Understanding foodborne pathogens and indicators from field to fork

by

Savannah C. Stewart

B.S., Kansas State University, 2016
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Food Science Institute
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Abstract

The global food supply is a complex network of interconnected industries, and opportunities for the introduction of foodborne pathogens are abundant throughout. While there is a fair amount of information available for some sectors of the food industry surrounding risk and mitigation techniques such as cleaning and sanitation standards, there is a lack of knowledge within industries that utilize or yield minimally-processed products. The purpose of this work is to generate a better understanding of potential routes that foodborne pathogens can take throughout the field-to-fork continuum as well as how different factors such as surface type, environmental conditions, and sanitizer type can impact how pathogen populations are able to survive.

In order to better understand resident bacteria within from Midwest feed mills, a multi-season survey of swine feed mills identified 121 resident isolates within the *Enterobacteriaceae*, *Pseudomonadaceae*, and *Enterococcoccaceae* families; a majority of isolates were identified in environmental samples from feed contact surfaces (32%, 37/121), non-feed contact surfaces (41%, 48/121), transient surfaces like brooms or workers' shoes (17%, 20/121), with the remaining 10% (12/121) identified directly from finished feed. The isolates were also analyzed for the presence of antimicrobial resistance genes (ARG) and metal tolerance genes (MTG).

To evaluate the efficacy of multiple commercially available sanitizers when used for the control of mature biofilms on surfaces commonly found within the tree-fruit harvesting process, multi-strain *E. coli* or *L. monocytogenes* biofilms were grown for 96 hours in a Centers for Disease Control (CDC) reactor at 25± 2˚C on high density polyethylene (HDPE), wood, or nylon. Bacteria were exposed to chlorine (500 ppm), peroxyacetic acid (PAA) (500 ppm), steam (75 psi), or silver-dihydrogen citrate (SDC) (4%) for 1 or 2 minutes; or chlorine dioxide gas

(ClO_2) (100 ppm) for 24 hours. Results were considered significant at $P < 0.05$ for both *E. coli* and *L. monocytogenes* Interactions between sanitizer, surface type, and exposure time were found to be significant, and all sanitizers with the exception of SDC were able to reduce population on all surfaces. ClO_2 was overall the most effective method of managing biofilm populations grown on all three surfaces.

After laboratory testing, a better understanding of how differing environmental conditions can impact sanitizer efficacy was needed. To do this, SDC and ClO_2 gas were tested against *E. coli* and *Listeria innocua* that was experimentally inoculated harvesting equipment at commercial orchards within the Midwest and Pacific Northwest (PNW) regions of the United States. Rifampicin resistant *E. coli* and *Listeria* were grown for either 24-hours (sessile form) or 96-hours (biofilm form) at 25± 2˚C on 10 cm^2 HDPE, wood, or nylon coupons. Surfaces were allowed to dry for one hour and then exposed to ClO_2 (100 ppm) for 24-hours or SDC (4%) for 2 minutes. Results were significant at $P < 0.05$. ClO_2 was the most effective treatment ($P < 0.05$) in controlling sessile *E. coli* and *Listeria* on HDPE and nylon in the Midwest and PNW. A lower level of inactivation was observed for biofilms grown on wood after ClO_2 treatment ($P < 0.05$). SDC did not reduce the population of sessile *E. coli* on HDPE in the PNW region. Neither *E. coli* bacterial forms were significantly reduced ($P > 0.05$) by SDS when grown on nylon, independently of the region. Conversely, biofilm population was reduced on HDPE after SDS exposure in both regions and bacteria tested ($P < 0.05$). Bacterial populations grown on wood surfaces were not reduced after exposure to SDC, regardless of form or bacteria type ($P > 0.05$).

Since differences in sanitizer impact on surviving biofilm populations were observed in the previous studies, and in order to better understand the relationship between sanitizer-induced stress and gene expression, analysis was performed on differentially expressed genes (DEGs) in

multi-strain *L. monocytogenes* biofilms after exposure to sub-lethal levels of chlorine, PAA, SDC, and lactic acid (LA) for 15 seconds. Biofilm-dwelling *L. monocytogenes* cells exhibited different transcriptome profiles in response to various sanitizers when compared to untreated biofilms. Few DEGs were noted in either the biofilms exposed to SDC and LA, indicating there was almost no change in the *L. monocytogenes* transcriptome. Exposure to PAA and chlorine caused relatively high levels of DEGs in *L. monocytogenes*, with 65 DEGs and 100 DEGs , respectively. Functional enrichment analysis found a 13-gene functional annotation cluster involved in the regulation of DNA-templated transcription was significantly regulated (*Q*-value = 0.0049) after exposure to chlorine. Both PAA and chlorine, which are categorized as oxidative sanitizers, had limited overlap in gene response. Only 10% (1/10) of the DEGs that were downregulated and 22% (28/126) of the upregulated DEGs were shared between the biofilms exposed to either.

The results obtained from these data underscore the importance of understanding how different surface types, sanitizers, and environments all should be considered when attempting to characterize risk within the food supply chain. The presence of resident bacteria within facilities could enable further transfer of ARGs and MTGs, which subsequently can impact both medical treatments for disease caused by pathogenic bacteria, as well as future developments with antimicrobial agents and sanitizers. Surface type, in addition to sanitizer type, can result in significant differences in how effective an antimicrobial is at managing bacterial populations. Further research is needed to better understand how differences with bacterial form, surface type, and how exposure to stress from sanitizer applications can impact bacterial populations within facilities that are harvesting and handling minimally processed products.

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Major Professor
Valentina Trinetta

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Dedication

To my family, past, present, and future.

Chapter 1 – Literature Review

There is a wide variety of bacteria that can be present within food manufacturing environments and even food products, some of which are even considered essential for the production of items like kimchi, yogurt, and some cheeses (Heller, 2001; Lee et al., 2020). However, not all bacteria found within food is beneficial- the presence of bacteria such as *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* can result in foodborne illness. Worldwide, it is estimated that bacteria are responsible for almost 350 million cases of foodborne illnesses annually (World Health Organization (WHO), 2024). Foodborne pathogens (including *L. monocytogenes* and *E. coli*) are considered ubiquitous in nature (Moshtaghi, Garg, & Mandokhot, 2003; Muniesa et al., 2006; Vivant, Garmyn, & Piveteau, 2013), so controlling exposure within the food supply chain can be challenging, especially within products and facilities that do not require extensive amounts of further processing or sanitation practices. Food systems have many interconnecting parts, and a variety of factors must be taken into consideration when attempting to understand the route that foodborne pathogens can take when entering into the food supply chain.

1.1 The role of bacteria as indicator organisms

Within the food industry, a variety of organisms are tested as indicators of either quality or safety concerns. Often referred to as "indicator" organisms, these tests allow for monitoring of potentially detrimental quality conditions or increased likelihood of pathogen presence that would impact food safety, without the need for highly specific testing. Indicator organisms that are commonly used within food and drink manufacturing include: aerobic plate count, coliforms, generic *Escherichia coli*, *Enterobacteriaceae*, yeast and molds, and generic *Listeria* spp.

(Tortorello, 2003). The use of indicator organisms is popular due to the low barrier to entry; methods are available that do not require extensive employee training, costly supplies, or the use of laboratory equipment (Rochell-Newall et al., 2015).

As previously stated, there is a variety of indicator organisms that can be utilized. Total coliform count is one method of testing for potential safety issues using indicator organisms. Public water systems routinely use the presence of members of the *Enterobacterales* order to test the likelihood of fecal contamination, utilizing measurements for total coliforms and generic *E. coli* (40 CFR Parts 141, 142). While they may not detect all bacteria present and can lack specificity, the use of these organisms as indicators of potential presence of fecal contamination has gained popularity as a method to evaluate hygiene and sanitation across multiple environments, including water systems (Elmund et al., 1999; Motlag & Yang, 2019), food manufacturing and handling facilities (Marriott et al., 2018) and even within feed manufacturing facilities (Stewart et al., 2020). There are a variety of rapid tests on the market for these organisms, which can help inform facility practices without having to test for specific pathogenic bacteria.

The *Enterobacteriaceae* family consists of Gram-negative, non-spore-forming bacteria, and is considered to be one of the most significant groupings of foodborne pathogens in terms of associated illnesses, with the inclusion of *Salmonella*, *E. coli*, and *Shigella* (Janda & Abbot, 2021). Many of the species naturally occur in a wide variety of environments. While not all members are typically associated with foodborne illness, monitoring of *Enterobacteriaceae* as a whole can be used within food handling facilities to measure postprocessing contamination (National Research Council (NRC), 1985). Testing for generic *E. coli* is also one method of utilizing *Enterobacteriaceae* as indicator organisms; it can be a more specific indicator of fecal

contamination compared to coliform testing (Motlag & Yang, 2019) and is currently used within the meat processing industry in the United States (9 CFR part 310 and 381) and for monitoring of water sources on produce farms (21 CFR Part 112). There is some ambiguity surrounding it's use as accurate indicator organism within water or processing facilities due to the ability to persist in a variety of environments and animals (Byppanahalli et al., 2003; van Elsas et al., 2011; Oliveira, Freire, & Pedroso, 2018).

1.2 The potential role of animal feed as a vehicle of transmission in the food supply chain

Feed mills, which produce animal feed for livestock such as poultry, swine, and cattle, are typically less controlled environments than facilities manufacturing human food, and little is known about how residential bacteria that could enter into the food supply via consumption by said livestock. Previous studies have characterized the presence of *Listeria*, *Salmonella* and *E. coli* within feed manufacturing environments (Whyte, McGill, & Collins, 2003; Torres et al., 2011; Magossi et al., 2020; Schwan et al., 2023), as well as characterized the presence of antimicrobial resistance (AMR) within isolates from feed mills (Hsieh et al., 2016; Schwan et al., 2023). While a solid connection between the presence of foodborne pathogens within animal feed and human infection, similar genetic profile between clinical isolates obtained from humans and isolates found in swine feed indicate that feed may serve as a method of introducing foodborne pathogens into the human food supply (Harrison et al., 2022). Both pathogenic bacteria and viruses impacting animal health have been shown to be present and able to circulate within feed manufacturing facilities (Pasik et al., 2014; Stewart et al., 2020), as well as transferred from feed mills to food animals, which can increase risk of contamination during

processing of that animal. Feed manufacturing facilities have constraints surrounding cleaning and sanitation inherently due to the types of processes occurring; this can lead to harborage of bacteria. The relationship between microbial presence within feed manufacturing facilities and the human food supply is complex, and presence of bacteria within a feed mill may not correlate to infection of livestock, or subsequently humans (Olson et al., 2023). Having a broader understanding of the microbial populations within feed manufacturing facilities, as well as how foodborne pathogens or bacteria with resistance genes can be transferred to other parts of the food chain could help lead to better understanding of risk further down the food-to-fork continuum.

1.3 The role of antimicrobial resistance and metal resistance in food safety

With the rise in awareness of antimicrobial resistance (AMR) within environmental bacteria populations, increased scrutiny has been placed on industries involved in all aspects of the food chain. AMR is considered to be a high-priority issue by multiple global health groups, including the World Health organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and World Organization for Animal Health (WHO, 2023). The presence of AMR bacteria can impact the ability to treat bacterial diseases (WHO, 2015). The presence of multi-drug resistance (MDR), which is typically defined as having resistance to more than one antimicrobial agent across three separate classes (Magiorakos et al., 2012), can make treatment of infection or disease even more complicated. While bacteria can be intrinsically, or inherently, resistant to some antimicrobials, acquired (extrinsic) resistance is typically blamed for AMR (Vats, Kaur, & Rishi, 2022). The presence of antimicrobial resistance genes (ARG) within non-pathogenic microbiota that share an environment with pathogenic bacteria can result in the

acquisition of resistance genes (Iwu, Korsten, & Okoh, 2020). Therefore, understanding what ARGs are present in environments with bacteria that can cause foodborne illness is important, especially throughout the many varied environments that make up the global food supply chain.

Multiple studies have shown the potential for ARGs to be present within bacteria found in animal-derived food products (Miranda et al., 2003; Bosco et al., 2012; Fakruddin et al., 2013; Li et al., 2016; Ramadan et al., 2020), and the use of prophylactic use of antimicrobials in animal feed may result in increased levels of AMR bacteria within feed manufacturing facilities, and subsequently diets of livestock (Brown et al., 2019). This can lead to further dissemination of ARGs across species as a result of horizontal or vertical gene transfer within bacterial populations circulating throughout the environment (da Costa, Loureiro, & Matos, 2013). With the rise in AMR and MDR bacteria, there has been a need to identify alternative antimicrobials; this has led to a growing interest in the use of certain metals and metal derivatives as antibacterial agents (Frei et al., 2023). While there is some promise in this approach, the presence of metal tolerance genes (MTGs) within bacterial populations has also been brought into focus. There is some evidence that the presence of MTGs can result in co-selection and cross-resistance to antimicrobials (Pal et al., 2017). The strong correlation between the presence of specific MTGs and ARGs identified within isolates obtained from groves (Glibota et al., 2020) further supports the link between MTG and ARG presence in agricultural settings. Further research characterizing the presence of MTG and ARG within the food chain could lead to a better understanding of what industries are at risk for furthering ARG presence.

1.4 Escherichia coli and Listeria monocytogenes: long road from field to fork

Since 2011, numerous outbreaks of foodborne pathogens like Shiga toxin-producing *E. coli* (STEC) or *Listeria monocytogenes* have been linked to fresh or minimally processed

produce such as leafy greens, melons, and apples (Table 1) (Centers for Disease Control (CDC)). Since most produce is minimally processed prior to consumption, foodborne pathogen contamination can be difficult to mitigate. STEC can be found in the intestinal tracts of ruminants (Hussein & Bollinger, 2005), but can also survive in soil and water, which can serve as potential routes of contamination (Fujioka et al., 1998; Truchado et al., 2018**)** .

Both *E. coli* and *L. monocytogenes* are considered ubiquitous within the environment. *L. monocytogenes*, a Gram-positive, rod-shaped bacteria, can be present in soil and vegetation found in agricultural settings and is able to survive and grow in a variety of temperature ranges, which can result in harborage within food production facilities (Osek, Lachtara, & Wieczorek, 2022). Listeriosis, the disease caused by *L. monocytogenes*, primarily presents in pregnant women, individuals with weakened immune systems, and neonates and has a wide range of symptoms depending on severity. Severe listeriosis infections are often characterized by symptoms associated with the central nervous system, and average case-fatality rate of listeriosis is between 20-30% (Swaminathan & Gerner-Smidt, 2007).

Like *L. monocytogenes*, STEC can be found within the environment, primarily within the gastrointestinal tracts of ruminant animals (Farrokh et al., 2013). STEC, like all *E. coli* are Gram-negative, rod-shaped member of the *Enterobacteriaceae* family. Strains of *E. coli* are characterized by their O and H antigens, which correspond to lipopolysaccharide and flagellin, respectively (Kaper, Nataro, & Mobley, 2004). There are many STEC serogroups, but O157 is the most commonly identified cause of infection within the United States (Tack et al., 2021). Severe STEC infections can lead to the development of hemolytic uremic syndrome, which is life-threatening (Karch et al., 1999).

Previous outbreak investigations involving extensive sampling have been able to link contaminated irrigation water to STEC outbreaks (CDC, 2018; CDC, 2019), however it can often be difficult to pinpoint how groundwater or irrigation water gets contaminated due to the ubiquitous nature of foodborne pathogens, especially in areas with wild and domestic ruminant animals. Previous studies have shown that contaminated soil, use of contaminated water for irrigation or washing, improper or misuse of organic fertilizers (manure), or contamination from workers during picking and packing are all potential routes of foodborne pathogen contamination for produce (Kowalska, 2023**;** Luna-Guevara et al., 2019). Like STEC, *L. monocytogenes* has been found in surface water samples on-farm (Townsend et al., 2021), further illustrating the need for farms and producers to be cognizant of water sources. In addition to potential exposure via contaminated water or soil, airborne particulate transmission, and insect transmission of STEC has been shown to be possible (Janisiewicz et al., 1999; Wasala et al., 2013**;** Berry et al., 2015, Berry et al., 2019).

1.5 The apple industry within the United States and related foodborne pathogen outbreaks

Within the United States, approximately 5 million tons of apples are grown, picked, and harvested annually, with primary growing regions including the states of Washington, New York, Michigan, Pennsylvania, and California (United States Department of Agriculture (USDA), 2024). There are over 26,000 farms nationwide, representing over 297,000 acres of land use (USApple, 2023; USDA, 2024). While many STEC and *L. monocytogenes* outbreaks are associated with other foods, there is a history of outbreaks associated with apple products, including apple cider and caramel apples (CDC, 1996; CDC, 1997**;** CDC, 2015; FDA, 2019). Between 2001-2024, there was a total of 11 *E. coli* and two *L. monocytogenes* outbreaks

associated with the consumption of apples or unpasteurized apple products (CDC). While not traditionally associated with foodborne pathogens, these outbreaks indicate that tree fruit and minimally processed byproducts should be considered at risk for pathogens associated with foodborne illness.

Recently, there has been an increase in concern surrounding foodborne pathogens presence in facilities that are part of the harvesting, packing, and storage processes for produce. For example, *Listeria* is able to grow at temperatures that are found in cold storage or packing houses, which could create reservoirs and increase the risk of contamination (Estrada et al., 2020; Chen et al., 2022). Due to common handling and storage practices within facilities involved in the produce supply chain, it can be difficult to maintain consistent cleaning and sanitation schedules for equipment and storage containers. Handling of produce during the harvesting and processing has been shown to increase the likelihood of contamination with foodborne pathogens (Bartz et al., 2017), which is of concern when the produce is typically not exposed to further heat processing prior to consumption. In addition to the risks associated with the harvesting process, many apple growers and packers utilize picking and storage materials such as nylon and wood, which are considered to be porous. There is little information available about the efficacy of different types of sanitizers on such surfaces, despite the environments within tree fruit harvesting and storage facilities having been identified as ideal for bacterial growth (Killinger & Adhikari, 2014).

1.6 Biofilms

Biofilms, which are communities of bacteria that have irreversibly attached to a surface while having formed a protective outer layer made up of extracellular polymeric substance (EPS) (Donlan, 2002), can form and survive on a wide range of surfaces and environments**.** The EPS,

which is composed of a combination of lipids, proteins, polysaccharides, and nucleic acids such as extracellular DNA (Di Martio, 2018) has been shown to act as a protective barrier for the underlying bacteria, and can improve survival after exposure to sanitizers (Chavant et al., 2014), and more mature biofilms may have even higher levels of resistance (Thuptimdang et al., 2015; Kim, Jyung, & Kang, 2022) . Biofilms are also characterized by having altered growth and gene transcription compared to sessile counterparts (Donlan & Costerton, 2002). Biofilm growth is typically marked with multiple phases that are cyclic; initial attachment of the bacteria to a surface, growth and accumulation of the EPS and other microorganisms, and final disbursement or detachment (Sauer et al., 2022).

Initial attachment of the bacteria to the surface (and continued attachment) is fairly dependent upon the physiochemical properties of the surface, as well as environmental factors (Srey, Jahid, & Ha, 2013). It should be noted that biofilms have been shown to be more likely to form when exposed to environments that are deficient in some aspect, instead of environments considered adequate for the bacteria present. Temperature conditions can also influence the speed of maturation of a biofilm (Kim, Jyung, & Kang, 2022); ideal formation temperatures are dependent on the bacteria present.

Within biofilms, proteins are often in charge of mediating interactions between different components, including between cells as well as between cells and the surrounding biofilm and may influence overall adhesion and formation (Latasa et al., 2006, Payne & Boles, 2015). Proteins can also regulate cell functions such as transcription and translation by binding genetic material present within the matrix (Martinez & Vadyvaloo, 2014). One important protein component of many bacterial biofilms are curli, an amyloid structure that helps with a variety of functions including attachment, cell aggregation, invasion, and motility of bacteria (Barhnart &

Chapman, 2006). Environmental conditions can impact the formation of curli due to changes in gene expression (Gualdi et al., 2008).

