed raw, which eliminates common sanitization practices like pasteurization and cooking (Benson, 2010). The U.S. Food and Drug Administration (FDA) recommends several practices such as separation of products (i.e. meat and produce), washing of hands and equipment, and washing of produce to prevent further contamination in the retail setting (U.S. Department of Health and Human Services, 2009).

Produce washes often contain sanitizing agents such as chlorine, ozone, and organic acids (Berger et al., 2010). The one of interest for this research is ozone, specifically aqueous ozone. Ozone has been demonstrated to be effective against both bacteria and viruses in suspension (Khadre and Yousef, 2001; Roy et al., 1982). There is research that shows the efficacy of ozone on bacteria on a food matrix (Bialka and Demirci, 2007; Koseki and Isobe, 2006; Selma et al., 2007). There is very little research on the efficacy of ozone against viruses on a food matrix with the only published article being Hirneisen and Kniel (2013) which investigated the inactivation of viruses by bubbling ozone on onions. There is no known published research on ozone inactivation of viruses in complex scenarios including microbe-microbe interactions.

The primary objectives of my research were to evaluate the efficacy of aqueous ozone 1) on the inactivation of viruses and bacteria on produce; 2) on the inactivation of viruses on stainless steel; and 3) against viruses in association with bacteria on produce surfaces.

To begin with, two types of produce, Boston bibb lettuce and cherry tomatoes, were inoculated with either a cocktail of viruses (murine norovirus (MNV) or MS2 bacteriophage) with two attachment times (24 h and 90 min) or bacteria (*Enterobacter cloacae* and *Bacillus cereus*). The produce was then washed in the batch wash ozone sanitation system (BWOSS) with

an ozone wash or a water only wash for 40 min with samples taken every 10 min. Log reductions were then calculated for each sample. It was determined there was not a significant difference in ozone efficacy between the two different attachment times for viruses. Ozone also did not have a significant impact on the reduction of viruses or bacteria in comparison to ozone. It is important to note there was a lot of variability among the viral samples which was not seen in the bacterial samples.

Due to the variability in viral data, virus inactivation on stainless steel was conducted to eliminate any added variability due to the food matrix. Stainless steel coupons were spot inoculated with a viral cocktail of MNV and MS2 bacteriophage and allowed to dry for 90 min prior to being placed into the BWOSS for either an ozone wash or a water only wash for a total of 10 min with samples taken at 0.5, 3, and 10 min. Log reductions were then calculated for each sample. The variability was reduced and log reductions increased over time; however, there was not a significant difference between the ozone and water washes. Due to these results it is hypothesized that there is a possible interaction that viruses have with the surface of produce that aids the virus from deactivation by ozone.

To evaluate inactivation of viruses in association with bacteria, Boston bibb was spot inoculated with a combination of MNV and *E. cloacae* or MNV and *B. cereus*. The virus and bacteria were allowed to associate for an hour prior to inoculating the produce. Again, the produce was placed in the BWOSS with either an ozone wash or a water only wash for 40 min with samples taken every 10 min. Viral log reductions were then calculated for each sample. There was no significant difference between the ozone and water wash for MNV with *E. cloacae*; however, there was a significant difference for MNV with *B. cereus*. This could indicate that if MNV is in association with *B.* cereus that the virus is more susceptible to

inactivation. When comparing both associations with the viral only wash results, there was no significant difference between ozone and water washes with any of the variables.

Overall, it can be hypothesized that there are interactions that occur between viruses and the surface of produce that are preventing complete viral inactivation. Further research is needed to fully understand the impact of a food matrix on the inactivation of viruses alone and in association with bacteria.

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Appendix

IBC Approval Letter

UNIVERSITY OF ARKANSAS

Office of Research Compliance

May 11, 2018

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: Bob Beitle, Acting Biosafety Committee Chair

RE: Protocol Renewal

PROTOCOL #: 15024

PROTOCOL TITLE: Efficacy of water efficient dipper well combined with ozone sanitizer for control of microbial persistence on stainless steel utensils

APPROVED PROJECT PERIOD: **Start Date** May 11, 2015 **Expiration Date** May 10, 2021

The Institutional Biosafety Committee (IBC) has approved your request, dated April 30, 2018, to renew IBC # 15024, "Efficacy of water efficient dipper well combined with ozone sanitizer for control of microbial persistence on stainless steel utensils".

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

1424 W. Martin Luther King, Jr. • Fayetteville, AR 72701
Voice (479) 575-4572 • Fax (479) 575-6527

IBC Registration for Research Project

University of Arkansas
Institutional Biosafety Committee

IBC Number: _____
For Committee Use Only.