In addition to proteins, polysaccharides play an important part in the formation and persistence of a biofilm. Polysaccharides are a structural component of the matrix, both when attached to bacterial cells and without attachment, and their synthesis and presence in biofilms is believed to be influenced by the specific bacterial strains present as well as environmental conditions (Payne & Boles, 2015; Zogaj et al., 2001; Serra, Richter, & Hengge, 2013). Exopolysaccharides are able to help provide mechanical stability, as well as retain moisture and impact nutrient availability (Yaron & Römling, 2014). The presence of polysaccharides, like cellulose, have also been shown to impact the ability of a biofilm to survive thermal and chemical stressors (Kim, Jyung, & Kang, 2022). Lipids play an important role in biofilms by providing additional levels of protection to the bacteria within when exposed to environmental stressors or antimicrobial agents compared to planktonic bacteria. Some types of bacteria have been shown to increase the concentration of saturated fatty acids within biofilm cells compared to planktonic forms (Dubois-Brissonnet, Trotier, & Briandet, 2016).

There are a variety of physiochemical properties that can impact how biofilms form, including things like surface properties as well as properties of the bacteria themselves. More and more research is being done to better understand how surface characteristics influence how bacteria start and maintain biofilm formation; the general conclusion across literature is that the interactions between different properties is complex, and the wide range of potential variables can lead to varied results. While some surface properties such as hydrophobicity and charge can greatly influence the initial rate of adhesion, the environmental conditions and interactions between bacterial cells can change the formation rate as well. A good amount of research has

been done on how surface hydrophobicity impacts cell adhesion. Some studies have indicated that surfaces that are hydrophilic have higher levels of adhesion than hydrophobic surfaces (Hemmatian, Lee, & Kim, 2021; Zhang et al., 2023), while others have shown contrasting or inconclusive results (Cerca et al., 2005; Silva et al., 2007). Charge density of the surface has been shown to impact bacterial adhesion in addition to hydrophobicity (Silva et al., 2007; Zhang et al., 2023). Charges and hydrophobic characteristics of the bacteria themselves may also impact the continued adhesion, as well as the rate at which the biofilm fully matures. Surface texture has also been shown to factor into biofilm adhesion. Surfaces with rougher, more "rugged" textures or surfaces that are more porous allow for better adhesion (Hemmatian et al., 2021; Zhang et al., 2023).

With the unique challenges and potentially inconsistent cleaning and sanitation practices for harvesting and storage equipment that are found within the tree fruit industry, formation of pathogenic biofilms could occur. Many of the surfaces that are commonly found within the picking and harvesting process, such as wood, nylon, and high-density polyethylene (HDPE) are more porous than surfaces that sanitizers are validated with. Such surfaces have been shown to be ideal for bacterial growth (Killinger & Adhikari, 2014; Haddad et al., 2021). Understanding how pathogens responsible for foodborne illnesses form biofilms, as well as how biofilm presence impacts sanitizer efficacy can help inform sanitation recommendations for facilities harvesting and handling produce. As stated above, the formation of biofilms has been shown to impact gene expression of the communal bacteria, as well alter the levels of resistance to sanitizers compared to sessile or planktonic bacteria. Characterization of the gene expression within biofilms can be done via transcriptome analysis, which identifies levels of gene expression. This facilitates a better understanding of gene regulation at the transcription level,

and can be used to identify resistance traits and characterize responses to certain conditions, such as stress induced from sanitizer exposure.

Understanding how sanitizer-induced stress impacts regulation of genes can help lead to a more comprehensive understanding of how foodborne pathogens respond to different antimicrobials; currently, little information is available on how gene expression within *L. monocytogenes* biofilms can be impacted by sanitizer-induced stress. Some evidence that sublethal exposure to sanitizers will impact gene expression (Kastbjerg et al., 2010; Kokkoni et al., 2021). Identification of potential differences between stress responses in biofilms could help inform sanitizer use in food handling facilities.

1.7 Cleaning and sanitation within the produce industry

After the introduction of the Food Safety Modernization Act (FSMA) and subsequent introduction of the Produce Safety Rule (PSR) (formally the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; 21 CFR 112) produce handling facilities were required to maintain certain levels of cleaning and sanitation. Improper sanitation practices have been potentially linked to foodborne disease outbreaks (Bartz et al., 2017; Angelo et al., 2017), and understanding how effective different sanitizers are against bacteria like *E. coli* and *L. monocytogenes* in unique environments like harvesting and packinghouses is essential.

Cleaning, or the physical removal of residues, dirt, and debris, is required prior to sanitation within food handling environments. While there are specific guidelines in place for different commodities and growing systems within the United States, the PSR provides baseline requirements, which include cleaning for both food contact and non-food contact surfaces. After

cleaning, sanitization is used to reduce the number of bacteria present on a given surface. It is important for facilities to fully evaluate and understand the unique risks associated with all aspects of their process when determining cleaning and sanitation procedures (Brackett, 1992). As previously stated, harvesting facilities and packinghouses within the fresh produce industry present challenges; while there is a wide variety of sanitizers approved for use within the fresh produce industry, there are limitations on efficacy that should be considered. The active ingredients and ideal application conditions of various sanitizers (Table 2), as well as potential interactions between chemicals and compounds found throughout the produce handling process should be taken into account.

Quaternary ammonium compounds (QAC) are commonly used sanitizers within the produce industry, but have been shown to be ineffective against biofilms grown on irregular food contact surfaces (Hua et al., 2019). Several oxidizing sanitizers, such as chlorine bleach, chlorine dioxide (ClO_2) gas, and peracetic acid (PAA), are approved for use within produce handling facilities. Oxidative sanitizers function on the general principle of disrupting the cell membrane via oxidation, which leads to structural changes and eventual cell death (Russel, 2003). While there is evidence that sanitizers like PAA can be used in the presence of liquids (Dery et al., 2021**,** Silva & Sabogal-Paz, 2021), there is little information comparing efficacy on wet or damp versus dry surfaces, which should be considered in the use case of a facility that wet cleans prior to sanitation. The type and physical characteristics of the surface to be treated also impact sanitizer efficacy (Yang et al., 2009; Bang et al., 2014; Park & Kang, 2017; Manville et al., 2023) with some evidence showing that PAA and ClO_2 are less impacted by surface type than other sanitizers (Hua et al., 2019).

The use of ClO_2 gas to reduce bacterial populations on both the surfaces of fresh produce has been extensively studied (Han et al., 2000; Du, Han, & Linton, 2002; Sy et al., 2005), as well as on a variety of surface types (Vaid et al., 2010; Trinetta et al., 2012; Kim & Park, 2022). While it consistently has shown a reduction in bacterial population present regardless of surface, there is an indication that relative humidity can impact efficacy (Kim & Park, 2022). Compared to other oxidative sanitizers, the gaseous form of ClO_2 may allow for better penetration of EPS when used to mitigate biofilm populations (Prodduk et al., 2014).

While oxidative sanitizers are commonly used in food processing environments, they can be harsh on equipment, and some may leave residual chemicals. The use of alternative chemical sanitizers, as well as non-chemical sanitizers to mitigate bacterial pathogens is also an option. One alternative sanitizer that has been successfully shown to reduce viral pathogens in a variety of environments is silver dihydrogen citrate (SDC) (Manuel, Moore, & Jaykus, 2017; Buckley et al., 2018). Recently, SDC has been investigated against bacterial pathogens, and has been shown to be able to significantly reduce populations of *E. coli* and *Listeria* when used alone (Masuku et al., 2014), or in combination with other sanitizers (Mendes-Oliveira et al., 2022**;** Manville et al., 2023). Steam has also been considered as an alternative to chemical sanitizers, working under the principle of thermal inactivation. Steam has been shown to be effective against both *Listeria* and *E. coli* biofilms on a variety of surfaces (Ban et al., 2014; Hua et al., 2021; Park & Kang, 2014) and may enhance chemical sanitizer efficacy when used in combination (Ban & Kang, 2016).

Table 1.1. **Produce-associated Shiga toxin-producing *E. coli* (STEC) and *L. monocytogenes* outbreaks within the United States, 2011-2024**

Pathogen	Year	Source	Illnesses	Hospitalizations	Deaths
L. monocytogenes	2011	Cantaloupes	147	143	33
L. monocytogenes	2014	Bean Sprouts	5	5	2

L. monocytogenes	2014	Caramel apples	35	34	7
L. monocytogenes	2016	Frozen vegetables	9	9	3
L. monocytogenes	2016	Packaged salads	19	19	1
L. monocytogenes	2017	Caramel apples	3	3	0
L. monocytogenes	2020	Enoki mushrooms	36	31	4
L. monocytogenes	2021	Packaged salads	10	10	1
L. monocytogenes	2021	Packaged salads	18	16	3
L. monocytogenes	2022	Enoki Mushrooms	5	5	0
L. monocytogenes	2023	Leafy greens	19	18	0
L. monocytogenes	2023	Peaches, nectarines, and plums	11	10	1
STEC	2011	Fruit	6	1	0
STEC	2011	Strawberries	15	7	2
STEC	2011	Apple cider, unpasteurized	14	0	0
STEC	2011	Romaine lettuce	60	35	0
STEC	2011	Lettuce	26	5	0
STEC	2011	Clover sprouts	29	7	0
STEC	2012	Vegetable-based salads	12	1	0
STEC	2012	Salads	9	7	0
STEC	2012	Apple cider, unpasteurized	3	0	0
STEC	2012	Leaf lettuce	8	4	0
STEC	2012	Prepackaged leafy greens	33	13	0
STEC	2012	Lettuce	16	6	0
STEC	2012	Romaine lettuce	52	-	-
STEC	2012	Leaf lettuce	24	-	-
STEC	2012	Spinach	10	-	0
STEC	2013	Salad	20	7	0
STEC	2013	Lettuce-based salads	6	4	0
STEC	2013	Prepackaged leafy greens	14	9	1
STEC	2013	Lettuce	94	22	0
STEC	2013	Kale	7	5	0
STEC	2013	Romaine lettuce	33	9	0
STEC	2013	Lettuce	9	8	0
STEC	2013	Green leaf lettuce	5	5	0
STEC	2013	Green beans	8	1	0
STEC	2013	Lettuce	26	5	0
STEC	2014	Spinach	4	1	0
STEC	2014	Clover sprouts	19	5	0
STEC	2014	Prepackaged salad	16	13	0
STEC	2014	Salad	11	2	0
STEC	2014	Cabbage	16	2	0

STEC	2015	Prepackaged leafy greens	7	5	0
STEC	2015	Romaine lettuce	16	10	0
STEC	2015	Apple cider, unpasteurized	2	1	0
STEC	2015	Apple cider, unpasteurized	2	0	0
STEC	2015	Prepackaged salad	5	3	0
STEC	2015	Apple cider	15	1	0
STEC	2016	Alfalfa sprouts	11	2	0
STEC	2016	Salad	11	4	0
STEC	2016	Iceberg lettuce	11	4	0
STEC	2016	Apple cider	56	10	0
STEC	2016	Cilantro	96	19	0
STEC	2017	Spinach	8	3	0
STEC	2017	Mixed green salad	3	3	0
STEC	2017	Spring salad; mizuna	68	18	0
STEC	2017	Leafy greens	25	9	1
STEC	2018	Romaine lettuce	10	5	0
STEC	2018	Romaine lettuce	239	104	5
STEC	2018	Menu items w/ lettuce	64	1	0
STEC	2018	Leafy greens	25	8	0
STEC	2018	Romaine lettuce	62	25	0
STEC	2018	Lettuce	30	0	0
STEC	2018	Iceberg lettuce	22	10	0
STEC	2019	Salad	4	3	0
STEC	2019	Romaine lettuce	23	11	0
STEC	2019	Romaine lettuce	15	3	0
STEC	2019	Spinach	10	3	0
STEC	2019	Prepackaged salad mix	6	0	0
STEC	2019	Clover sprouts	23	1	0
STEC	2019	Romaine lettuce	172	88	0
STEC	2019	Leafy greens	24	7	0
STEC	2019	Prepackaged salad	11	4	0
STEC	2019	Romaine lettuce	21	7	0
STEC	2019	Iceberg lettuce	11	5	0
STEC	2020	Leafy greens	40	20	0
STEC	2020	Clover sprouts	51	3	0
STEC	2020	Leafy greens	40	4	0
STEC	2021	Packaged salads	10	4	0
STEC	2021	Spinach	15	4	0
STEC	2022	Romaine lettuce	109	52	0
STEC	2024	Organic walnuts	13	7	0

Table 1.2. **Information on selected sanitizers.**

Sanitizer	**Active compound**	**Ideal pH application range**	**Mode of action**
Sodium hypochlorite (chlorine bleach)	Sodium hypochlorite (3-7%) Sodium chlorine (1-5%) Sodium hydroxide (>0.1-<1%)	6.5-7.0	Oxidation
Chlorine dioxide (ClO_2) gas	Sodium Chlorite, Chlorous Acid, Sodium Salt, Active Sodium Chlorite (1-15%)	N/A	Oxidation
Peracetic acid (PAA)	Hydrogen peroxide (21.5-22.5%) Acetic acid (15.8-16.6%) Peroxyacetic acid (14.8-15.7%)	3.0-7.5	Oxidation
Silver dihydrogen citrate (SDC)	4.85% citric acid 0.003% silver	< 4.0	Disruption of cellular membrane and inhibition of division
Steam	water	N/A	Thermal

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Chapter 2 – Research Importance

Currently, there is a gap surrounding underestimated risks that contribute to the food safety of the food supply chain from field to fork. Little information is available about the bacteria that can be found within the feed manufacturing facilities, and understanding what type of bacteria, as well as the gene expression characteristics, can be beneficial to prevent possible contamination and prevent potential entry routes for pathogens. Additionally, characterizing the presence antimicrobial resistance (AMR) and metal tolerance genes (MTG) found within resident bacteria can help obtain a holistic understanding of what resistance is present and has the potential to enter into the food chain, even in bacterial populations that are not considered to cause foodborne illness. Among the top foodborne pathogens threats in the United States, *Escherichia coli* and *Listeria monocytogenes* are considered of high concern within the food supply chain, especially within the produce industry, since products are minimally processed by the end consumers. Both bacteria are able to form biofilms, and understanding how the surfaces commonly found within harvesting and packing facilities can impact biofilm formation and sanitizer efficacy is important for ensuring effective food safety practices. There is a lack of information surrounding how different sanitizers could impact any remaining bacterial cells after exposure, or what changes in gene expression could result. Understanding how gene expression within surviving populations of *L. monocytogenes* biofilms change after exposure to commercially used sanitizers may help better understand sanitizer efficacy.

Therefore, the main objectives of this research were to:

1. Characterize resident *Enterobacterales* collected during sampling surveys conducted at seasonal timepoints in Midwest feed mills, including descriptions of antimicrobial resistance and metal tolerance genes.

2. Evaluate the efficacy of multiple commercially available sanitizers (chlorine dioxide gas (ClO_2), steam, silver dihydrogen citrate (SDC), peracetic acid (PAA), and liquid chlorine) when used for the control of mature, multi-strain *Listeria monocytogenes* and *Escherichia coli* grown on food contact surfaces commonly found within the tree-fruit harvesting process.
3. Validate and understand how environmental conditions in small- and large-scale facilities in two unique regions of the United States impact the efficacy of selected the sanitizers previously tested in laboratory condition.
4. Identify differences between gene expression from *L. monocytogenes* biofilms after sublethal exposure to four commercially available sanitizers (chlorine, PAA, silver dihydrogen citrate (SDC), and lactic acid).

Chapter 3 - Genomic Characterization of Resident Bacteria from Selected Swine Feed Mills in the Midwest Region of the United States

3.1 Abstract

There is a lack of data on resident bacteria present within animal feed manufacturing facilities. Knowledge of the bacteria present in such facilities, as well as the understanding of antimicrobial resistance genes (ARGs) they harbor, can help inform sanitation and cleaning practices to reduce the microbial risk for the animal and human food chains. A survey of selected swine feed mills within the Midwest region of the United States at multiple seasonal timepoints identified 121 resident bacterial isolates, classified into 7 genera, consisting of 66 *Enterobacter*, 34 *Citrobacter*, 9 *Klebsiella*, 4 *Cronobacter*, 4 *Pseudomonas*, 3 *Proteus*, and 1 *Enterococcus*, in addition to 33 *Salmonella* and 30 *Escherichia coli* characterized previously. Among the 121 isolates discussed within the current work, only 10% (12/121) of samples came directly from finished feed, while a majority came from environmental samples taken from either feed contact surfaces (31%, 37/121), non-feed contact surfaces like floors (40%, 48/121), or transient surfaces like brooms or workers' shoes (17%, 20/121). The isolates were also analyzed for the presence of ARGs and metal tolerance genes (MTGs). This research provides an initial framework to understand the diversity of resident flora in feed mill facilities which helps identify areas to focus on housekeeping and sanitization within mills. This information is crucial to develop feed safety strategies and prevent antimicrobial resistance from spreading in the farm-to-table continuum.

3.2 Introduction

Understanding the diversity of resident bacteria in animal food processing environments during operation and after cleaning and sanitation is important to assess the risk for animal food

and human food safety. These bacteria can grow and survive production and storage, along with foodborne pathogens. Several studies indicated that the survival of foodborne pathogens during processing and storage is heavily influenced by non-pathogenic resident bacteria (Giaouris et al., 2015). Furthermore, resident bacteria might play a role in the spread of antimicrobial resistance genes (ARG) (Verraes et al., 2013).

Resident bacteria are usually isolated from non-selective media and defined as the bacteria community that persists over time in production facilities (Moretro and Langrud, 2017). During routine operations, facilities might perform environmental sampling to verify the effectiveness of cleaning procedures. Resident bacteria such as *Enterococcus* and *Escherichia coli* are usually used as indicators for hygiene and sanitation (Marriott et al., 2018). A recent survey that sampled 647 pet food and 378 animal feed samples reported that the overall prevalence ranged from 12.5% for *E. coli* to 45.2% for *Enterococcus* spp., and 11.2% of samples had both organisms. Regardless of bacterial genus, animal feed samples reported overall a statistically significant higher prevalence than pet food samples (Ge et al., 2020).

Resident bacteria may also have a role in ARG spread in animal food-related commodities. ARG is a growing concern for both human and animal health, and it is considered to be a pressing issue by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organization for Animal Health (WHO, 2023). Essentially, ARG impacts the ability to treat diseases, resulting in treatment failures and severe infections (WHO, 2015). Since animal feed is at the beginning of the farm-to-table continuum, investigating ARG in animal food-related commodities is of key importance. In the meantime, metal tolerance has become an emerging issue that may compromise sanitation and cause cross-resistance to antimicrobials (Pal et al., 2017). While characterization of

antimicrobial resistance genes (ARGs) and metal tolerance genes (MTGs) has been done on specific bacterial pathogens present within feed mills and ingredients (Davies and Wales, 2010, Torres et al., 2010, Burns et al., 2015, Munoz et al., 2021, Magossi et al., 2020), little is known about the overarching bacterial presence and associated ARG and metal tolerance within facilities.

In our previous study (Magossi et al., 2020), 405 samples were collected from six swine feed mills in the north central part of the United States (Midwest region). Twelve sampling sites were selected within each mill, and samples were analyzed for the presence of resident bacteria. A total of 212 positive isolates were selected for further studies. Our previous research (Schwan et al., 2023) focused primarily on the genotypic and phenotypic characterization of *Salmonella* and *Escherichia coli.* In this study, we characterized other resident bacteria collected from the survey.