Registration for Research Projects
Form 1: GENERAL INFORMATION

Principal Investigator Name: First Kristen M.I. E Last Gibson

Please check the boxes for any of the forms below that are applicable to the research project you are registering. *The General Information Form (Form 1) MUST be completed on all submitted project* registrations, regardless of the type of research.

- [x] **General Information (MUST BE COMPLETED) (Form 1)**
- [ ] **Recombinant and/or synthetic nucleic acid molecules (rDNA; Even if EXEMPT from the NIH Guidelines) (Form 2)**
- [x] **Risk Group 2 or 3 Organisms (pathogenic to humans/plants/animals) (Form 3)**
- [ ] **Biological Toxins (Form 4)**
- [ ] **Human Materials/nonhuman primate materials (Form 5) IRB # (if applicable):** _____
- [ ] **Animals or animal tissues and any of the above categories; transgenic animals; wild vertebrates or tissues (Form 6) AUP # (if applicable):** _____
- [ ] **Plants, plant tissues, or seed any of the above categories; transgenic plants, plant tissues, or seeds (Form 7)**
- [x] **Notice to Pat Walker Health Center (Form 8)**

1. To initiate the review process, you must attach and send all completed registration forms via email to: ibc@uark.edu . ALL REGISTRATION FORMS MUST BE SUBMITTED ELECTRONICALLY.

2. To complete your registration, you must print out page 1 of this form, sign, date (by the Principal Investigator), scan and email it to ibc.uark.edu. You may also mail to: Compliance Coordinator-IBC, 109 MLKG, Fayetteville, AR 72701 or FAX it to 479-575-6527.

As Principal Investigator:

- [x] I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee in a timely manner.
- [x] I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to, the NIH *Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines)* and *Biosafety in Microbiological and Biomedical Laboratories*.
- [x] I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- [x] If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- [x] I will submit a written report to the Institutional Biosafety Committee and the Office of Science Policy at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems pertaining to the implementation of biological and physical containment procedures; or violations of the NIH Guidelines.
- [x] I agree that no work will be initiated prior to project approval by the Institutional Biosafety Committee.
- [x] I will submit in a timely fashion my annual progress report to the IBC.

Signature (PI): Kristen Gibson Digitally signed by Kristen Gibson Date: 2018.04.30 14:11:23 -05'00' **Date:** 4/30/2018

CONTACT INFORMATION

Principal Investigator:

Name: Kristen Gibson Title: Associated Professor

Department: FDSC Campus Address: 2650 N Young Ave

Phone#: A/C [ ] Phone 5-6844 E-Mail keg005@uark.edu

Fax#: A/C [ ] Phone 5-6936

After hours phone number (required if research is at Biosafety Level 2):

Phone#: A/C [ ] Phone 410-419-6620

Co-Principal Investigator:

Name: [ ] Title: [ ]

Department: [ ] Campus Address: [ ]

Phone#: A/C [ ] Phone [ ] E-Mail [ ]

Fax#: A/C [ ] Phone [ ]

After hours phone number (required if research is at Biosafety Level 2):

Phone#: A/C [ ] Phone [ ]

PROJECT INFORMATION

Have you registered ANY project previously with the Institutional Biosafety Committee? (*Check one*) ☑ Yes ☐ No

Are you registering a new project or renewing a previous project registration? "

☐ New project ☑ Modification ☑ Renewal (*Mandatory after 3 years*)

Project Title: Evaluation of RinseWell™ Batch Wash Ozone Sanitation System (BWOSS) for Reduction of Microbes on Fresh Produce

Project Duration: Start Date 5/14/2018 End Date 5/31/2019

Indicate what containment conditions you propose to use (check all that apply):

☑ Biosafety Level 1 *(2,3)*
☐ Animal Biosafety Level 1 *(2,3a,3b)*
☐ Plant Biosafety Level 1 *(3)*
☐ Biosafety Level 2 *(2,3)*
☐ Animal Biosafety Level 2 *(2,3a,3b)*
☐ Plant Biosafety Level 2 *(3)*

Note: When I checked this box, it marked all of them so made my own check mark.

NOTE: Hyperlinks for references throughout this form are available on the last page of this form. **References for Biosafety criteria (click to view)**:

(1) University of Arkansas Biological Safety Manual
(2) Biosafety in Microbiological and Biomedical Laboratories (BMBL) - 5th edition. CDC/NIH
(3) NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules

If working at BSL-2, has your laboratory been inspected by the Biosafety Officer or a member of the IBC?

☑ Yes Date (if known) [ ] ☐ No (*If No* - schedule the inspection with the BSO)

Please provide the following information on the research project **(Please DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal)**

Project Abstract:

Fresh produce is vulnerable to microbial contamination from farm-to-fork as evidenced by the incidence of foodborne disease outbreaks and recalls related to fresh produce each year. While many products are sold packaged and "ready to eat" (RTE) to food service operations (FSO), numerous fresh produce commodities require washing prior to preparation. The U.S. Food and Drug Administration (FDA) guidance specifically states not to rewash RTE produce, but there is anecdotal evidence that these steps still occur in FSOs. Under ideal conditions, the microbial quality and "pathogen-free" status of fresh produce would be maintained from the farm gate to the consumer's plate. However, there are several indications that transportation, handling, and preparation practices can impact the levels of generic and pathogenic microbes on fresh produce. Other studies have reported that 55% of leafy green shipments to FSOs were received with product temperatures >5°C and 30% of those >7°C. These temperature abuse scenarios are well-known risk factors for bacterial growth resulting in both accelerated spoilage and increased pathogen levels in the presence of "decay juices" . Even though FDA recommends not rewashing RTE produce, FSO employees may observe these "decay juices", remove the unusable portion, and rewash the product to avoid food loss. There are a variety of standard operating procedures (SOP) employed by FSOs during preparation of fresh produce including the application of an antimicrobial treatment during batch washing or soaking. Since water washing alone can result in cross contamination of fresh produce, validating the effectiveness of a batch recirculating wash system with integrated ozone sanitizer for the inactivation of bacterial and viral pathogens on fresh produce commodities under ideal and "real world" scenarios would allow for implementation of best practices in FSOs; thus, preserving the microbial safety of fresh produce through the final steps in the supply chain.

Specific Aims:

1. Evaluate the efficacy of a batch wash ozone sanitation system (BWOSS) to inactivate viruses and bacteria on fresh produce.
2. Investigate the effectiveness of the BWOSS to improve the microbial quality and safety of fresh produce after temperature abuse and occurrence of decayed product.
3. Compare the BWOSS to currently used SOPs in FSOs established by preliminary survey data.

Relevant *Materials and Methods*: *(this information should be specific to the research project)*

The following fresh produce types will be inoculated with bacteria and viruses for evaluation of BWOSS efficacy: iceberg lettuce, butterhead lettuce, shredded iceberg lettuce, hearts of romaine, loose leaf lettuce, strawberries, cherry tomatoes, peppers, and culinary herbs such as cilantro.

The following microorganisms will be used: Escherichia coli C-3000, Salmonella Typhimurium LT2, Listeria innocua, Enterobacer cloacae, Bacillus cereus, MS2 bacteriophage, murine norovirus (MNV).

Bacteria cultures are prepared as follows:
Each bacteria will be recovered from 50% glycerol stock and streaked individually onto the appropriate media plate (TSA or BHI). Plates will be placed at 37C overnight for growth. A single CFU of each microbe from the plate will be selected and inoculated into 10mL of either TSB or BHI broth. The broth cultures will be incubated overnight at 37C with shaking at 125 rpm. Following overnight growth, 5mL of culture will be centrifuged at 5,000xg for 10 min in order to pellet the bacterial cells. The supernatant will be decanted and the pellet resuspended in 5mL of 1xPBS, vortexed, and centrifuged and repeated 2 more times for washing of bacterial cells with final resuspension in 5mL of buffered peptone water (BPW). The prepared bacteria will then be cultured to determine final concentrations and a cocktail will be prepared to give 10^6 CFU total of each bacteria in the cocktail.

Preparation of MS2 bacteriophage:
MS2 stock is produced by inoculating MS2 with a lawn of bacterial host, E. coli C-3000. MS2 is recovered using 1xPBS with vortexing followed by centrifugation to remove phage from bacterial cell lysate. MS2 concentration is determined by performing the double agar layer assay again using log phase E. coli C-3000 as the bacterial host. The prepared MS2 will be added to the cocktail at a concentration of 10^7 PFU total,

Preparation of MNV:
MNV stock is produced by infecting a murine macrophage cell line (RAW 264.7) at a 1:10 ratio. MNV is harvested from the cells via a freeze-thaw cycle followed by centrifugation to separate MNV from cell lysate. MNV concentration is determined by performing plaque assay using the RAW 264.7 cells. The prepared MS2 will be added to the cocktail at a concentration of 10^6 PFU total.