3.3 Materials and Methods

3.3.1 Sample collection and isolation

This study is part of a previous sampling survey conducted in commercial swine feed mills located in the Midwest region of the United States during 2018-2019 (Magossi et al., 2020). A total of 405 samples were collected across different sampling sites, including feed, floors, brooms, and feed manufacturing equipment. Each site was analyzed for the presence of resident bacteria, following previous protocols used in our laboratory (USDA-FSIS, 2014). From all the presumptive positive samples, at least two colonies were selected for further investigation as described in Schawn et al. (2023). Isolates were stored in Trypticase Soy Broth (TSB; Hardy Diagnostics, Santa Maria, CA, USA) containing glycerol at -80°C until analysis. On day 1 of the

experiments, isolates were retrieved from -80°C and grown on Trypticase Soy Agar (TSA, Hardy Diagnostics, Santa Maria, CA, USA) or Blood Agar (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C overnight.

3.3.2 Bacterial speciation

The VITEK 2 Compact system (bioMérieux, Marcy l'Etoile, France) or matrix-assisted laser desorption/ionization time-of- flight (MALDI-TOF) mass spectrometry on the MALDI Biotyper (Bruker, Billerica, MA) were used for the identification of bacterial genera and species following the manufacturers' instructions.

3.3.3 Antimicrobial susceptibility testing and multidrug resistance

Minimal inhibitory concentrations (MICs) were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) standard guidelines M100-S28 (CLSI, 2019) using the Sensititre automated antimicrobial susceptibility system (Thermo Fisher Scientific) with the National Antimicrobial Resistance Monitoring System (NARMS) Gram-negative CMV4AGNF plate (FDA, 2015). Fourteen antimicrobials were tested as previously described (Schwan et al., 2023).

The MICs were interpreted using the CLSI standards and NARMS breakpoints to categorize MIC results as susceptible or resistant (FDA, 2015; CLSI, 2019). Multidrug resistance (MDR) was accounted for when resistance to ≥ 3 antimicrobial classes was identified (NARMS, 2021).

3.3.4 WGS characterization

DNA extraction, library preparation, sequencing, and *in silico* analysis were performed as previously described in Domesle et al. (2021) with some modifications. Genomic DNAs were extracted using the Qiagen DNeasy Blood & Tissue kit (Germantown, MD, USA). DNA concentrations were measured with a Qubit Fluorometer 4.0 using the dsDNA HS assay kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). DNA extracts were stored at -20°C until analysis. Nextera XT Library Preparation kit (Illumina, San Diego, CA, USA) was used for preparing paired-end libraries and WGS was carried on the MiSeq sequencer, using a 2×300-cycle MiSeq reagent V3 kit (Illumina). Trimming and *de novo* assemblies were obtained with Shovill version 9 (https://github.com/tseemann/shovill), available in the GalaxyTrakr pipeline (http://galaxytrakr.org/) with default parameters (Afgan et al., 2018)(Zhang et al., 2015). The National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Automatic Annotation pipeline (https://www.ncbi.nlm.nih.gov/refseq/annotation_prok/) was used to annotate draft genomes of each isolate.

3.3.5 Detection of ARGs and MTGs

ARGs and MTGs were identified using AMRFinderPlus v3.12.8 (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) with default parameters.

3.3.6 Data availability

The accession numbers and additional genomic information for the isolates can be found under the BioProject number PRJNA561052 at the National Library of Medicine.

3.4 Results and Discussion

3.4.1 Bacterial species characterization

All isolates, characterized by culture-based tests as Enterobacterales (212/405) were analyzed by WGS and classified into 16 genera. Of these 212 isolates, 28 isolates were not assembled due to poor sequence quality. *E. coli* (*n* = 30) and *Salmonella* (*n* = 33) isolates were further characterized in an earlier report (Schwann et al., 2023). The contigs N50, total length of assembly, and number of contigs of all the isolates belonging to this study (*n* = 121) are available in *Supplementary Table 1*.

In the present research only 121 isolates were investigated further: 66 *Enterobacter*, 34 *Citrobacter*, 9 *Klebsiella*, 4 *Cronobacter*, 3 *Proteus*, and 1 *Enterococcus* isolates. The research conducted previously by Schawn et al. (2023) characterizes the isolates of *Salmonella enterica* and *Escherichia coli*.

3.4.2 Isolate location profile within animal feed manufacturing facilities

Currently, there are little data on profiling resident bacteria within animal feed manufacturing facilities, and not much is known about the bacteria present in these environments. The isolates obtained in this study were primarily found through environmental swabbing, with only 12 of the 121 samples (9.9%) coming from complete feed (Table 1). The majority of the environmental samples were taken on hard stationary surfaces, with slightly more (51/121, 43.1%) isolates coming from non-feed contact surfaces like floors than those on feed contact surfaces (38/121, 31.4%) such as receiving grates, fat inlet valves, pellet mills, and load

out equipment within the different facilities. Twenty (20/121, 16.5%) isolates were obtained from transient surfaces like worker shoes and brooms.

Out of the 66 *Enterobacter* isolates, six different species were identified as *Enterobacter hormaechei* (32/66), *Enterobacter cloacae* (21/66), *Enterobacter asburiae* (5/66), *Enterobacter bugandensis* (3/66), *Enterobacter cancerogenus* (1/66), *Enterobacter kobei* (1/66), and *Enterobacter roggenkampii* (1/66), and two (2/66) isolates unable to be further characterized. Fifty-nine isolates identified (89%) were grouped into the *Enterobacter cloacae* complex (ECC), a polyphyletic group of five or six species (*Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis*) that has been identified as able to produce infections within humans (Mezzatesta et al., 2012). Five (5/66) of the total *Enterobacter* spp. isolates came from whole feed samples, with the remaining samples coming from environmental swabs. Most of the isolates were found on either direct feed contact (24/66) or non-feed contact surfaces (30/66), with only 7 (7/66) coming from transient surfaces such as shoes or brooms.

Among the 34 *Citrobacter* isolates, 6 different species were identified; *Citrobacter freundii* (14/34), *Citrobacter werkmanii* (5/34), *Citrobacter braakii* (4/34), , *Citrobacter koseri* (1/34), *Citrobacter rodentium* (1/34), *Citrobacter amalonaticus* (1/34), with an additional 8 isolates that could not be speciated. Of the speciated isolates, 25 belong to the *Citrobacter freundii complex* (*C. freundii*, *C. werkmanii*, *C. braakii*, *C. koseri*, and *C. rodentium*) (Brenner et al., 1993). *Citrobacter* spp. are found in soil, water, and intestinal tracts of animals and humans, and some species have been documented to cause infections in humans (Ranjan and Ranjan, 2013). The majority of the *Citrobacter* spp. isolates were from environmental swabs from non-

feed contact surfaces (12/34), feed contact surfaces (9/34), and transient surfaces (11/34). Two (2/34) isolates were obtained from finished feed samples.

Three species of *Klebsiella* (*n* = 9) were identified in this study as *Klebsiella pneumoniae* (5/9), *Klebsiella oxytoca* (3/9) and *Klebsiella aerogenes* (1/9). Three of the *K. pneumoniae* isolates were obtained from finished feed (3/5), with the other two (2/5) found on surfaces within raw ingredient receiving areas (1 feed contact, 1 non-feed contact). Two (2/3) *K. oxytoca* isolates were found on workers' shoes; the remaining one was found in a non-feed contact surface sample. One *K. aerogenes* isolate was obtained from a non-feed contact surface within an ingredient receiving area. *Klebsiella* spp. are commonly found in animal and human intestinal tracts, as well as plants, soil, and water (Bagley, 1985). *Klebsiella* spp. has been previously documented within feed samples (Zadoks et al., 2011).

Four *Cronobacter* species were identified within the samples taken for this study, one coming from finished feed (1/4), with the other three (3/4) isolated from environmental samples taken within the receiving area of two different facilities. Of the environmental samples, two (2/4) were from the same facility, but were identified at different sampling timepoints and sites (ingredient receiving pit vs receiving area floor). *Cronobacter* spp. can be found naturally within a variety of environments, and has been known to cause severe illness within humans (Healy et al., 2010).

Of the four *Pseudomonas* isolates found, three were *Pseudomonas aeruginosa*, and one was *Psuedomonas putida*. Three of the four isolates were identified from environmental swabs on non-feed contact surfaces, while one *Psuedomonas aeruginosa* isolate was found from an environmental swab taken on a feed contact surface. *Pseudomonas* are commonly found in soil and water and while often linked to food spoilage, they can also cause infection in humans and

plants (Raposo et al., 2017). Raw vegetables, especially those that are grown near or in soil, have been found to harbor *Pseudomonas* spp. (Ruiz- Roldán et al., 2021).

Only one Proteus species was identified. All three *Proteus mirabilis* isolates were identified in separate facilities with one isolate coming from finished feed (1/3), one from a feed contact surface (1/3), and one from a non-feed contact surface (1/3). This bacterium is considered commensal within gastrointestinal tracts, but it can be found also in water and soil. Foodborne illness due to *P. mirabilis* consumption in contaminated meat and seafood has been documented (Gong et al., 2019; Ma et al., 2022).

Only one *Enterococcus faecalis* was isolated in this study from a non-feed contact surface. *Enterococcus spp.* are commonly found in intestinal tracts of animals and humans, and has been found in some animal-derived food products (Byappanahalli et al., 2012; Ben Braïek & Smaoui, 2019). Previous studies surveying stored product insect populations within midwestern feed mills have shown the presence of multidrug resistant *Enterococcus* spp. (Larson et al., 2008a). *E. faecalis* is generally considered to be commensal, nevertheless it has been shown to be skilled at horizontal gene transfer, both within strains and to other pathogens (Manson et al., 2010, Palmer et al., 2010).

3.4.3 Presence of ARG

Overall, a wide range of ARGs were identified within the isolates of this study; indicative of resistance to a total of 13 classes of antimicrobials (Table 2). All but one isolate had at least one gene indicative of resistance to either a critically important or highly important class of antibiotics (WHO, 2019). The most common classes implicated included β-lactams (110/121,

90.9%), phenicols (75/121, 62%), quinolones, (72/121, 59.5%), fosfomycins (59/121, 48.8%), and tetracyclines (36/121, 29.8%).

A variety of ARGs were identified within *Enterobacter* spp. isolates. Almost all isolates contained at least one β-lactam resistance gene (97%, 64/66) and 77% (51/66) contained at least one fosfomycin resistance gene. Tetracycline resistance from either *tet(A), tet(B*), or *tet(C)* was found in 33% (22/66) of isolates. Several aminoglycoside resistance genes, *aph(3')-Ia, aph(3")-Ib,* and *aph(6)-Id,* were identified in 15% (10/66) isolates.

All *Citrobacter* spp. isolates were found to have at least one ARG, and 94% (32/34) contained a gene indicative of β-lactam resistance. The second most common gene identified was *qnrB*, with 41% (14/34) indicating quinolone resistance. Additionally, 21% (7/34) of isolates were found to have aminoglycoside resistance, and 24% (8/34) with tetracycline resistance.

The nine different *Klebsiella* spp. isolates contained genes indicative of β-lactam resistance (9/9). All samples also expressed *oqxA* and/or *oqxB,* which are considered intrinsic within *Klebsiella pneumoniae.* indicating phenicol and quinolone resistance (Lam et al., 2021). Fosfomycin resistance from *fosA* was found in all but one *Klebsiella pneumoniae* (4/5), and one sample also expressed sulfonomide, aminoclycoside, and tetracycline resistance with *sul2, aph(6)-Id,* and *aph(3")-Ib,* and *tet(A)* and *tet(B),* respectively.

Within the four *Cronobacter* spp. identified, all contained a gene indicative of β-lactam resistance (4/4). Three isolates contained additional resistance genes, including 50% (2/4) with a gene indicative of phenicol/quinolone resistance, as well as 25% (1/4) having aminoglycoside, fosfomycin, quinonlone, and tetracycline resistance, indicated by the *aph(6)-Id* or *aph(3")-Ib, fosA2, qnrB12,* and *tet(B)* genes, respectively.

All three *Proteus mirabilis* isolates showed tetracycline resistance with the *tet(J)* gene, due to the intrinsic resistance to tetracycline (Danilo de Oliveira et al., 2021). Two isolates also contained *catA*, indicating phenicol resistance, with one also containing *aadA31*, indicative of resistance to aminoglycosides.

The lone *Pseudomonas putida* isolate contained only the *tetA* gene, indicative of tetracycline resistance. All *Pseudomonas aeruginosa* isolates contained three separate genes indicative of increased efflux pump activity *(mexA*, *mexE*, *mexX*) and resistance to phenicol (*catB7*), fosfomycin (*fosA*), aminoglocosides [*aph(3')-Iib)*], and multiple genes for β-lactams (genes within the *blaOXA* family, as well as *blaPDC-3*).

The *Enterococcus faecalis* isolate was found to have six ARG: *catA, erm(B), lnu(A), lsa(A), tet(L), and tet(M),* indicating resistance to phenicol, lincosamide/macrolide/streptogramin, lincosamide/streptogramin, and tetracycline.

3.4.4 Phenotypic antimicrobial susceptibility and presence of MDR

All 121 isolates were tested for susceptibility against fourteen antimicrobials (Table 3): gentamicin (GEN), streptomycin (STR), amoxicillin/clavulanic acid (2:1 ratio; AMC), meropenem (MER), cefoxitin (FOX), ceftriaxone (AXO), ampicillin (AMP), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (COT), azithromycin (AZI), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline (TET). These represent antimicrobial classes defined by the CLSI (CLSI, 2019). None of the isolates tested were resistant to GEN.

Because of production of AmpC β-lactamase, *Enterobacter* are considered to be inherently resistant to a variety of antibiotics including ampicillin, amoxicillin-clavulanate, and cefoxitin (Bouza & Cercenado, 2002), which was mirrored within the phenotypic susceptibility

results of this study. Of the 66 *Enterobacter* isolates, only 5 (8%, 5/66) were not resistant to any of the three antibiotics listed previously. The most common resistance indicated outside of AMC, FOX, and AMP for *Entereobacter* isolates was TET (35%, 23/66). After the exclusion of intrinsic resistance, one *E. cloacae* isolate was found to exhibit MDR.

Citrobacter spp. are considered intrinsically resistant to cephalosporins (Pepperel et al., 2002; Liu et al., 2017) and ampicillin (Wanger et al., 2017). Specific strains, such as *C. freundii*, have been found to be intrinsically resistant to ampicillin-sulbactam and cephalosporins (Wanger et al., 2017). The data obtained in this study showed that 62% (21/34) of the *Citrobacter* isolates were FOX resistant, 50% (17/34) AMP resistant, 24% (8/34) TET resistant, and 20% (7/34) CIP resistant. Almost all of the *C. freundii* isolates (79%, 11/14) were AMC resistant. Additionally 5/34 (15%) isolates were STR resistant, 6% (2/34) were FIS resistant, and 1 isolate (3%) was resistant to AMC. None of the *Citrobacter* isolates were found to have MDR.

Klebsiella spp. are resistant to penicillins (such as AMP) intrinsically (Bouza & Cercenado, 2002). Of the nine *Klebsiella* isolates from this study, 67% (6/9) were AMP resistant. Two isolates (22%, 2/9) were resistant to STR and TET, and resistance to AMC, FOX, FIS, and CIP were found in individual isolates (11%, 1/9). Two isolates (one *K, oxytoca* and one *K. pneumoniae*) were found to be MDR. The *K. oxytoca* isolate showed resistance to STR, CIP, and TET, while the *K. pneumoniae* isolate was resistant to STR, FIS, and TET in addition to the intrinsic AMP resistance.

Specific *Cronobacter* spp. have been found to have intrinsic resistance to antibiotics such as AMP, and first and second generation cephalosporins such as FOX (Donnenberg, 2015), and other studies have shown a high level of resistance to AMC (Pakbin et al., 2022). A majority (75%, 3/4) of the isolates from this study were resistant to both AMC and FOX; two (50%, 2/4)

of those isolates were also resistant to AMP. Additionally, CIP and TET resistance were found in one separate isolate (25%, 1/4), respectively. None of the *Cronobacter* spp. isolates were MDR, and one isolate (25%, 1/4) was resistant to all antimicrobials tested.

Intrinsic resistance of TET (Danilo de Oliveira et al., 2021; Ngeum et al., 2021); all *Proteus mirabilis* isolates (100%, 3/3) in this study were resistant. Additionally, all isolates showed resistance to AZI, and one isolate was also resistant to STR. None of the three isolates exhibited MDR after the exclusion of intrinsic resistance.

Pseudomonas is well regarded as having significant amounts of intrinsic resistance to a variety of antimicrobials, and are generally adept at acquiring resistance (Breidenstein et al., 2011; Pang et al., 2019). All (100%, 4/4) of the *Pseudomonas* isolates in this study were resistant to nine of the antimicrobials tested (STR, AMC, AXO, AMP, COT, AZI, CHL, CIP, and NAL). Additionally, 75% (3/4) were resistant to TET, 50% (2/4) to FIS, and 25% (1/4) to MER.

Similarly to the *Pseudomonas* spp. identified, the *Entereococus faecalis* isolate identified in this study also showed resistance to nine of the tested antimicrobials (STR, FOX, AXO, FIS, AZI, CHL, CIP, NAL, and TET). *E. faecalis* has intrinsic resistance to a variety of antimicrobials, including aminoglycosides such as STR and CIP (Arsene & Leclercq, 2007; Kristich, Rice, & Arias, 2014). Overall, these two species exhibited the highest diversity of phenotypic resistance against the antimicrobials tested.

3.4.5 Presence of MTG

The presence of at least one MTG was identified within each individual isolate analyzed in this study, excluding *Pseudomonas* isolate (Table 4). Research has indicated that the presence of MTGs can be indicative of selective pressures being applied to bacteria from the environment,

which may lead to co-selection of genes that confer metal tolerance and antimicrobial resistance (Nguyen et al., 2023). Identifying the presence of MTG, in addition to the ARGs found within environmental isolates could lead to a more robust understanding of the connection between the genes.

All isolates other than the four *Pseudomonas* spp., three *Proteus* spp. and *Enterococcus faecalis* isolate (113/121) contained the *fieF* gene, an efflux modulator. A study of select multi-drug resistant clinical isolates sourced within a hospital found similar presence of *fieF* within a majority of the Enterobacterales identified (Yaikhan et al., 2024), excluding *Proteus* spp., which when combined with the results of this study indicate an increased likelihood of presence within the order. Overall, 41% (59/121) isolates contained genes indicative of tolerance to copper/silver; individually, genes responsible for copper tolerance were found in 30% (37/121) of isolates and silver tolerance genes in 31% (38/121) of isolates. Arsenic tolerance genes were found in 36% (43/121) of isolates.

Enterobacter spp. isolates displayed joint Copper/Silver tolerance within 71% (47/66) of isolates. Standalone tolerance to silver was indicated within 41% (27/66) of isolates, as well as copper in 39% (26/66). Arsenic tolerance was indicated in 36% (24/66) of isolates. Genes indicating tolerance to tellurium were found in 29% (19/66) isolates, and mercury tolerance was detected in 6% of the isolates (4/66).

The most prevalent tolerance genes present within the *Citrobacter* spp. isolates were to arsenic, with 44% (15/34) isolates containing at least one gene. Copper/silver tolerance genes were present in 29% (10/34) of isolates, with the same isolates also having genes indicative of standalone silver tolerance and 27% (9/34) standalone copper tolerance. A small portion of the isolates (9%, 3/34) contained genes indicative of tellurium tolerance.