Treatment of produce in BWOSS and detection of microbes:
Produce will be spot inoculated with the 1mL total of the cocktail and placed in covered autoclavable pan at 4C overnight prior to treatment in BWOSS. Inoculated produce samples will be placed in BWOSS (already filled with water at appropriate level of ozone (~0.8ppm free ozone) and at a specific temperature). Produce will be submerged completed. Duplicate samples will be collected at specific time points over a 30 to 40 minute period. Samples will be place in stomacher bag containing 100mL of BPW, processed, and the eluate will be diluted in BPW for detection of microorganisms using the appropriate media and assay as described in the preparation of the inoculum.

** This information can be attached as an electronic document (Word format) or entered in the space provided (If you add an attachment, to your e-mail or FAX. Please indicate "see attached" and list the file name in the space provided):*

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name: (first and last) **- POSITION** (Title, academic degrees, certifications, and material field ofexpertise)	**QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE** Describe previous work or training with biohazardous and/or rDNA and include Biosafety Levels)
Example: Bob Biohazard - Associate Professor, PhD Microbiology	14 yrs working with *E. coli* at BSL1, *Salmonella enterica* at BSL2, 8 yrs working with transgenic mice.
Kristen Gibson - Associate Professor, PhD Environmental Health Sciences	16 years working with BL1 and BL2 organisms including viruses, bacteria, and protozoa
Cailin Dawley - MS student, BS Food Science	1.5 years working with BL1 and BL2 bacteria and viruses
Giselle Almeida - Program Technician, BS Biotechnology	20 years working with BL1 and BL2 bacteria and viruses and 5 years with cell culture

Additional Personnel Information (if needed):

List all the laboratories/facilities where research is to be conducted (specify building, room number and category for each **(e.g. laboratories, cold/warm rooms, animal care facilities or farms, growth chambers and greenhouses, biological material storage areas, tissue culture rooms.)**

	Building	Room Number	Category	New Biohazard Door Sign?
1.	BIOR	117	laboratory	☑ Yes
2.	BIOR	101	autoclave/biostorage	☑ Yes
3.				☐ Yes
4.				☐ Yes
5.				☐ Yes
6.				☐ Yes
7.				☐ Yes
8.				☐ Yes
9.				☐ Yes
10.				☐ Yes
11.				☐ Yes
12.				☐ Yes

Additional Laboratory/Facility Information (if needed):

* **Biohazard signs are required for entrances to Biosafety Level 2 areas (including Animal Biosafety Level 2 areas). The Office of Environmental Health & Safety will supply these signs.**

If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign in addition to what is being registered. Describe below:

SAFETY PROCEDURES:

Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures requiring handling or manipulation of the registered biological materials.

GLOVES:

☑ Latex ☐ Vinyl ☑ Nitrile ☐ Leather ☐ Other (specify) ☐

FACE & EYE PROTECTION:

☐ Face Shield ☐ Safety Goggles ☑ Safety Glasses ☐ Other (specify) ☐

CLOTHING PROTECTION:

☐ Disposable clothing protection
☑ Re-usable Lab Coat
☐ Re-usable Coverall
☐ Other (specify)

How will protective clothing be cleaned once dirty or contaminated? (Check all that apply)

☑ Autoclaved prior to laundering or disposal
☐ Laundered with qualified commercial service
☐ Laundered in on-site facilities with bleach
☐ Other (specify)

Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials (Autoclaving or fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify exceptions):

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into biohazard bags for autoclaving.

Describe waste disposal methods employed for all biological and recombinant materials used. (Please include methods involving the following types of waste)**

Sharps:	Policies and procedures will be instituted for handling and disposing of sharp objects (e.g. broken glass). Broken glassware is not handled directly but is removed by mechanical devices (eg. brooms, forceps) and placed in a
Cultures, stocks, and disposable labware:	All materials and disposables contaminated with pathogens will be disposed of into biohazard bags. The biohazard waste, bags, and glassware will be autoclaved at 121C and 15psi for 45 minutes by trained personnel
Pathological Waste:	Pathological waste will be collected in biohazard bags. Biohazard bags and contaminated glassware will be autoclaved at 121C and 15psi for 45 minutes
Other:	

** **For more information, please reference the Biological Safety Manual (click to view).**

Indicate autoclave location(s) used for waste disposal and describe autoclave validation procedures:

BIOR 101 autoclaves are checked monthly using SteriGage test strips (3M) and SporAmpule vials to ensure autoclaves completely sterilize all bacterial life forms including spores.

Will you be using a biological safety cabinet? *(choose one)* ☑ Yes ☐ No

If yes, please provide the following information:

SERIAL #	FAMA #	CERTIFICATION EXPIRATION DATE	LOCATION OF UNIT (Bldg./room)	MAKE/MODEL
SP7537V	UA011-B	1/17/19	BIOR 117	Baker/VBM600
104294-5978	UA009-B	1/17/19	BIOR 136	Thermo/1284
104294-5978	UA010-B	1/17/19	BIOR 136	Thermo/1371

Indicate if any of the following aerosol-producing procedures will occur:

- [x] Centrifuging
- [ ] Grinding
- [ ] Blending
- [x] Vigorous shaking or mixing
- [ ] Sonic disruption
- [x] Pipetting
- [ ] Dissection
- [x] Stomacher
- [ ] Inoculating animals intranasally

Other (please describe):

Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:

All pipetting of biological material will take place either in the BSC when in BIOR or using aseptic technique on the lab benchtop. A mechanical pipette aid will always be used to pipette. Lab coats buttoned over street clothes, gloves and glasses or goggles will be worn. Centrifuged cultures or viral suspensions will be contained in a closed centrifuge screw-capped polypropylene or polystyrene tube with gasket seals to prevent aerosol exposure. Cultures to be vortexed will be contained in screw-capped polypropylene or polystyrene tubes.

EMERGENCY PROCEDURES

In the event of **personnel** exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment.

In the event of accidental exposure of personnel the person exposed should notify the laboratory supervisor immediately. Treatable exposures will be treated by use of the first aid kit containing antimicrobial agents. Mucous membrane exposure or puncture with contaminated material will result in the person being taken to the Pat Walker Health Center for medical evaluation and treatment if necessary.

In the event of **environmental** contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.

For a spill inside the biological safety cabinet, alert nearby people and inform laboratory supervisor. The area will be secured to prevent spreading. Safety goggles, lab coat buttoned over street clothes and latex gloves should be worn during clean up. If there are any sharps they will be picked up with tongs, and the spill covered with paper towels. Carefully pour disinfectant (freshly made 10% bleach) around the edges of the spill, then into the spill without splashing. Let sit for 20 minutes. Use more paper towels to wipe up the spill working inward from the edge. Clean the area with fresh paper towels soaked in disinfectant. Place all contaminated towels in a biohazard bag for autoclaving. Remove personal protective clothing and wash hands thoroughly.
For a spill in the centrifuge turnoff motor, allow the machine to be at rest for 30 minutes before opening. If breakage is discovered after the machine has stopped, reclose the lid immediately and allow the unit to be at rest for 30 minutes. Unplug centrifuge before initiating clean up. Wear strong, thick rubber gloves and other personal protective equipment (PPE) such as gloves, safety goggles, N95 respirator mask, coat, etc., before proceeding with clean up. Flood centrifuge bowl with disinfectant. Place paper towels soaked in a disinfectant over the entire spill area. Allow 20 minute contact time. Use forceps to remove broken tubes and fragments. Place them in a sharps container for autoclaving and disposal as infectious waste. Remove buckets, trunnions and rotor and place in disinfectant for 24 hours or autoclave.
Unbroken, capped tubes may be placed in disinfectant and recovered after 20 minute contact time or autoclaved. Use mechanical means to remove remaining disinfectant soaked materials from centrifuge bowl and discard as infectious waste. Place paper towels soaked in a disinfectant in the centrifuge bowl and allow it to soak overnight, wipe down again with disinfectant, wash with water and dry. Discard disinfectant soaked materials as infectious waste. Remove protective clothing used during cleanup and place in a biohazard bag for autoclaving. Wash hands whenever gloves are removed.
For a spill outside the biological safety cabinet or centrifuge have all laboratory personnel evacuate. Close the doors and use clean up procedures as above.

TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS

As per the Department of Transportation **49 CFR Parts 171-173** (5), some biological materials are regulated as hazardous materials and require special training of all personnel involved in shipping.
Will you be transporting or shipping any of the following off campus? *(Yes* ***or*** *No)*

☐ Yes ☑ No

If yes, check all that apply.

☐ Cultures of human or animal pathogens

☐ Environmental samples known or suspected to contain a human or animal pathogen

☐ Human or animal material (including excreta, secreta, blood and its components, tissue or tissue fluids, and cell lines) containing or suspected of containing a human or animal pathogen

Have you or anyone in your lab involved in packaging, labeling, or completing/signing paper work received training to ship infectious substances or diagnostic specimens within the past three years?

☐ Yes

☐ No

If yes, please provide the following information.

Name	Date Trained	Certified Shipping Trainer

REFERENCES (click to view)

1. Office of Research Compliance
2. Biosafety in Microbiological and Biomedical Laboratories (BMBL) - 5th ed. CDC and NIH
3. NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines, April 2016)
4. University of Arkansas Office of Environmental Health and Safety
5. Department of Transportation - Hazardous Materials: Standards for Infectious Substances