All three of the *Klebsiella oxytoca* isolates were tolerant to arsenic, making up 33% (3/9) of the overall *Klebsiella* samples. One *Klebsiella pneumoniae* isolate contained a gene indicative of tellurium tolerance. Of the *Cronobacter* spp. identified in this experiment, two (50%, 2/4) contained at least one gene indicative of copper/silver tolerance, with one of those isolates also containing genes for standalone copper and silver. One isolate (25%, 1/4) contained an arsenic tolerance gene. All three (100%) *Proteus mirabilis* contained *terD* and *terZ* genes for tellurium tolerance. The individual *Enterococcus faecalis* isolate contained *tcrB*, indicative of copper tolerance.

3.5 Conclusion

Several challenges currently exist in order to obtain a clearer picture of the resident flora throughout feed manufacturing facilities. Industry stakeholders are usually hesitant to allow data collection within facilities, and it can be difficult to access ideal sampling locations once in a mill. While foodborne pathogens of concern such as pathogenic *E. coli*, *Salmonella*, and viral agents may be of higher concern to producers and feed manufacturing facilities, knowing the other bacteria present, especially those that can indicate fecal contamination, might be of key importance to develop feed safety strategies, as well as prevent ARG and MTG spread in the farm-to-table continuum. This research provides an initial framework to understand possible inherent resident bacteria of feed mill facilities and help identify areas to focus housekeeping and sanitization within mills.

3.6 Acknowledgements

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Table 3.1. Sample metadata

Genus	*Species*	Sample ID	BioSample	Facility	Sample Location	Sample Type	Season
Enterobacter	*asburiae*	CVM 45449	SAMN12619252	1	Pellet mill	Feed Contact	Spring
		CVM 45463	SAMN12619266	3	Loadout auger	Feed Contact	Spring
		CVM 45616	SAMN14410557	1	Pellet Mill	Feed Contact	Summer
		CVM 45636	SAMN14410577	3	Receiving grate	Feed Contact	Summer
		CVM 45647	SAMN14410588	3	Worker shoe	Transient	Summer
	bugandensis	CVM 45640	SAMN14410581	3	Control room floor	Non-feed Contact	Summer
		CVM 45668	SAMN14410609	5	Pellet mill	Feed Contact	Summer
		CVM 45670	SAMN14410611	5	Loadout auger	Feed Contact	Summer
	cancerogenus	CVM 45638	SAMN14410579	3	Loadout auger	Feed Contact	Summer
	cloacae	CVM 45414	SAMN12619217	2	Finished Feed	Finished Feed	Fall
		CVM 45418	SAMN12619221	3	Loadout auger	Feed Contact	Fall
		CVM 45420	SAMN12619223	3	Manufacturing floor	Non-feed Contact	Fall
		CVM 45421	SAMN12619224	4	Receiving grate	Feed Contact	Fall
		CVM 45452	SAMN12619255	1	Warehouse floor	Non-feed Contact	Spring
		CVM 45453	SAMN12619256	2	Pellet mill	Feed Contact	Spring
		CVM 45458	SAMN12619261	2	Warehouse floor	Non-feed Contact	Spring
		CVM 45462	SAMN12619265	3	Discharge bin	Feed Contact	Spring
		CVM 45474	SAMN12619277	4	Broom	Transient	Spring
		CVM 45475	SAMN12619278	5	Receiving pit	Feed Contact	Spring

	CVM 45477	SAMN12619280	5	Receiving floor	Non-feed Contact	Spring
	CVM 45494	SAMN12619297	4	Receiving pit	Feed Contact	Fall
	CVM 45504	SAMN12619307	2	Finished Feed	Finished Feed	Spring
	CVM 45631	SAMN14410572	2	Manufacturing floor	Non-feed Contact	Summer
	CVM 45642	SAMN14410583	3	Control room floor	Non-feed Contact	Summer
	CVM 45645	SAMN14410586	3	Warehouse floor	Non-feed Contact	Summer
	CVM 45646	SAMN14410587	3	Warehouse floor	Non-feed Contact	Summer
	CVM 45652	SAMN14410593	4	Fat intake valve	Feed Contact	Summer
	CVM 45658	SAMN14410599	4	Manufacturing floor	Non-feed Contact	Summer
	CVM 45660	SAMN14410601	4	Warehouse floor	Non-feed Contact	Summer
	CVM 45681	SAMN14410622	6	Control room floor	Non-feed Contact	Summer
hormaechei	CVM 45434	SAMN12619237	5	Finished Feed	Finished Feed	Fall
	CVM 45435	SAMN12619238	5	Finished Feed	Finished Feed	Fall
	CVM 45440	SAMN12619243	6	Discharge bin	Feed Contact	Fall
	CVM 45444	SAMN12619247	6	Manufacturing floor	Non-feed Contact	Fall
	CVM 45450	SAMN12619253	1	Receiving floor	Non-feed Contact	Spring
	CVM 45454	SAMN12619257	2	Loadout auger	Feed Contact	Spring
	CVM 45457	SAMN12619260	2	Manufacturing floor	Non-feed Contact	Spring
	CVM 45464	SAMN12619267	3	Control room floor	Non-feed Contact	Spring
	CVM 45488	SAMN12619291	2	Receiving floor	Non-feed Contact	Fall
	CVM 45505	SAMN12619308	3	Worker shoe	Transient	Spring

CVM 45508	SAMN12619311	4	Control room floor	Non-feed Contact	Fall
CVM 45608	SAMN14410549	6	Manufacturing floor	Non-feed Contact	Summer
CVM 45609	SAMN14410550	6	Worker shoe	Transient	Summer
CVM 45617	SAMN14410558	1	Discharge bin	Feed Contact	Summer
CVM 45619	SAMN14410560	1	Control room floor	Non-feed Contact	Summer
CVM 45624	SAMN14410565	1	Worker shoe	Transient	Summer
CVM 45643	SAMN14410584	3	Receiving floor	Non-feed Contact	Summer
CVM 45650	SAMN14410591	4	Receiving pit	Feed Contact	Summer
CVM 45653	SAMN14410594	4	Discharge bin	Feed Contact	Summer
CVM 45656	SAMN14410597	4	Receiving floor	Non-feed Contact	Summer
CVM 45659	SAMN14410600	4	Manufacturing floor	Non-feed Contact	Summer
CVM 45661	SAMN14410602	4	Warehouse floor	Non-feed Contact	Summer
CVM 45662	SAMN14410603	4	Worker shoe	Transient	Summer
CVM 45663	SAMN14410604	4	Worker shoe	Transient	Summer
CVM 45666	SAMN14410607	5	Receiving pit	Feed Contact	Summer
CVM 45669	SAMN14410610	5	Discharge bin	Feed Contact	Summer
CVM 45671	SAMN14410612	5	Control room floor	Non-feed Contact	Summer
CVM 45674	SAMN14410615	5	Warehouse floor	Non-feed Contact	Summer
CVM 45675	SAMN14410616	5	Warehouse floor	Non-feed Contact	Summer
CVM 45680	SAMN14410621	6	Loadout auger	Feed Contact	Summer
CVM 45682	SAMN14410623	6	Control room floor	Non-feed Contact	Summer

		CVM 45684	SAMN14410625	6	Receiving floor	Non-feed Contact	Summer
	kobei	CVM 45628	SAMN14410569	2	Pellet mill	Feed Contact	Summer
	roggenkampii	CVM 45651	SAMN14410592	4	Receiving pit	Feed Contact	Summer
	spp.	CVM 45523	SAMN14410528	3	Manufacturing floor	Non-feed Contact	Fall
		CVM 45654	SAMN14410595	4	Finished Feed	Finished Feed	Summer
Citrobacter	*amalonaticus*	CVM 45409	SAMN12619212	1	Receiving floor	Non-feed Contact	Fall
	braakii	CVM 45639	SAMN14410580	3	Finished Feed	Finished Feed	Summer
		CVM 45644	SAMN14410585	3	Receiving floor	Non-feed Contact	Summer
		CVM 45649	SAMN14410590	3	Broom	Transient	Summer
		CVM 45602	SAMN14410543	1	Receiving floor	Non-feed Contact	Summer
	freundii	CVM 45411	SAMN12619214	1	Worker shoe	Transient	Fall
		CVM 45413	SAMN12619216	2	Discharge bin	Feed Contact	Fall
		CVM 45419	SAMN12619222	3	Control room floor	Non-feed Contact	Fall
		CVM 45469	SAMN12619272	4	Loadout auger	Feed Contact	Spring
		CVM 45481	SAMN12619284	5	Broom	Transient	Spring
		CVM 45604	SAMN14410545	3	Finished Feed	Finished Feed	Summer
		CVM 45626	SAMN14410567	1	Broom	Transient	Summer
		CVM 45627	SAMN14410568	2	Receiving pit	Feed Contact	Summer
		CVM 45635	SAMN14410576	3	Receiving pit	Feed Contact	Summer
		CVM 45637	SAMN14410578	3	Discharge bin	Feed Contact	Summer
		CVM 45641	SAMN14410582	3	Control room floor	Non-feed Contact	Summer

		CVM 45657	SAMN14410598	4	Receiving floor	Non-feed Contact	Summer
		CVM 45664	SAMN14410605	4	Broom	Transient	Summer
		CVM 45679	SAMN14410620	6	Discharge bin	Feed Contact	Summer
	koseri	CVM 45676	SAMN14410617	5	Broom	Transient	Summer
	rodentium	CVM 45693	SAMN14410634	6	Manufacturing floor	Non-feed Contact	Spring
	spp.	CVM 45412	SAMN12619215	2	Receiving pit	Feed Contact	Fall
		CVM 45437	SAMN12619240	5	Manufacturing floor	Non-feed Contact	Fall
		CVM 45466	SAMN12619269	3	Worker shoe	Transient	Spring
		CVM 45467	SAMN12619270	3	Broom	Transient	Spring
		CVM 45476	SAMN12619279	5	Control room floor	Non-feed Contact	Spring
		CVM 45479	SAMN12619282	5	Manufacturing floor	Non-feed Contact	Spring
		CVM 45480	SAMN12619283	5	shoe	Transient	Spring
		CVM 45531	SAMN14410536	5	Broom	Transient	Fall
	werkmanii	CVM 45618	SAMN14410559	1	Loadout auger	Feed Contact	Summer
		CVM 45620	SAMN14410561	1	Receiving floor	Non-feed Contact	Summer
		CVM 45625	SAMN14410566	1	Worker shoe	Transient	Summer
		CVM 45667	SAMN14410608	5	Receiving pit	Feed Contact	Summer
		CVM 45672	SAMN14410613	5	Manufacturing floor	Non-feed Contact	Summer
Klebsiella	*aerogenes*	CVM 45485	SAMN12619288	1	Receiving floor	Non-feed Contact	Fall
	oxytoca	CVM 45459	SAMN12619262	2	Worker shoe	Transient	Spring
		CVM 45487	SAMN12619290	1	Receiving floor	Non-feed Contact	Fall

	pneumoniae	CVM 45498	SAMN12619301	4	Worker shoe	Transient	Fall
		CVM 45486	SAMN12619289	1	Receiving floor	Non-feed Contact	Fall
		CVM 45506	SAMN12619309	4	Finished Feed	Finished Feed	Spring
		CVM 45603	SAMN14410544	2	Receiving pit	Feed Contact	Summer
		CVM 45687	SAMN14410628	3	Finished Feed	Finished Feed	Fall
		CVM 45692	SAMN14410633	6	Finished Feed	Finished Feed	Spring
Cronobacter	*spp.*	CVM 45441	SAMN12619244	6	Finished Feed	Finished Feed	Fall
		CVM 45461	SAMN12619264	3	Receiving pit	Feed Contact	Spring
		CVM 45468	SAMN12619271	4	Receiving pit	Feed Contact	Spring
		CVM 45655	SAMN14410596	4	Receiving floor	Non-feed Contact	Summer
Proteus	*mirabilis*	CVM 45451	SAMN12619254	1	Manufacturing floor	Non-feed Contact	Spring
		CVM 45470	SAMN12619273	4	Finished Feed	Finished Feed	Spring
		CVM 45678	SAMN14410619	6	Receiving pit	Feed Contact	Summer
Psuedomonas	*aeruginosa*	CVM 45433	SAMN12619276	5	Loadout auger	Feed contact	Fall
		CVM 45471	SAMN12619274	4	Control room floor	Non-feed contact	Spring
		CVM 45472	SAMN12619275	4	Receiving floor	Non-feed contact	Spring
	putuida	CVM 45438	SAMN12619241	5	Warehouse floor	Non-feed contact	Fall
Enterococcus	*faecalis*	CVM 45611	SAMN14410552	1	Manufacturing floor	Non-feed Contact	Fall

Table 3.2. Antimicrobial resistance data

			ARG Class											
Genus	**Species**	**Sample ID**	**SU**	**ST**	**A**	**F**	**C**	**BL**	**P**	**Q**	**T**	**L**	**L/M/S**	**L/S**
Enterobacter	*asburiae*	CVM 45449				X		X	X	X				
		CVM 45463				X		X	X	X				
		CVM 45616				X		X	X	X				
		CVM 45636				X		X	X	X				
		CVM 45647				X		X	X	X				
	bugandensis	CVM 45640				X		X	X	X	X			
		CVM 45668				X		X	X	X				
		CVM 45670				X		X	X	X				
	cancerogenus	CVM 45638				X		X						
	cloacae	CVM 45414				X		X	X	X				
		CVM 45418				X		X	X	X				
		CVM 45420				X		X						
		CVM 45421				X		X	X	X				
		CVM 45452				X		X	X	X				
		CVM 45453				X		X	X	X				
		CVM 45458			X						X			
		CVM 45462				X		X	X	X				
		CVM 45474				X		X	X	X				
		CVM 45475				X		X	X	X				
		CVM 45477				X		X	X	X				
		CVM 45494			X			X	X	X	X			
		CVM 45504				X		X						
		CVM 45631				X		X	X	X				

	CVM 45642	X	X		X	X	X	X	
	CVM 45645		X		X	X	X		
	CVM 45646		X		X	X	X		
	CVM 45652		X		X	X	X		
	CVM 45658	X	X		X	X	X	X	
	CVM 45660		X		X	X	X		
	CVM 45681	X	X		X	X	X	X	
hormaechei	CVM 45434				X	X	X		
	CVM 45435				X	X	X		
	CVM 45440		X		X	X	X		
	CVM 45444	X			X	X	X	X	
	CVM 45450		X		X	X	X		
	CVM 45454		X		X	X	X		
	CVM 45457		X		X	X	X	X	
	CVM 45464	X			X	X	X	X	
	CVM 45488				X	X	X	X	
	CVM 45505		X		X	X	X		
	CVM 45508		X		X	X	X	X	
	CVM 45608		X		X	X	X	X	
	CVM 45609		X		X	X	X	X	
	CVM 45617		X		X	X	X		
	CVM 45619		X		X	X	X	X	
	CVM 45624		X		X	X	X		
	CVM 45643		X		X	X	X		
	CVM 45650				X	X	X		
	CVM 45653		X		X	X	X		
	CVM 45656				X	X	X		
	CVM 45659				X	X	X	X	

		CVM 45661			X			X	X	X	X
		CVM 45662				X		X	X	X	
		CVM 45663				X		X	X	X	
		CVM 45666						X	X	X	
		CVM 45669				X	X	X	X	X	X
		CVM 45671				X		X	X	X	
		CVM 45674						X	X	X	X
		CVM 45675			X	X		X	X	X	X
		CVM 45680				X		X	X	X	X
		CVM 45682				X		X	X	X	X
		CVM 45684			X			X	X	X	X
	kobei	CVM 45628				X		X	X	X	
	roggenkampii	CVM 45651				X		X	X	X	
	spp.	CVM 45523									
		CVM 45654				X		X	X	X	
Citrobacter	*amalonaticus*	CVM 45409	X		X						
	braakii	CVM 45602						X			
		CVM 45639						X			
		CVM 45644						X			
		CVM 45649						X			
	freundii	CVM 45411				X		X			
		CVM 45413						X			
		CVM 45419						X			X
		CVM 45469						X			
		CVM 45481				X		X			
		CVM 45604						X			
		CVM 45626						X			
		CVM 45627						X			

		CVM 45635				X			
		CVM 45637				X			
		CVM 45641				X		X	
		CVM 45657		X		X		X	
		CVM 45664				X			
		CVM 45679		X		X		X	
	koseri	CVM 45676	X	X		X		X	
	rodentium	CVM 45693				X			
	spp.	CVM 45412				X			
		CVM 45437		X		X		X	
		CVM 45466				X			
		CVM 45467				X			
		CVM 45476				X			
		CVM 45479				X			
		CVM 45480		X		X			
		CVM 45531	X	X		X		X	
	werkmanii	CVM 45618				X			
		CVM 45620				X			
		CVM 45625				X		X	
		CVM 45667				X			
		CVM 45672				X			
Klebsiella	*aerogenes*	CVM 45485			X	X	X	X	
	oxytoca	CVM 45459				X	X	X	
		CVM 45487				X	X	X	
		CVM 45498				X	X	X	
	pneumoniae	CVM 45486			X	X	X	X	
		CVM 45506			X	X	X	X	
		CVM 45603			X	X	X	X	

		CVM 45687			X		X	X	X					
		CVM 45692	X		X		X	X	X	X				
Cronobacter	*spp.*	CVM 45441					X							
		CVM 45461			X		X	X	X					
		CVM 45468					X							
		CVM 45655			X		X	X	X	X				
Proteus	*mirabilis*	CVM 45451			X			X		X				
		CVM 45470								X				
		CVM 45678						X		X				
Pseudomonas	*aeruginosa*	CVM 45433			X	X	X							
		CVM 45471			X	X	X							
		CVM 45472			X	X	X							
	putida	CVM 45438								X				
Enterococcus	*faecalis*	CVM 45611						X		X	X	X	X	

SU = sulfonamide, ST = streptothricin, A = aminoglycoside, F = fosfomycin, C = colistin, BL = beta-lactam, P = phenicol, Q = quinonlone, T = tetracycline, L = lincosamide, L/M/S = lencosamide/macrolide/streptogramin, L/S = lincosamide/streptogramin

X denotes presence of genes indicative of resistance within ARG class.

The following genes were included when considering resistance to the corresponding class:
Sulfonamides- *sul2*; **Streptothricin**- *sat2*; **Aminoclycosides**- *aac(2')-IIa, aac(6')-If, aadA1, aadA31, aph(3')-Ia, aph(3'')-Ib, aph(3')-IIb, aph(6)-Id*; **Fosfomycin**- *fosA, fosA2, fosA7; Colistin- mcr-10.1*; **Beta-Lactam**- *ampC, blaACT, blaACT-15, blaACT-17, blaACT-25, blaACT-37, blaACT-43, blaACT-46, blaACT-65, blaACT-76, blaACT-90, blaCMH, blaCMH-3, blaCMY, blaCMY-101, blaCMY-105, blaCMY-152, blaCMY-70, blaCMY-83, blaCSA, blaLEN-13, blaMIR, blaMIR-10, blaOXA-486, blaOXA-494, blaOXA-926, blaOXY-2-7, blaOXY-6-1, blaPDC-3, blaSED, blaSHV-1, blaSHV-108, blaSHV-11, blaTEM-1*; **Phenicol**- *catA, catB7*; **Quinolone**- *qnrB, qnrB12, qnrB34, qnrB38, qnrB71, qnrB50, qnrB69, qnrE*; **both Phenicol and Quinolone**- *oqxA, oqxB, oqxB19, oqxB25*; **Tetracycline**- *tet(A), tet(B), tet(C), tet(J), tet(L), tet(M*); **Lincosamide**- *lnu(A*); **Lincosamide/Macrolide/Streptogramin**- *erm(B*); **Lincosamide/Streptogramin**- *lsa(A)*

Table 3.3. Antimicrobial susceptibility

			Aminoglycosides		Beta-lactam/beta-lactam inhibitor		Cephems		Penicillin	Folate pathway inhibitor		Macrolide	Phenicol	Quinolones		Tetracycline
Genus	Species	Sample ID	GEN	STR	AMC	MER	FOX	AXO	AMP	FIS	COT	AZI	CHL	CIP	NAL	TET
Enterobacter	*asburiae*	CVM 45449			X		X		X					X		
		CVM 45463			X		X		X							
		CVM 45616			X		X		X							
		CVM 45636			X		X									
		CVM 45647			X		X		X							
	bugandensis	CVM 45640			X		X		X							X
		CVM 45668			X		X		X							
		CVM 45670			X		X		X							
	cancerogenus	CVM 45638			X		X		X							
	cloacae	CVM 45414			X		X		X							
		CVM 45418			X		X		X							X
		CVM 45420			X		X		X							
		CVM 45421			X		X		X							
		CVM 45452			X		X		X							

		CVM 45453			X	X	X				
		CVM 45458		X	= 4						X
		CVM 45462			X	X	X				
		CVM 45474			X	X	X				
		CVM 45475			X	X	X				
		CVM 45477			X	X	X				
		CVM 45494		X	X	X			X		X
		CVM 45504			X	X	X				
		CVM 45631			X	X	X				
		CVM 45642		X	X	X	X				X
		CVM 45645									
		CVM 45646			X	X	X				
		CVM 45652			X	X	X				
		CVM 45658		X	X	X	X				X
		CVM 45660			X	X	X				
		CVM 45681			X	X	X				X
	hormaechei	CVM 45434			X	X	X				

	CVM 45435			X	X	X		
	CVM 45440			X	X	X		
	CVM 45444		X	X	X	X		X
	CVM 45450			X	X	X		
	CVM 45454			X	X	X		
	CVM 45457			X	X			X
	CVM 45464		X	X	X	X		X
	CVM 45488			X	X			X
	CVM 45505							
	CVM 45508			X	X	X		X
	CVM 45608			X	X	X		X
	CVM 45609			X	X	X		X
	CVM 45617			X	X	X		
	CVM 45619			X	X	X		X
	CVM 45624			X	X	X		
	CVM 45643			X	X	X		
	CVM 45650			X	X	X		

	CVM 45653			X	X	X			
	CVM 45656			X	X	X			
	CVM 45659					X			X
	CVM 45661		X	X	X				X
	CVM 45662								
	CVM 45663			X	X	X			
	CVM 45666			X	X	X			
	CVM 45669			X	X	X			X
	CVM 45671			X	X	X			
	CVM 45674			X	X	X			X
	CVM 45675		X	X	X	X			X
	CVM 45680			X	X	X			X
	CVM 45682			X	X	X			X
	CVM 45684		X	X	X				X
kobei	CVM 45628			X	X	X			
roggenkampii	CVM 45651			X	X	X			
spp.	CVM 45523					X			

		CVM 45654												
Citrobacter	*amalonaticus*	CVM 45409					X							
	braakii	CVM 45602				X								
		CVM 45639		X		X								
		CVM 45644				X								
		CVM 45649												
	freundii	CVM 45411			X	X	X					X		
		CVM 45413			X	X								
		CVM 45419				X								X
		CVM 45469				X				X				
		CVM 45481			X	X						X		
		CVM 45604			X	X	X					X		
		CVM 45626			X	X								
		CVM 45627			X	X	X					X		
		CVM 45635		X	X	X								
		CVM 45637		X		X								
		CVM 45641			X	X	X							X

	CVM 45657		X	X		X		X					X
	CVM 45664			X		X							
	CVM 45679			X		X		X					X
koseri	CVM 45676							X	X	X			X
rodentium	CVM 45693												
spp.	CVM 45412							X					
	CVM 45437		X					X					X
	CVM 45466							X					
	CVM 45467							X					
	CVM 45476							X					
	CVM 45479							X					
	CVM 45480							X					
	CVM 45531							X	X				X
werkmanii	CVM 45618							X					
	CVM 45620					X						X	
	CVM 45625					X						X	X
	CVM 45667					X						X	

		CVM 45672				X								
Klebsiella	*aerogenes*	CVM 45485			X		X		X					
	oxytoca	CVM 45459		X									X	X
		CVM 45487							X					
		CVM 45498												
	pneumoniae	CVM 45486							X					
		CVM 45506												
		CVM 45603							X					
		CVM 45687							X					
		CVM 45692		X					X	X				X
Cronobacter	*spp.*	CVM 45441												
		CVM 45461			X		X		X					
		CVM 45468			X		X		X				X	
		CVM 45655			X		X							X
Proteus	*mirabilis*	CVM 45451		X							X			X
		CVM 45470									X			X
		CVM 45678									X			X

Pseudomonas	*aeruginosa*	CVM 45433	X	X			X	X	X	X	X	X	X	X	X
		CVM 45471	X	X			X	X		X	X	X	X	X	X
		CVM 45472	X	X			X	X		X	X	X	X	X	
	putida	CVM 45438	X	X	X		X	X	X	X	X	X	X	X	X
Enterococcus	*faecalis*	CVM 45611	X			X	X		X		X	X	X	X	X

X indicates resistance to antimicrobial.

Table 3.4. Metal tolerance genes (MTGs)

			Tolerance type						
Genus	**Species**	**Sample ID**	Arsenic	Copper	Copper/Silver	Silver	Tellurium	Mercury	Cadmium
Enterobacter	*asburiae*	CVM 45449			X				
		CVM 45463			X				
		CVM 45616			X				
		CVM 45636			X				
		CVM 45647			X				
	bugandensis	CVM 45640	X		X				
		CVM 45668							
		CVM 45670							
	cancerogenus	CVM 45638							
	cloacae	CVM 45414			X				
		CVM 45418			X				
		CVM 45420							
		CVM 45421	X		X				
		CVM 45452	X	X	X	X			

		CVM 45453			X			
		CVM 45458		X	X	X	X	
		CVM 45462			X			
		CVM 45474	X		X			
		CVM 45475	X		X			
		CVM 45477						
		CVM 45494		X	X	X	X	
		CVM 45504						
		CVM 45631			X		X	
		CVM 45642		X	X	X	X	
		CVM 45645		X	X	X	X	
		CVM 45646			X			
		CVM 45652			X			
		CVM 45658	X	X	X	X	X	X
		CVM 45660			X			
		CVM 45681	X	X	X	X		
	hormaechei	CVM 45434						
		CVM 45435						
		CVM 45440						
		CVM 45444		X	X	X		
		CVM 45450						
		CVM 45454						
		CVM 45457	X	X	X	X		
		CVM 45464	X	X	X	X		
		CVM 45488	X	X	X	X	X	
		CVM 45505	X	X	X	X		
		CVM 45508	X	X	X	X	X	
		CVM 45608	X	X	X	X	X	
		CVM 45609	X	X	X	X	X	

		CVM 45617						
		CVM 45619		X	X	X		
		CVM 45624	X	X	X	X	X	X
		CVM 45643						
		CVM 45650						
		CVM 45653						
		CVM 45656	X	X	X	X	X	
		CVM 45659	X	X	X	X		
		CVM 45661	X	X	X	X	X	X
		CVM 45662						
		CVM 45663						
		CVM 45666						
		CVM 45669	X	X	X	X	X	
		CVM 45671						
		CVM 45674	X	X	X	X	X	
		CVM 45675	X	X	X	X	X	X
		CVM 45680	X	X	X	X	X	
		CVM 45682		X	X	X		
		CVM 45684		X	X	X	X	
	kobei	CVM 45628	X		X			
	roggenkampii	CVM 45651			X		X	
	spp.	CVM 45523	X		X	X		
		CVM 45654			X			
Citrobacter	*amalonaticus*	CVM 45409	X	X	X	X		
	braakii	CVM 45602						
		CVM 45639						
		CVM 45644						
		CVM 45649						
	freundii	CVM 45411						

		CVM 45413						
		CVM 45419	X					
		CVM 45469						
		CVM 45481	X					
		CVM 45604						
		CVM 45626	X					
		CVM 45627	X					
		CVM 45635	X				X	
		CVM 45637						
		CVM 45641	X		X	X		
		CVM 45657	X	X	X	X	X	
		CVM 45664						
		CVM 45679	X	X	X	X		
	koseri	CVM 45676	X	X	X	X		
	rodentium	CVM 45693	X					
	spp.	CVM 45412		X	X	X		
		CVM 45437	X	X	X	X	X	
		CVM 45466						
		CVM 45467						
		CVM 45476		X	X	X		
		CVM 45479		X	X	X		
		CVM 45480						
		CVM 45531	X	X	X	X		
	werkmanii	CVM 45618						
		CVM 45620						
		CVM 45625	X					
		CVM 45667	X					
		CVM 45672						
Klebsiella	*aerogenes*	CVM 45485						

	oxytoca	CVM 45459	X							
		CVM 45487	X							
		CVM 45498	X							
	pneumoniae	CVM 45486								
		CVM 45506								
		CVM 45603								
		CVM 45687								
		CVM 45692						X		
Cronobacter	*spp.*	CVM 45441								
		CVM 45461			X					
		CVM 45468	X							
		CVM 45655		X	X	X				
Proteus	*mirabilis*	CVM 45451						X		
		CVM 45470						X		
		CVM 45678						X		
Pseudomonas	*aeruginosa*	CVM 45433								
		CVM 45471								
		CVM 45472								
	putida	CVM 45438								X
Enterococcus	*faecalis*	CVM 45611		X						

X denotes presence of genes indicative of tolerance within MTG type

The following genes were included when considering resistance to the corresponding metal**: Arsenic**- *arsA, arsB, arsC, arsD, arsR*; **Copper**- *pcoA, pcoB, pcoC, pcoD, pcoE, pcoR, pcoS, tcrB*; **Copper/Silver**- *silA, silB, silC, silF, silR, silS*; **Silver**- *silE, silP*; **Tellurium**- *terB, terD, terC, terE, terW, terZ*; **Mercury**- *merD, merE, merP, merR, merT*; **Cadmium**- *cadR*

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Chapter 4 - The effect of commercial sanitizers on experimentally inoculated *Listeria monocytogenes* biofilms grown on materials commonly used during tree-fruit harvesting

4.1 Abstract

This study compares the efficacy of commercially available sanitizers in reducing *L. monocytogenes* biofilms formed on surfaces commonly used during tree fruit harvesting. Biofilms were grown for 96 hours in a Centers for Disease Control (CDC) reactor at 25± 2˚C on high density polyethylene (HDPE), wood, or nylon. Bacteria were exposed to chlorine (500 ppm), peroxyacetic acid (500 ppm), steam (75 psi), or silver-dihydrogen citrate (SDC) (4%) for 1 or 2 minutes; or exposed to chlorine dioxide gas (ClO_2) (100 ppm) for 24 hours. Coupons were neutralized, and remaining cells enumerated. Treatment times were randomized, and all treatments were replicated six times. Results were considered significant at $P < 0.05$. When applied to *Listeria* biofilms, steam, chlorine, PAA, and ClO_2 significantly reduced the population as compared to untreated coupons for all surface types ($P < 0.05$). SDC affected population on HDPE at either contact time ($P < 0.05$), but not on wood or nylon coupons. Interactions between sanitizer, surface type, and exposure time should be considered by tree fruit harvesting facilities when determining what sanitation strategies should be implemented to reduce potential presence of foodborne pathogens.

4.2 Introduction

Harvesting and storage facilities often have ideal environmental conditions for bacterial growth (Killinger and Adhikari, 2014), making proper sanitation at the time of harvest an

important tool for preventing contamination. The presence of persistent foodborne pathogen, such as *Listeria monocytogenes,* pose additional risks when surfaces that are difficult to clean are used for harvesting and storage (Haddad et al., 2021).

Like many types of bacteria, *L. monocytogenes* can form biofilms on solid surfaces. Biofilms are defined as a group of microbial cells strongly attached to a surface and enclosed in an Extracellular Polymeric Substance (EPS) (Donlan, 2002). The EPS, in combination with other molecules such as proteins, polysaccharides, teichoic acids, extracellular DNA (eDNA), and flagella compose the extracellular matrix of *L. monocytogenes* biofilms (Combrouse et al., 2013; Colagiorgi et al., 2016). The physical structure of *L. monocytogenes* biofilms differs from that of some other bacterial species- *L. monocytogenes* biofilms generally do not form thick multilayered biofilms, and instead have been shown to form a variety of structures, but most strains will eventually result in honeycomb-like structures that are spread across a surface (Gram et al., 2007; Pilchová et al., 2014; Guilbald et al., 2015).

Biofilm growth differs throughout biofilm development; a study by Mosquera-Fernández et al. (2016) found that the thickness of *L. monocytogenes* biofilms, as well as the overall volume of biofilm composed of bacterial cells (biovolume), started to decrease after biofilms reached 72-96 hours old, although this was somewhat strain-dependent. Biovolume and biofilm thickness have been positively correlated (Guilbald et al., 2015); while both attributes are strain-dependent, the typical thickness after 48- hours of growth is 10- 50 µm (Guilbald et al., 2015; Mosquera-Fernández et al., 2016). Thickness and biovolume of biofilms, as well as the overall structure of EPS, can impact the ability for antimicrobials and sanitizers to effectively interact with bacterial cells within (Stewart, 2003; Chavant et al., 2004), thus offering a level of protection against population control techniques that sessile and planktonic bacteria are not afforded.

Recent foodborne outbreak episodes associated with tree fruit, including an apple recall in 2019 (FDA, 2019) and kiwifruit recall in 2023 (FDA, 2023), have been linked to *L. monocytogenes*. Often originating from soil and vegetation, *L. monocytogenes* can be present in agricultural environments where sanitation practices can be difficult to implement or maintain. The ability of this foodborne pathogen to form biofilms is a threat for Ready-to-Eat or minimally processed food such as produce. Furthermore, several outbreaks related to fruit and vegetables have been linked to poor sanitation practices during harvesting and handling (e.g. harvesting tools and equipment, traffic flow, worker health and overall facility design). Researchers have shown that *L. monocytogenes* was able to survive on experimentally inoculated and stored apples (Macarisin et al., 2019) and within packing and storage facilities (Chen et al., 2022). Tree fruit growers and packers commonly utilize harvesting containers made of porous materials like wood or nylon that can allow for increased bacterial growth, and are more difficult to sanitize. The use of chemical sanitizers represents the most common practice used to prevent microbial contamination. The variety of materials used in the produce industry, as well as physical limitations at facilities can make sanitation efforts difficult. Ensuring that information on different interventions tested on multiple surfaces is essential to helping guide picking and packing facilities to establish food safety procedures. There is limited knowledge and information available to producers on sanitation practices for the unique surfaces and equipment that are found within the harvesting and storage process.

The objective of this study was to evaluate the effectiveness of commercially available sanitizers (chlorine, peracetic acid, silver dihydrogen citrate, steam, and chlorine dioxide gas) in controlling *L. monocytogenes* biofilms formed on food contact surfaces commonly encountered during the tree fruit harvesting process (e.g. wood, nylon and plastic).

4.3 Materials and Methods

4.3.1 Bacterial strains and inoculum preparation

Three *L. monocytogenes* strains, LM 390-2 (serotype 1/2a), LM 390-6 (serotype 1/2b), environmental isolates from the 2011 cantaloupe outbreak and LM 573-035 (serotype 4b), a clinical isolate from the 2014-2015 caramel apple outbreak were used in this study. Strains were obtained from the University of Georgia and stored on Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA) plates at 4˚C until use. A bacterial cocktail was obtained as previously described (Manville et al., 2023) to reach an initial population of ~ 1 x 10^8 CFU/ml.

4.3.2 Food-contact materials

Three different materials were used for this study: wood, HDPE (high density polyethylene), and nylon fabric. These surfaces were selected due to prevalence of use among fresh fruit harvesting and storage facilities. HDPE coupons of 1.27 cm in diameter were purchased from BioSurface Technologies Corporation (Bozeman, MT, USA). The other two surfaces, unfinished basswood plywood obtained from a local store, and nylon coupons cut from a used field harvesting bag, were cut into 1.27cm diameter coupons. Biofilm growth areas were 1.27cm in diameter with populations forming on both sides of the coupons.

4.3.3 Biofilm growth

Biofilms were grown in a Center for Disease Control and Prevention (CDC) Biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) as previously described in Manville et

al., 2023. Each biofilm reactor holds eight rods: one coupon for each of the food contact surfaces being tested were placed into rods in randomly assigned positions. After a total of 96-h incubation, sanitizer treatments were applied.

4.3.4 Sanitizer treatments

Five different sanitization methods were studied to verify effectiveness on mature *Listeria* biofilms: 500 ppm liquid chlorine (Diversey, Fort Mill, SC, USA), 500 ppm peracetic acid (PAA) (Pace International, Wapato, WA, USA), Silver Dihydrogen Citrate (SDC) with 0.003% silver and 4.85% citric acid (Pure Bioscience, El Cajon, CA, USA), 75 psi steam generated by a Hill Injection™ steamer (Dupray, USA), and 100 ppm chlorine dioxide gas (ICA Trinova, Newnan, GA, USA). Fresh solutions of chlorine and PAA were made the day of application, and concentration checked using test kits (ChemWorld.com, Kennesaw, GA, USA and MCI, Wood Dale, IL, USA, respectively). The pH levels of the fresh sanitizer solutions were also confirmed to be 7.0 ± 0.5 prior to use. Steam was applied at a distance of 4 cm from the surface, and throughout application water temperature was monitored via internal sensors within the steamer to reach 174°C prior to each application. Steam temperature at both the coupon and nozzle were measured using a thermocouple (Traceable, Weber, TX, USA). ClO_2 gas generation was achieved by mixing 0.5g of precursor and 0.5g of activator in a sachet, humidity and temperature levels were recorded in 2- minute intervals using a combination hygrometer/thermometer (Traceable, Thermo-Fischer Scientific, Waltham, MA, USA).

Application time for sanitizers was determined after discussions with industry stakeholders. All liquid sanitizers (chlorine, PAA, and SDC) were applied at a rate of 400 ml/min using a diaphragm benchtop pump attached to a food-grade spray nozzle for either 1- or 2-

minute exposure times. Rods containing the coupons rotated halfway through (Figure 1). Steam was applied following manufacturer's use instructions for either 1- or 2- minute intervals, with coupons rotated halfway through the treatment. Due to manufacturer recommendations to ensure employee safety, chlorine dioxide gas was applied for a 24- hour period: coupons in a rod were placed into a 4.7L plastic container (Produce Keeper, OXO, New York, NY, USA) which was then completely sealed during exposure time.

4.3.5 Recovery and enumeration

After exposure to sanitizing agents, coupons were individually transferred into 10ml of Dey Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA, USA) for liquid chlorine, PAA, and SDC or neutralizing buffer (Difco, Sparks, MD, USA) for chlorine dioxide gas. Neutralizing assays were performed prior to starting experiments. Control coupons and steam treated coupons were instead transferred into sterile phosphate-buffered saline (PBS, VWR, Solon, OH, USA). Cells were then recovered by 3 rounds of alternating 30s sonication (40kHz and 100% amplitude, with a resulting 110W power) and 30s vortexing, following an ASTM standard method (ASTM, 2019). Serial dilutions were performed in 0.1% peptone water (Difco, Sparks, MD, USA) and spread plate on Modified Oxford Media (Difco, Sparks, MD, USA). Colonies were counted and reported as both log CFU/coupon after 24-30 hours of incubation at 37°C.

4.3.6 Experimental design and statistical analysis

For biofilm treatment, each sanitizer was given 6 replications per surface, and each sample was plated in duplicate. Untreated coupons (control) were also replicated 6 times. Data

were analyzed via PROC GLIMMIX in SAS (Cary, NC, USA). Data were considered significant at $P \leq 0.05$.

4.4 Results

The effect of sanitizer, surface type, and application time were evaluated on mature *L. monocytogenes* biofilms, with a three-way interaction between surface, treatment time, and sanitizer, two-way interaction between sanitizer and surface type, and individual effects of sanitizer, application time, and surface were found to be significant ($P \leq 0.05$) (Table 1). Untreated coupons for each surface type (HDPE, nylon, and wood) were enumerated, and the following populations (log CFU/coupon) recovered: 7.06 ± 0.25 on HDPE, 7.83 ± 0.25 on nylon, and 8.22 ± 0.25 on wood. The effect of chlorine, PAA, SDC, steam, on nylon, HDPE, and wood coupons for either 1- or 2- min of exposure time, and ClO_2 at 24- hour on *Listeria* biofilm population is shown in Table 2.

Untreated coupons for each surface type (HDPE, nylon, and wood) were enumerated, and the following populations (log CFU/coupon) recovered: 7.06 ± 0.25 on HDPE, 7.83 ± 0.25 on nylon, and 8.22 ± 0.25 on wood. The effect of chlorine, PAA, SDC, steam, on nylon, HDPE, and wood coupons for either 1- or 2- min of exposure time, and ClO_2 at 24- hour on *Listeria* biofilm population is shown in Table 2.

Application time did not significantly impact survival across surface type when chlorine was applied. No significant difference in population on wood was seen at either time (7.85 ± 0.32 log CFU/coupon at 1-min, 7.49 ± 0.32 log CFU/coupon at 2-min, $P = 0.37$ and $P = 0.73$, respectively). Conversely, the remaining populations on nylon coupons (6.52 ± 0.32 log CFU/coupon at 1-min, 5.87 ± 0.32 log CFU/coupon at 2-min) treated with chlorine was

significantly different as compared to the recovered population on wood (P = 0.002 and P <.0001, respectively). Similarly, a significant difference in recovered population was also observed on HDPE coupons when chlorine was applied: 3.52 ± 0.32 log CFU/coupon at 1-min (P <0.001), and 3.16 ± 0.32 log CFU/coupon at 2-min (P <0.001).

When compared to untreated coupons, differences in *Listeria* biofilm population were observed when PAA was used: nylon exposed to PAA for 1-min (4.94 ± 0.25 log CFU/coupon) had lower population (P <.0001 for both exposure times) than wood surfaces at either time point.

Different application time for SDC did not result in significant reduction of *Listeria* biofilm population on either wood (7.82 ± 0.32 log CFU/coupon at 1-min, 7.80 ± 0.32 log CFU/coupon at 2-min) or nylon (7.37 ± 0.32 log CFU/coupon at 1-min, 7.33 ± 0.32 log CFU/coupon at 2-min). Conversely, recovered populations on HDPE (5.53 ± 0.32 log CFU/coupon at 1-min, 5.82 ± 0.32 log CFU/coupon at 2-min) was statistically different than the control, but this reduction was not impacted by application times (P> 0.05).

HDPE surfaces had the lowest levels of recovered population after exposure to steam, with no difference between contact times (3.66 ± 0.32 log CFU/coupon at 1-min, 2.99 ± 0.32 log CFU/coupon at 2-min, P= 0.88). Reduction amounts on nylon and wood coupons after steam treatment were also not impacted by contact time. The population recovered on nylon after 1- and 2-min treatment were 6.31 ± 0.32 log CFU/coupon and 6.13 ± 0.32 log CFU/coupon respectively (P= 0.65); while 7.10 ± 0.32 log CFU/coupon and 6.41 ± 0.32 log CFU/coupon were the counts observed after treating wood for 1 and 2 min (P = 0.09).

Chlorine dioxide gas was overall the most effective method of controlling *Listeria* biofilms: regardless of surface, a reduction below detectable levels (2.40 log CFU/coupon) was observed after 24 hours (P <.0001).

4.5 Discussion

The results of this study indicate that interactions between surface, sanitizer, and contact time are important to consider when assessing cleaning and sanitation strategies to control *L. monocytogenes* biofilms on harvesting bins and picking bags. Across all sanitizers and application times, excluding ClO_2, wood surfaces generally showed lower levels of reduction in comparison to HDPE. Three oxidative sanitizers (chlorine, PAA, and ClO_2) were tested in our study: PAA and chlorine statistically reduced the population on HDPE. Hua et al., (2019) found that increasing sanitizer contact time on food contact surfaces such as stainless-steel, low-density polyethylene (LDPE), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) resulted in larger reduction in *L. monocytogenes* biofilm population. Our data did not show a difference in population between exposure time on HDPE surfaces ($P > 0.05$), a difference on nylon surfaces after treatment with PAA was noted over time. This could suggest that contact time during use of liquid oxidative sanitizers on more textured surfaces like nylon should be considered to mitigate *Listeria* biofilms.

Like the other oxidative sanitizers tested in our study, research on the efficacy of ClO_2 gas used against bacterial pathogens including *L. monocytogenes* on a variety of surfaces and contact times is readily available (Vaid et al., 2010; Trinetta et al., 2012; Kim & Park, 2022) , and consistently shows lower populations recovered post-exposure. This is consistent with the results of this study, where after a 24-hr contact time a complete reduction of the *L. monocytogenes* biofilms on all surfaces was observed. ClO_2 has a higher oxidation potential than other oxidative sanitizers (Bernarde et al., 1965), which could explain the more extensive cell death than other sanitizers tested in this study. The use of ClO_2 might be an effective way to control biofilm growth, on harvesting and storage surfaces and could be used at facilities to treat

large quantities of equipment at one time. Unlike spray application antimicrobials, there is limited need for manpower during the actual treatment process.

Our study also tested two non-oxidative sanitizers, including a chemical (SDC) and non-chemical (steam) option. The liquid application of SDC disrupts cell membrane proteins and results in cell death (Pure Bioscience Inc., 2016). Previous studies have shown that the use of SDC on stainless steel and aluminum surfaces inoculated with sessile *Listeria spp*. resulted in a significant population reduction (Masuku et al., 2014). In another study, SDC was used in combination with glycerin and lactic acid and a significant decrease of *L. monocytogenes* and *E. coli* on produce was observed (Mendes-Oliveira et al., 2022). Nevertheless, the data obtained in our experiments indicated limited effect on experimentally inoculated surfaces that were more porous, like nylon and wood. This may be due in part to the different characteristics of the surfaces and how that impacts biofilm adherence as reported by Manville et al. (2023).

Steam is a non-chemical sanitizer that uses thermal inactivation. Previous studies by Ban et al. (2014) and Hua et al. (2021) have investigated the efficacy of dry, or saturated steam, on *Listeria* biofilms grown on a variety of food contact surfaces, including stainless steel, polyvinyl chloride (PVC), polyethylene terephthalate (PET), low density polyethylene (LDPE), and rubber. The authors found that the use of saturated steam at 175°C on 7-day old *L. monocytogenes* biofilm resulted in a 1.86-4.84 log CFU/coupon and 3.72- 4.49 log CFU/coupon reduction on PVC and stainless-steel coupons, respectively, after exposure times between 5-30 seconds. The authors noted that on both surfaces at the longest treatment time (30 seconds), the remaining populations were below the limit of detection. Hua et al. (2021) also tested the efficacy of saturated steam: at 100°C reduction of 2.7- 4.5 log CFU/coupon on PVC, 4.0- 6.4 log CFU/coupon on stainless steel, 2.8- 4.2 log CFU/coupon on LDPE, 3.0- 4.8 log CFU/coupon on

PET and 2.6- 3.3 log CFU/coupon on rubber were observed after treatment times ranging between 6 and 180 seconds. While our study utilized unsaturated steam, all surfaces tested showed a reduction in biofilm population compared to untreated control at both timepoints tested. While these results demonstrate that steam may be an effective, non-oxidative method for biofilms control on a variety of surfaces, the small size of area being treated should be considered as a limitation in our study. Additionally, the method of production and type of steam being used for treatment should be considered.

4.6 Conclusion

Understanding how sanitizers impact *L. monocytogenes* populations in both biofilm form on surfaces commonly used within tree fruit harvesting facilities can help inform sanitation practices and techniques. In our study we observed that the three different oxidative sanitizers (chlorine, PAA, and chlorine dioxide) and steam resulted in statistically significant population differences on *Listeria* biofilms on all three surfaces at all timepoints tested. Characterization of the efficacy of such products on surfaces that have potential to harbor *Listeria* and form biofilms can help harvesting and packaging facilities make informed decisions surrounding sanitation practices as well as what surfaces to allow use of during the picking and packing processes.

Table 4.1. Type 3 fixed effects comparing experimental factors when *L. monocytogenes* biofilms were exposed to different sanitizers (1- or 2- minute contact time of steam, chlorine, peracetic acid, or silver dihydrogen citrate or 24-hour contact time for chlorine dioxide).

Effect	**Pr>F**
Sanitizer	<0.05
Application Time	<0.05
Sanitizer*Application Time	0.25
Surface	<0.05
Sanitizer*Surface	<0.05
Application Time*Surface	0.07
Sanitizer*Application Time*Surface	0.01

Table 4.2, Log (CFU/coupon) of *L. monocytogenes* biofilm on common food contact surfaces when treated with sanitizers.

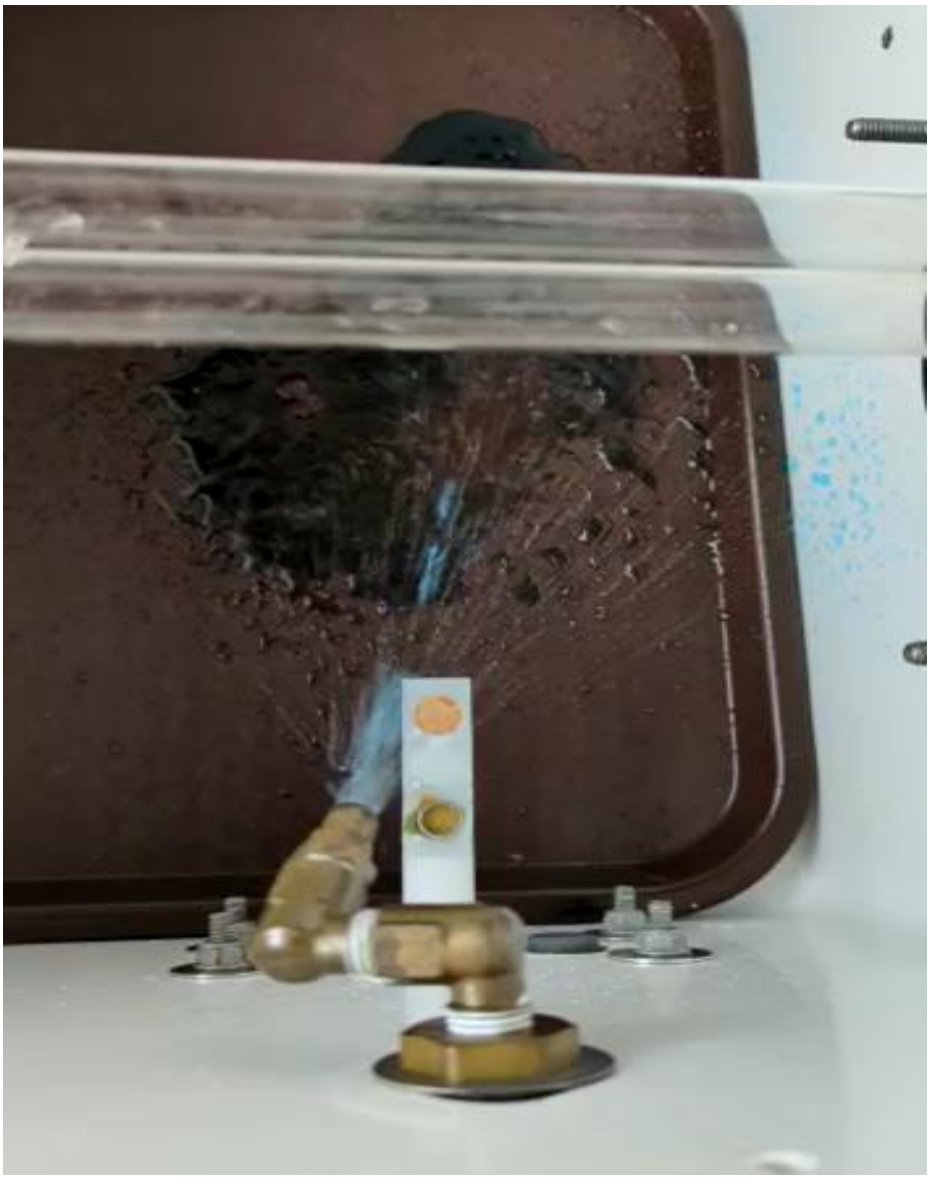
Sanitizer	Treatment Time	Surface	Mean Log (CFU/coupon)
Untreated		HDPE	7.06 ± 0.25
		NYLON	7.83 ± 0.25
		WOOD	8.22 ± 0.25
Chlorine	1 min	HDPE	$3.52 \pm 0.32^{*e}$
		NYLON	$6.52 \pm 0.32^{*bc}$
		WOOD	7.85 ± 0.32^{a}
	2 min	HDPE	$3.16 \pm 0.32^{*ef}$
		NYLON	$5.87 \pm 0.32^{*cd}$
		WOOD	7.49 ± 0.32^{a}
Peracetic acid	1 min	HDPE	$2.61 \pm 0.25^{*ef}$
		NYLON	$4.94 \pm 0.25^{*c}$
		WOOD	$7.30 \pm 0.25^{*a}$
	2 min	HDPE	$3.27 \pm 0.25^{*de}$
		NYLON	$3.16 \pm 0.25^{*def}$
		WOOD	$7.02 \pm 0.25^{*ab}$
Silver dihydrogen citrate	1 min	HDPE	$5.53 \pm 0.32^{*d}$
		NYLON	7.37 ± 0.32^{b}
		WOOD	7.82 ± 0.32^{ab}
	2 min	HDPE	$5.82 \pm 0.32^{*cd}$
		NYLON	7.33 ± 0.32^{b}
		WOOD	7.80 ± 0.32^{ab}
Steam	1 min	HDPE	$3.66 \pm 0.32^{*e}$
		NYLON	$6.31 \pm 0.32^{*cd}$
		WOOD	$7.10 \pm 0.32^{*ab}$
	2 min	HDPE	$2.99 \pm 0.32^{*ef}$
		NYLON	$6.13 \pm 0.32^{*d}$
		WOOD	$6.41 \pm 0.32^{*bcd}$
Chlorine Dioxide	24 hr	HDPE	$2.40 \pm 0.25^{*a}$
		NYLON	$2.40 \pm 0.25^{*a}$
		WOOD	$2.40 \pm 0.25^{*a}$

* indicates significant difference from control of same surface type.

Lowercase letters compare surface type and application time within sanitizer.

$P \leq 0.05$ is considered significant

Figure 4.1. . Rod after removal from CDC biofilm reactor with 3 coupons inserted into spray application box.



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Chapter 5 - The effect of commercial sanitizers on experimentally inoculated *E. coli* biofilms grown on materials commonly used during tree-fruit harvesting

5.1 Abstract

This study compares the efficacy of five commercially available sanitizers in reducing *E. coli* biofilms formed on surfaces commonly used for tree fruit harvesting. Biofilms were grown for 96 hours in a Centers for Disease Control (CDC) reactor at 25± 2˚C on high density polyethylene plastic (HDPE), wood, or nylon. Bacteria were exposed to chlorine (500 ppm), chlorine dioxide gas (ClO_2) (100 ppm), peroxyacetic acid (500 ppm), steam (75 psi) and silver-dihydrogen citrate (SDC) (4%) at varying timepoints (1- or 2- minute for all sanitizers other than ClO_2, which had a 24- h contact time). Coupons were neutralized, and remaining cells enumerated. Treatments were randomized, and replicated six times. A significant interaction between sanitizer, application time, and surface ($P = 0.0011$) was observed. All treatments at all exposure times resulted in a significant reduction ($P< 0.05$) in recovered population as compared to untreated controls for the same surface type. Overall recovered population was lower on HDPE surfaces than untreated control ($P< 0.05$) for all treatments with the exception of 2-min application of PAA. Fruit tree harvesting and packing facilities should consider any potential interactions between sanitizer, surface type, and exposure time when implementing strategies to reduce potential foodborne pathogens.

5.2 Introduction

The presence of *Escherichia coli* (*E. coli*) in production facilities has been of growing concern within the fresh produce industry for the last decade. While many outbreaks have been linked to leafy greens (Marshall et al., 2020), concern surrounding the ability of pathogenic *E. coli* to contaminate crops grown on trees has also increased. Preliminary research done after multiple *E. coli* outbreaks in the 1990's linked to unpasteurized apple cider has indicated that the bacteria is able to be spread by pests and grow on the surfaces of apples (Janisiewicz et al., 1999). Between the years of 2001-2021, the CDC reported 11 *E. coli* outbreaks linked to either consumption of apples or unpasteurized apple cider (CDC), indicating that tree fruit and minimally processed byproducts should be considered a food safety risk.

Biofilms, which are groups of microbial cells that are strongly attached to a surface while being encapsulated within an Extracellular Polymeric Substance (EPS) (Donlan, 2002), can form when bacteria come into contact with a surface. EPS has been shown to impact sanitizer efficacy against bacteria within biofilms, allowing for survival after exposure (Chavant et al., 2004). Research has shown that fresh produce that is handled is more likely to become contaminated (Bartz et al., 2017); this is of concern for commodities that are handled during the harvesting process but are unlikely to undergo a kill step, such as tree fruit, leafy greens, or melons.

Tree fruit growers and packers typically utilize harvesting containers made of surfaces that are considered to be porous, such as wood or nylon. It has been showing that surfaces and conditions within tree fruit harvesting and storage facilities are ideal for bacterial growth (Killinger & Adhikari, 2014), so understanding the specific sanitation challenges presented by use of those surfaces is important when establishing effective cleaning and sanitation protocols. Utilizing both chemical and non-chemical sanitizers is common practice within other areas of

fresh produce handling and processing; however, there is limited knowledge surrounding the efficacy of such sanitizers on surfaces like nylon, wood, and high-density polyethylene (HDPE), which are commonly used during the tree fruit harvesting process.

The objective of this study was to evaluate the efficacy of multiple commercially available sanitizers (chlorine dioxide gas (ClO_2), steam, silver dihydrogen citrate (SDC), peracetic acid (PAA), and liquid chlorine) on controlling multi-strain *E. coli* biofilms formed on food contact surfaces such as HDPE, nylon and wood, which commonly found during the tree fruit harvesting process.

5.3 Materials and Methods

5.3.1 Bacterial strains and inoculum preparation

Three strains of Shiga toxin-producing *E coli* (STEC) were used for this study: *E. coli* O157:H7 KSU 31 from an apple juice outbreak, *E. coli* O104:H4 a clinical isolate from the 2011 German sprouts outbreak, and *E. coli* O45:H2 from a clinical isolate. Strains were obtained from the Kansas State University- Food Safety and Microbiology Laboratory collection and stored on Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA) plates at 4˚C until use. A bacterial cocktail was obtained as previously described (Manville et al., 2023) to reach an initial population of ~ 1 x 10^8 CFU/ml.

5.3.2 Food contact materials

Three different materials were used for this study: HDPE (high density polyethylene), wood, and nylon fabric. These surfaces were selected due to prevalence of use among fresh fruit harvesting and storage facilities. HDPE coupons of 1.27 cm in diameter were purchased from

BioSurface Technologies Corporation (Bozeman, MT, USA). The other two surfaces, unfinished basswood plywood obtained from a local store, and nylon coupons were cut from a used field harvesting bag, into 1.27cm diameter coupons. Biofilm growth areas were 1.27cm in diameter with populations forming on both sides of the coupons.

5.3.3 Biofilm growth

Biofilms were grown in a Center for Disease Control and Prevention (CDC) Biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) for 96 hours at 25±3 ˚C. The reactor has eight rods holding 3 coupons each. Each of the tested food contact surfaces were randomly assigned a position in each rod. One ml of the cocktail obtained from the three *E. coli* strains was used to inoculate the reactor. An initial batch phase was maintained for 24-h in 1.5 g/L TSB (Tryptic Soy Broth, BD Difco, Sparks, MD) with constant agitation at 70 rpm. Subsequently a continuous flow of sterile 0.5 g/L TSB nutrient at 7 ml/min was provided. The reactor was maintained with constant agitation for an additional 72-h. After a total of 96-h incubation, rods were removed and sanitizer treatments were applied.

5.3.4 Sanitizer treatments

A variety of sanitization methods were utilized within this study; three with liquid spray application (500 ppm liquid chlorine (Diversy, Fort Mill, SC, USA), 500 ppm peracetic acid (Pace International, Wapato, WA, USA), and silver dihydrogen citrate (SDC) with 0.003% silver and 4.85% citric acid (Pure Bioscience, El Cajon, CA, USA)), 75 psi steam generated by a Hill Injection™ steamer (Dupray, USA), and 100 ppm chlorine dioxide (ClO_2) gas (ICA Trinova, Newnan, GA, USA). Prior to application to mature *E. coli* biofilm, fresh solutions of chlorine

and peracetic acid were made and concentration checked using test kits, and pH levels were measured to be 7.0 ± 0.5 (ChemWorld.com, Kennesaw, GA, USA and MCI, Wood Dale, IL, USA, respectively). Steam application temperature was monitored via internal sensors within the steamer to reach 174°C prior to each application. Thermocouples (Traceable, Weber, TX, USA) located at both the coupon and nozzle were used to measure steam temperature at those locations throughout application. Throughout steam application, the nozzle was held 4 cm away from the surface being treated. Generation of ClO_2 was achieved by mixing 0.5g of precursor and 0.5g of activator in a sachet. Humidity and temperature levels were recorded in 2- minute intervals throughout exposure time using a combination hygrometer/thermometer (Traceable, Thermo-Fischer Scientific, Waltham, MA, USA).

All liquid sanitizers (chlorine, peracetic acid, and SDC) were applied at a rate of 400 ml/min using a diaphragm benchtop pump attached to a food-grade spray nozzle for either 1- or 2-minute exposure times, with the rods containing the coupons rotated halfway through. Steam was applied following manufacturer's use instructions for either 1- or 2- minute intervals, with coupons rotated halfway through treatment time. Treatment with ClO_2 gas was performed for a 24- hour period. Gas was generated by mixing 0.5g of precursor and 0.5g of activator in a sachet, humidity and temperature levels were recorded in 2- minute intervals using a combination hygrometer/thermometer (Traceable, Thermo-Fischer Scientific, Waltham, MA, USA). Coupons in a rod were placed into a 4.7L plastic container (Produce Keeper, OXO, New York, NY, USA) which was then completely sealed during exposure.

5.3.5 Recovery and enumeration

After exposure to sanitizing agents, coupons were individually transferred into 10ml of Dey Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA, USA) for liquid chlorine, peracetic acid, and SDC or neutralizing buffer (Difco, Sparks, MD, USA) for chlorine dioxide gas. Neutralizing assays were performed prior to starting experiments. Control coupons and steam treated coupons were instead transferred into sterile phosphate-buffered saline (PBS, VWR, Solon, OH, USA). Cells were then recovered by 3 rounds of alternating 30s sonication (40kHz and 100% amplitude, with a resulting 110W power) and 30s vortexing, following an ASTM standard method (ASTM, 2019). Serial dilutions were performed in 0.1% peptone water (Difco, Sparks, MD, USA) and spread plate on Tryptic Soy Agar (Difco, Sparks, MD, USA). Colonies were counted and reported as both log CFU/coupon and log CFU/cm^2 after 24-30 hours of incubation at 37°C.

5.3.6 Experimental design and statistical analysis

For biofilm treatment, each sanitizer was given 6 replications per surface, and each sample was plated in duplicate. Untreated coupons (control) were also replicated 6 times. Data were analyzed via PROC GLIMMIX in SAS (Cary, NC, USA). Results were considered significant at $P \leq 0.05$.

5.4 Results

Evaluation of mature biofilm recovered populations after exposure to sanitizers were performed. For *E. coli* biofilms, a significant interaction was noted between sanitizer, application time, and surface type ($P \leq 0.05$) (Table 1). Across exposure timepoints, all surfaces, and all

sanitizers, biofilm populations were significantly different than untreated controls grown on HDPE, nylon, and wood (7.70 ± 0.27, 8.83 ± 0.27, and 8.56 ± 0.27 log CFU/coupon, respectively) (Table 2). Recovered population was lowest for surfaces exposed to ClO_2 for a 24-hour period (2.40 ± 0.31 log CFU/coupon for all surfaces).

Nylon and wood surfaces did not have a difference in population after increasing exposure time for steam from 1- to 2- minutes (5.13 ± 0.32 vs 5.63 ± 0.32 log CFU/coupon for nylon, 5.72 ± 0.32 vs 6.11 ± 0.32 log CFU/coupon wood at 1- and 2- min exposure, respectively). On HDPE, recovered biofilm populations were statistically different after 2-min exposure (2. 54 ± 0.32 log CFU/coupon) compared to 1-min exposure (3.44 ± 0.32 log CFU/coupon).

Recovered biofilm populations after treatment with SDC were significantly different when compared to untreated controls. An increase of exposure time to 2-min did result in a significant population difference on wood (6.96 ± 0.32 log CFU/coupon). Exposure time to SDC did not impact recovered biofilm population on HDPE (6.20 ± 0.32 log CFU/coupon at 1-min vs 6.06 ± 0.32 log CFU/coupon at 2-min), but longer exposure time did reduce recovered populations grown on nylon (7.56 ± 0.32 log CFU/coupon at 1-min vs 6.77 ± 0.32 log CFU/coupon at 2-min).

Biofilms grown on HDPE had similar recovered population at both 1- and 2-min PAA exposure times (2.44 ± 0.32 vs. 2.40 ± 0.32 log CFU/coupon). Similar recovered populations were observed on wood (6.19 ± 0.32 vs. 6.65 ± 0.32 log CFU/coupon). Exposure time did impact recovered population for biofilms grown on nylon, with 2-min exposure resulting in a decreased population when compared to 1-min (2.45 ± 0.32 log CFU/coupon and 4.75 ± 0.32 log CFU/coupon, respectively).

Similarly to the biofilm populations recovered after exposure to PAA, there was not a difference in population between exposure times for chlorine when biofilms were grown on HDPE (4.05 ± 0.32 log CFU/coupon at 1-min vs 3.81 ± 0.32 log CFU/coupon at 2-min) or wood (7.61 ± 0.32 log CFU/coupon at 1-min and 7.29 ± 0.32 log CFU/coupon at 2-min). Longer exposure to chlorine did result in a lower population of biofilms on nylon surfaces (6.59 ± 0.32 log CFU/coupon after 1-min vs 5.44 ± 0.32 log CFU/coupon after 2-min).

5.5 Discussion

The use of ClO_2 as a mitigation strategy for bacterial growth on fresh produce has been well documented (Han et al., 2000; Du, Han, & Linton, 2002; Sy et al., 2005). The use of ClO_2 against biofilms grown on food contact surfaces has also been investigated. Kim & Park, 2022, found that exposure to 50 ppmv ClO_2 for 20 minutes was able to reduce population levels below detectable limits on both stainless steel and HDPE surfaces, but also noted that relative humidity did factor into efficacy. Our data, which was obtained after 24-h exposure at 80% relative humidity, showed similar levels of population reduction across all three surfaces tested to below the limit of detection. Chlorine dioxide is known to be a strong oxidizer; the gaseous form may allow for better penetration of the biofilm structure compared to aqueous chlorine application (Prodduk et al., 2014), which could explain why we were unable to recover viable cells.

Steam inactivation has been tested against mature multi-strain *E. coli* biofilms grown on both stainless steel and PVC coupons, with lower levels of recovered population noted on stainless steel surfaces versus PVC (Park & Kang, 2014). Similarly, our data showed lower recovered populations on HDPE when compared to nylon and wood coupons, indicating that surface texture and porosity should be considered prior to implementing steam treatment as a

sanitation technique. Steam has also been shown to have potentially synergistic effects when combined with chemical sanitizer use against biofilms (Ban & Kang, 2016), which could be further investigated in future research.

Instead of utilizing oxidation, SDC application disrupts proteins within the cell membrane, resulting in cell death (Pure Bioscience Inc., 2016). Previous studies (Mendes-Oliveira et al., 2022) have shown that when used in combination with glycerin and lactic acid, a significant population reduction of *E. coli* on experimentally inoculated lettuce was observed. Our study looked only at the efficacy of SDC alone, and was able to result in a difference in population after both 1- and 2- min exposure time for HDPE, wood, and nylon surfaces. Previous studies investigating the use of SDC on *Listeria* biofilms have shown that surface characteristics such as porosity can impact biofilm adherence (Manville et al., 2023); biofilm growth on wood surfaces compared to the HDPE and nylon surfaces used in this study could contribute to the differences in efficacy observed.

The efficacy of two different liquid oxidative sanitizers were tested for this study; PAA and chlorine. PAA has been shown to be effective when used in a wash step applied directly to fresh produce (Banach et al., 2020), as well as on worn polyethylene surfaces (Beltrame et al., 2016). The other oxidative sanitizer tested for this study was liquid chlorine. Wang et al., 2012 analyzed the efficacy of chlorine applied at 200 ppm to mature single-strain *E. coli* biofilms grown on polystyrene. They observed that efficacy of chlorine did seem to be strain-dependent: while some individual strains of *E. coli* in the Wang et al. study showed similar recovery of population between 2- min exposure vs untreated surfaces to our HDPE results, other strains had either higher or lower recovery. It should be noted that our data was obtained utilizing a 3-strain cocktail of *E. coli*, as opposed to single-strain data. Our data show that for both PAA and

chlorine, exposure time did not impact recovered population on HDPE or wood surfaces, but did result in lower recovered population after 2-min of exposure on nylon; this could be due to the differences in surface characteristics between the materials.

5.6 Conclusion

Understanding how surface type impacts sanitizer efficacy can help inform sanitation practice decisions for facilities that are harvesting and storing tree fruits. The data obtained in this study indicated that while almost all sanitizers and timepoints tested did result in a difference in recovered population on all surfaces as compared to the controls, the remaining population after exposure was impacted by surface type. For all spray applications tested, HDPE surfaces had significantly lower recovered populations than wood surfaces, indicating that the use of wood within tree fruit harvesting may increase the difficulty to eliminate any potential bacterial growth present. In comparison, populations recovered on nylon surfaces were less predictable and differences between nylon and other surface type populations were dependent upon sanitizer type. Facilities should consider different sanitation practices based on the type of materials used for produce harvesting and storage.

Table 5.1. Type 3 fixed effects comparing experimental factors when *E. coli* biofilms were exposed to different sanitizers (1- or 2- minute contact time of steam, chlorine, peracetic acid, or silver dihydrogen citrate or 24-hour contact time for chlorine dioxide).

Effect	**Pr>F**
Sanitizer	<0.0001
Application Time	0.0018
Sanitizer*Application Time	0.2825
Surface	<0.0001
Sanitizer*Surface	<0.0001
Application Time*Surface	0.0081
Sanitizer*Application Time*Surface	0.0011

Table 5.2. Log (CFU/coupon) of *E. coli* biofilm on common food contact surfaces when treated with sanitizers.

Sanitizer	Treatment Time	Surface	Mean Log (CFU/coupon)
Untreated		HDPE	7.70 ± 0.27
		NYLON	8.83 ± 0.27
		WOOD	8.56 ± 0.27
Chlorine	1 min	HDPE	$4.05 \pm 0.32^{*ef}$
		NYLON	$6.59 \pm 0.32^{*bc}$
		WOOD	$7.61 \pm 0.32^{*a}$
	2 min	HDPE	$3.81 \pm 0.32^{*f}$
		NYLON	$5.44 \pm 0.32^{*d}$
		WOOD	$7.29 \pm 0.32^{*ab}$
PAA	1 min	HDPE	$2.44 \pm 0.32^{*d}$
		NYLON	$4.75 \pm 0.32^{*c}$
		WOOD	$6.19 \pm 0.32^{*ab}$
	2 min	HDPE	$2.40 \pm 0.32^{*d}$
		NYLON	$2.45 \pm 0.32^{*d}$
		WOOD	$6.65 \pm 0.32^{*abc}$
SDC	1 min	HDPE	$6.20 \pm 0.32^{*de}$
		NYLON	$7.56 \pm 0.32^{*ab}$
		WOOD	$7.42 \pm 0.32^{*abc}$
	2 min	HDPE	$6.06 \pm 0.32^{*e}$
		NYLON	$6.77 \pm 0.32^{*bcde}$
		WOOD	$6.96 \pm 0.32^{*abcd}$
Steam	1 min	HDPE	$3.44 \pm 0.32^{*e}$
		NYLON	$5.13 \pm 0.32^{*cd}$
		WOOD	$5.72 \pm 0.32^{*abc}$
	2 min	HDPE	$2.54 \pm 0.32^{*f}$
		NYLON	$5.63 \pm 0.32^{*bcd}$
		WOOD	$6.11 \pm 0.32^{*ab}$
ClO_2	24 hr	HDPE	$2.40 \pm 0.31^{*a}$
		NYLON	$2.40 \pm 0.31^{*a}$
		WOOD	$2.40 \pm 0.31^{*a}$

* indicates significant difference from control of same surface type.

Lowercase letters compare surface type and application time within sanitizer.

$P \leq 0.05$ is considered significant

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Chapter 6 - A validation study for the tree fruit industry: the effectiveness of silver dihydrogen citrate and chlorine dioxide gas (ClO2) as sanitation strategies for picking bags and storage bins

6.1 Abstract

Foodborne outbreaks and recalls within the tree fruit industry are making producers re-evaluate appropriate cleaning and sanitation practices during harvesting. Without effective sanitation, bacteria can create niches and eventually form biofilms. This study evaluates the efficacy of silver dihydrogen citrate (SDC) and chlorine dioxide (ClO_2) gas to control *Escherichia coli* (*E. coli*) and *Listeria innocua* on experimentally inoculated harvesting equipment at commercial orchards within the Midwest and Pacific Northwest (PNW) regions. Rifampicin resistant *E. coli* and *Listeria* were grown for either 24-hours (sessile form) or 96-hours (biofilm form) in 80μg/ml rifampicin tryptic soy broth (TSB) at 25± 2˚C on high density polyethylene plastic (HDPE), wood, or nylon coupons. Surfaces were allowed to dry for one hour and then exposed to ClO_2 (100 ppm) for 24-hours or SDC (4%) for 2 minutes. Coupons were swabbed, and surviving populations were enumerated. Experiments were conducted in triplicate and results significance was established at $P< 0.05$. ClO_2 was the most effective treatment ($P<0.05$) in controlling sessile *E. coli* and *Listeria* on HDPE and nylon in the Midwest and PNW. A lower level of inactivation was observed for biofilms grown on wood after ClO_2 treatment ($P<0.05$). SDC did not reduce the population of sessile *E. coli* on HDPE in the PNW region. Neither *E. coli* bacterial forms were significantly reduced ($P > 0.05$) by SDS when grown on nylon, independently of the region. Conversely, biofilm population was reduced on HDPE after SDS exposure in both regions and bacteria tested ($P < 0.05$). Bacterial populations grown

on wood surfaces were not reduced after exposure to SDC, regardless of form or bacteria type ($P > 0.05$). Validating sanitation procedures in various field conditions can help producers understand the most effective and convenient strategies to mitigate microbial risks for harvesting bins and picking bags sanitation.

6.2 Introduction

Recent foodborne illness outbreaks spread via tree fruits, such as the 2019 multi-state apple recall due to possible *Listeria* contamination (FDA, 2019), has resulted in increased scrutiny and growing concern surrounding sanitation practices during harvesting and storing produce. Little information is available surrounding sanitizer efficacy for surfaces commonly found within apple harvesting facilities. While research projects have focused on microbial post-harvest mitigation (Pietrysiak, Smith, & Ganjyal, 2019), there is still the need to identify interventions for use within harvesting and packinghouse environments. Current industry practices involve storing harvested fruit in bins for 9-12 months at a time in controlled environments, then moving empty bins to outdoor storage until needed for subsequent harvest. The constant use can create challenges for regular cleaning and sanitation of equipment, which has been identified as a potential cause of previous foodborne illness outbreaks (Carstens, Salazar, & Darkoh, 2019).

Because of the unique challenges that facility harvesting and storage practices, as well as the surfaces themselves, gaining an understanding of how a variety of sanitizers with different application methods and modes of action can be utilized to mitigate foodborne pathogens is important. Based on previous laboratory studies (data not published) where we evaluated the effectiveness of five commercially available sanitizers to control the growth of *Listeria, E. coli,*

and *Salmonella* on wood, nylon, and high-density polyethylene coupons, two strategies were selected for this validation study: silver dihydrogen citrate (SDC), a non-oxidative liquid sanitizer, and exposure to oxidative ClO_2 gas to control *E. coli* and *L. innocua* in both sessile and biofilm forms on experimentally inoculated harvesting equipment at commercial orchards within the Midwest and Pacific Northwest (PNW) regions.

Located within the Pacific Northwest region of the United States, the state of Washington is responsible for approximately two-thirds of the national domestic apple crop (UDSA, 2024). The region has multiple large-scale production facilities with high throughput and orchards may also handle other tree fruit as well. Two facilities within this region were identified as host sites for this validation study to represent high-throughput facilities. Smaller-scale apple and tree fruit production is spread throughout various other regions of the United States, in areas that have different environmental and climate factors than the Pacific Northwest (PNW). In order to account for differences within large- and small-scale operations, as well as potential climate impacts, two small-scale facilities located within the Midwest region (Kansas and Iowa) were also selected for this validation study. While apple production within regions outside of the PNW and Eastern US accounts for much less of the overall national production, there are numerous small orchards spread throughout the Midwest. The Midwest climate can present unique challenges for apple production, however there are numerous small family-owned orchards throughout the region. By testing sanitizers at both small- and large-scale facilities in two unique regions of the country, we hope to be able to provide information on sanitizer efficacy when applied within harvesting and packing facilities in order help inform sanitation strategies.

6.3 Materials AND Methods

6.3.1 Bacterial strains and inoculum preparation

Two strains of BSL-1 rifampicin-resistant bacteria were used for this trial: *E. coli* TVS 353 and *L. innocua* ATCC 51742 were obtained from the University of Georgia and stored on CyroCare Bacteria Preserver beads (Key Scientific, Stamford, TX) and kept at −80 °C until the experiment. Prior data has validated strains as surrogate strains for shiga toxin-producing *E. coli* and *Salmonella enterica,* and *Listeria monocytogenes*, respectively. One week before experiments, rifampicin-resistance was verified by plating strains on TSA with 80 µg/ml rifampicin. Isolates were maintained in TSA plates with 80 µg/ml rifampicin at 4˚C in dark conditions until use. Initial inoculum was obtained by placing a colony into Tryptic Soy Broth (TSA, Difco, Sparks, MD, USA) with 80 µg/ml rifampicin and incubating for 24 hours at 37 ± 2˚C in the dark to reach an initial population of ~ 1 x 10^8 CFU/ml.

6.3.2 Field trial locations

Four different facilities were recruited to take part in this study. A vertically integrated facility harvesting multiple species of tree fruit, and one large scale apple facility with automated packing lines processing over 7 million boxes annually were selected in the Pacific Northwest (PNW) region. Within the Midwest, smaller family-owned sites were involved in this research: a 35-acre tree fruit facility, and a 15-acre facility growing cider variety apple trees. All facilities provided access to a portion of their facilities that allowed sanitizers to be tested on equipment within the facility environments that would be found during typical operation.

6.3.3 Food contact materials

Three different materials were used for this study: wood, HDPE (high density polyethylene), and nylon fabric. These surfaces were selected due to the prevalence of use among fresh fruit harvesting and storage facilities. Coupons that measured 10 cm × 10 cm were cut from facility-used HDPE produce bin, wooden bins, or from nylon field harvesting bags.

6.3.4 Sessile growth

Cleaned and sanitized coupons were placed into individual stomacher bags (Nasco, Pleasant Prairie, WI, USA). Subsequently, 100ml TSB containing 80 µg/ml rifampicin and 1 ml of either *E. coli* or *L. innocua* inoculum was added into each bag with a coupon. Each bag was closed and placed so that coupons were submerged within the liquid. Bags containing the coupons were incubated for 24-h at 25 ± 2˚C in an opaque container. Once at the facility, coupons were removed aseptically and hung or positioned onto harvesting bags or bins made up of the respective surface type. Coupons were allowed to dry for 1-hr at ambient temperature prior to sanitizer application.

6.3.5 Biofilm growth

Cleaned and sanitized coupons were placed into sterilized glass containers (Pyrex, Rosemont, IL, USA). At the start of the experiment, 200 mL TSB containing 80 µg/mL rifampicin and 2 mL of either the *E. coli* or *L. innocua* inoculum was placed into each container with a coupon. Containers were sealed and placed into an opaque container. Surfaces were incubated at 72-h at 25 ± 2˚C . Once at the facility, coupons were removed aseptically and hung

or positioned as needed. Coupons were allowed to dry for 1-hr at ambient temperature prior to sanitizer application.

6.3.6 Sanitizer treatment

Coupons were either exposed to Silver Dihydrogen Citrate (SDC) with 0.003% silver and 4.85% citric acid (Pure Bioscience, El Cajon, CA, USA) for 2-minutes or 100 ppm chlorine dioxide (ClO_2) gas (ICA Trinova, Newnan, GA, USA) for 24-hours. SDC was applied to coupons clipped to their respective surface utilizing a Chapin™ SureSpray™ 1 Gallon Tank Sprayer (Chapin, Batavia, NY, USA) (Figure 1) at a rate of 400 ml/min for one minute, then allowed to sit an additional minute prior to recovery. Chlorine dioxide gas was applied for a 24-hour period: nylon surfaces were clipped to picking bags and then placed into a 50-gal plastic tote (Sterilite, Townsend, MA, USA) with reagent sachets (Figure 2) and sealed. For both HDPE and wooden bins, a 10-mil polyethylene vapor barrier (Farm Plastic Supply, Addison, IL, USA) was used to cover bins after sachet activation and sealed (Image 3). After exposure, coupons were swabbed utilizing sponge-stick swabs hydrated with 10 mL of DE broth. All samples were kept on ice, and remaining populations enumerated. Temperature and relative humidity conditions at time of sanitizer application were noted as reported by Weather Underground (wunderground.com).

6.3.7 Recovery and enumeration

Prior to quantification, each sponge was squeezed several times to ensure homogenization, then 1 mL was extracted for use in serial dilutions. Dilutions were performed in 0.1% peptone water (Difco, Sparks, MD, USA) and spread plate on Tryptic Soy Agar (Difco,

Sparks, MD, USA) with 80 µg/ml rifampicin. Colonies were counted and reported as CFU/coupon after 24-30 hours of incubation at 37± 2˚C.

6.3.8 Experimental design and statistical analysis

Due to differences in production practices, only HDPE and nylon were tested at facilities in the Midwest, while the PNW tests also included wood. Experiments were conducted in triplicate and results considered significant at $P < 0.05$. Data were analyzed via PROC GLIMMIX in SAS (Cary, NC, USA) utilizing a Sidak adjustment for multiple comparisons.

6.4 Results

Three-way fixed effects were evaluated within each pairing of microorganisms (*Listeria* and *E. coli*) and bacterial form (sessile and biofilm) within the Midwest and PNW (Table 1) regions. Significant interactions ($P < 0.05$) between sanitizer and surface were noted for all combinations. Evaluation of both mature biofilm and sessile populations after exposure to both sanitizers in both the Midwest (Table 2) and PNW (Table 3) regions were performed.

A lower level of inactivation was observed after 24-h ClO_2 exposure in the PNW for all surfaces except for sessile bacteria grown on HDPE (1.95 log CFU/coupon for both bacteria), and *E. coli* biofilms (2.19 log CFU/coupon). Both sessile bacteria on nylon showed lower populations when treated with ClO_2 than control (5. 96 log CFU/coupon for *E. coli,* 6.25 log CFU/coupon for *Listeria*), as well as in the biofilm form (3.55 log CFU/coupon for *E. coli,* 2.34 log CFU/coupon for *Listeria*). Sessile bacteria grown on wood surfaces did have lower populations after exposure to ClO_2 (5.75 log CFU/coupon for *E. coli,* 4.51 log CFU/coupon for *Listeria*), but biofilms grown on wood had much higher populations (8.7 log CFU/coupon for

E. coli, 7.84 log CFU/coupon for *Listeria*). After 2-min exposure to SDC, lower biofilm populations on HDPE for both bacteria in the PNW (6.37 log CFU/coupon for *E. coli*, 5.12 log CFU/coupon for *Listeria*) were observed. SDC exposure did not reduce population of sessile *E. coli* on HDPE in the PNW (9.04 log CFU/coupon), or on nylon (9.39 log CFU/coupon sessile, 9.28 log CFU/coupon biofilm). SDC exposure at the PNW facilities did result in lower recovered population on nylon surfaces inoculated with *Listeria* (7.91 log CFU/coupon), while no difference in population for *Listeria* biofilms was noted (9.54 log CFU/coupon).

Within the Midwest, ClO_2 was the most effective treatment ($P<0.05$) in controlling populations of *E. coli* and *Listeria* on both surfaces. A complete population reduction to the limit of detection (1.95 log CFU/coupon) was observed for *Listeria* in both forms and sessile *E. coli* on both surfaces tested with a final count near the limit of detection (1.95 log CFU/coupon) noted for biofilm *E. coli* (2.28 log CFU/coupon on HDPE and 2.03 log CFU/coupon on nylon). When bacteria were treated with SDC, a lower population of *E. coli* was observed on HDPE surfaces as compared to nylon (for sessile- 4.60 log CFU/coupon on HDPE and 7.38 log CFU/coupon on nylon; for biofilm- 5.80 log CFU/coupon on HDPE and 7.66 log CFU/coupon on nylon). These results were mirrored in the Midwest; after SDC treatment, recovered populations of sessile *Listeria* were 4.39 log CFU/coupon on HDPE and 6.61 log CFU/coupon on nylon, and biofilm populations were 5.70 log CFU/coupon on HDPE and 7.37 log CFU/coupon on nylon.

These results of this study indicate that, in addition to interactions between surface type and sanitizer, sanitizer efficacy may be dependent upon facility location as well. This study also sought to determine how bacterial form (sessile or biofilm) could impact the effectiveness of the two sanitizers tested when grown on surfaces found within harvesting facilities. It is generally

recognized that biofilms are known to be more resistant to antimicrobials than their sessile counterparts (Stewart & Costerton, 2001), and the data obtained from the Midwest region facilities supported that; particularly when looking at the remaining populations of sessile and biofilm bacteria after SDC application. Within the Midwest, the ClO_2 exposure resulted in similar remaining populations in both forms of *Listeria* and *E. coli*; the same cannot be said for the data obtained in the PNW facilities. Sessile and biofilm populations varied inconsistently when compared for each bacteria and sanitizer combination; *E. coli* grown on nylon and wood had similar recovered populations in both sessile and biofilm after exposure to SDC, while *Listeria* populations on nylon and wood did have lower recovered populations for sessile coupons compared to biofilm coupons. On HDPE in the PNW, both *Listeria* and *E. coli* biofilm recovered populations decreased after exposure to SDC than the respective sessile populations. Population differences between bacterial forms after exposure to ClO_2 were also variable for both bacteria, but the highest recovered populations (8.70 ± 0.28 log CFU/coupon for *E. coli* and 7.84 ± 0.36 log CFU/coupon for *Listeria*) after the 24-h exposure time were from biofilms grown on wood. These data could at least in part be due to differences in environmental conditions between the two regions; while there is a direct comparison between regions for HDPE and nylon, due to differences in production practices wood was not tested in the Midwest.

6.5 Discussion

Due to the extensively documented use of ClO_2 as a bacterial growth mitigation strategy on the surfaces of fresh produce (Han et al., 2000; Du, Han, & Linton, 2002; Sy et al., 2005) as well as other food contact surfaces (Trinetta et al., 2012; Vaid et al., 2010) testing the efficacy on food contact surfaces found within the harvesting process was worth investigation. Within the

Midwest, use of ClO_2 resulted in significant reduction in recovered population for sessile bacteria and biofilms grown on both HDPE and nylon surfaces. In contrast, treatment with ClO_2 in the PNW region resulted in less difference in population on HDPE and nylon surfaces, and no difference in recovered population levels on *E. coli* and *Listeria* biofilms grown on wood. As previously mentioned, relative humidity can impact efficacy of ClO_2 treatment (Kim & Park, 2022), and the differences in results between the PNW and Midwest facilities, and general environmental characteristics of those growing regions corroborate that.

Relative humidity has been shown to impact efficacy of ClO_2 treatment (Kim & Park, 2022), and differences in surface adherence have also been shown to impact sanitizer efficacy in laboratory studies on similar surfaces (Bang et al., 2014; Manville et al., 2023; Yang et al., 2009). Researchers have also found that surface hydrophobicity can also impact sanitizer efficacy, and texture patterns that are similar in size and shape to pathogens on food and food contact surfaces can also impact sanitizer efficacy (Park & Kang, 2017). While some hydrophobicity data is available for similar surfaces to those tested within this current study (Manville et al., 2023), additional characterization of actual field-used food contact surfaces may help to better understand food safety challenges associated with them.

While environmental conditions like humidity may have the ability to impact ClO_2 application more than traditional sanitizers, it should be noted that one distinct advantage to its use is the lower level of manpower needed for application. Instead of having to apply liquid sanitizers to each individual bin or bag, it would be possible to treat batches of equipment at once. There is also no residue left over after treatment; researchers noted that SDC application within the facilities used in this study did sometimes result in residue that needed to be further rinsed off of equipment.

As an alternative to oxidative sanitizers, SDC causes cell death by disrupting membrane proteins (Pure Bioscience Inc., 2016). SDC has been shown to be effective against viral pathogens present on stainless steel (Manuel, Moore, & Jaykus, 2017), however, little data is available on SDC efficacy against pathogens on "non-traditional" surfaces such as nylon or wood. SDC has been demonstrated to reduce viral pathogens on surfaces containing nylon, but at a reduced level compared to less porous surfaces like glass (Buckley et al., 2018). Previous laboratory studies have shown that SDC can reduce populations of *E. coli* and *Listeria* when used in combination with other sanitizers (Mendes-Oliveira et al., 2022) or alone (Masuku et al., 2014). Our results show that when scaled up, SDC does result in a significant difference in recovered population on biofilms grown on HDPE, but results against sessile bacteria and bacteria present on nylon and wood are inconsistent.

6.6 Conclusion

Historically, little data has been available regarding sanitizer efficacy on surfaces that are commonly found within apple harvesting environments. The data from this study demonstrates that surface type can impact sanitizer efficacy and should be considered when developing food safety practices. More porous surfaces such as wood and nylon were able to retain higher bacterial populations after exposure to SDC when compared to HDPE. Environmental conditions may also need to be considered when selecting sanitizers for use, as demonstrated in the differences in recovered populations after ClO_2 exposure between the facilities located in the Midwest versus the PNW region. Understanding unique sanitation challenges presented by the surface types within a facility can help fruit tree harvesting and packing facilities determine what practices should be implemented to help mitigate pathogen presence.

Table 6.1. Type 3 fixed effects comparing experimental factors when *E. coli* and *Listeria* in two different forms (sessile and biofilm) were exposed to different sanitizers (2- minute contact time of silver dihydrogen citrate or 24-hour contact time for chlorine dioxide) for the Midwest and PNW regions

	Pr > F, Midwest Region				**Pr > F, PNW Region**			
	E. coli		*Listeria*		*E. coli*		*Listeria*	
Effect	Sessile	Biofilm	Sessile	Biofilm	Sessile	Biofilm	Sessile	Biofilm
Sanitizer	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Surface	0.0006	0.0035	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Sanitizer*Surface	0.0003	0.0008	<0.0001	0.0004	<0.0001	<0.0001	0.0178	<0.0001

Table 6.2. Log (CFU/coupon) of *E. coli* and *Listeria* populations on HDPE and nylon surfaces when treated with sanitizers in the Midwest Region.

		Sessile			Biofilm		
		Control	*SDC*	*ClO_2*	*Control*	*SDC*	*ClO_2*
E. coli	HDPE	7.76 ± 0.40^{a}	4.60 ± 0.34^{b}	1.95 ± 0.34^{c}	7.99 ± 0.43^{a}	5.80 ± 0.41^{b}	2.28 ± 0.41^{c}
	Nylon	8.17 ± 0.40^{a}	7.38 ± 0.34^{a}	1.95 ± 0.34^{c}	8.38 ± 0.43^{a}	7.66 ± 0.41^{a}	2.03 ± 0.41^{c}
Listeria	HDPE	8.54 ± 0.22^{a}	4.39 ± 0.18^{c}	1.95 ± 0.18^{d}	8.45 ± 0.20^{b}	5.70 ± 0.18^{d}	1.95 ± 0.18^{e}
	Nylon	9.03 ± 0.22^{a}	6.61 ± 0.18^{b}	1.95 ± 0.18^{d}	9.31 ± 0.20^{a}	7.37 ± 0.18^{c}	1.95 ± 0.18^{e}

Superscripts indicate significant differences ($P < 0.05$) within bacteria and bacterial form groupings

Table 6.3. Log (CFU/coupon) of *E. coli* and *Listeria* populations on HDPE, nylon, and wood surfaces when treated with sanitizers in the PNW Region.

		Sessile			Biofilm		
		Control	*SDC*	*ClO_2*	*Control*	*SDC*	*ClO_2*
E. coli	HDPE	10.02 ± 0.39^{ab}	9.04 ± 0.39^{b}	1.95 ± 0.39^{c}	8.75 ± 0.28^{b}	6.37 ± 0.28^{c}	2.19 ± 0.28^{e}
	Nylon	10.09 ± 0.39^{a}	9.39 ± 0.39^{ab}	5.96 ± 0.39^{c}	9.80 ± 0.28^{a}	9.28 ± 0.28^{ab}	3.55 ± 0.28^{d}
	Wood	9.94 ± 0.39^{ab}	9.49 ± 0.39^{ab}	5.75 ± 0.39^{c}	9.90 ± 0.28^{a}	9.30 ± 0.28^{ab}	8.70 ± 0.28^{b}
Listeria	HDPE	9.03 ± 0.60^{ab}	6.59 ± 0.60^{c}	1.95 ± 0.60^{e}	8.60 ± 0.36^{bcd}	5.12 ± 0.36^{e}	3.64 ± 0.36^{f}
	Nylon	10.59 ± 0.60^{a}	7.91 ± 0.60^{bc}	6.25 ± 0.60^{c}	10.05 ± 0.36^{a}	9.54 ± 0.36^{ab}	2.34 ± 0.36^{g}
	Wood	7.91 ± 0.60^{bc}	7.36 ± 0.60^{bc}	4.51 ± 0.60^{d}	9.06 ± 0.36^{abc}	8.08 ± 0.36^{cd}	7.84 ± 0.36^{d}

Superscripts indicate significant differences ($P < 0.05$) within bacteria and bacterial form groupings

Figure 6.1. Nylon and HDPE coupons positioned SDC spray application.

Figure 6.2. Nylon, wood, and HDPE coupons prepared for ClO_2 treatment.

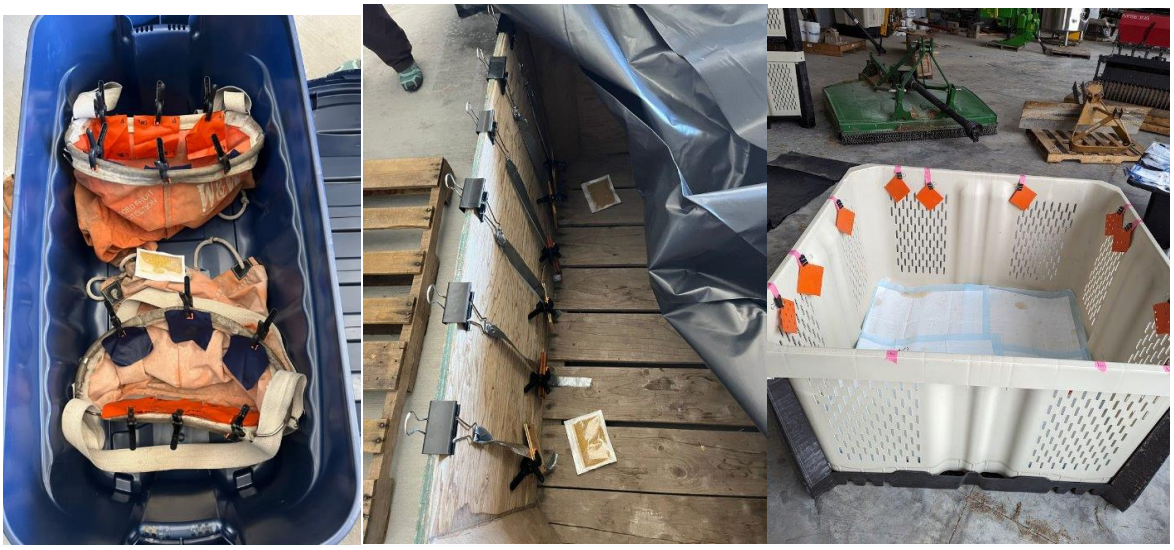

Figure 6.3. Bins covered with polyethylene vapor barrier for ClO_2 treatment.

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Chapter 7 - Transcriptomic characterization of *Listeria monocytogenes* biofilms after exposure to commercially available sanitizers

7.1 Abstract

Listeria monocytogenes is a foodborne pathogen that is capable of forming biofilms. There is little information available about how stress induced by exposure to sublethal levels of sanitizers impacts gene expression within *L. monocytogenes* biofilms. Having a better understanding of how sanitizers commonly used in food handling facilities impact gene expression can help further understanding of how those sanitizers interact with biofilms, therefore aid in developing effective strategies of sanitizer utilization for removal of biofilm. This study analyzed differentially expressed genes (DEGs) in multi-strain *L. monocytogenes* biofilms after exposure to sub-lethal levels of chlorine, PAA, silver dihydrogen citrate (SDC), and lactic acid (LA) for 15 seconds. Biofilm-dwelling *L. monocytogenes* cells exhibited different transcriptome profiles in response to various sanitizers when compared to untreated biofilms. After 15 seconds exposure to SDC and LA, there is almost no change in the *L. monocytogenes* transcriptome, with only six DEGs after exposure to SDC, and one DEG after exposure to 0.4% LA. Exposure to 10 ppm PAA and 10 ppm chlorine for 15 seconds caused relatively high levels of DEGs in *L. monocytogenes*, with 65 DEGs and 100 DEGs , respectively. Functional enrichment analysis found a 13-gene functional annotation cluster involved in the regulation of DNA-templated transcription was significantly regulated (*Q*-value = 0.0049) after exposure to chlorine. Both PAA and chlorine, which are categorized as oxidative sanitizers has limited overlap in gene response. Only 10% (1/10) of the DEGs that were downregulated and 22% (28/126) of the upregulated DEGs were shared between the biofilms exposed to either. This data

suggested different sanitizers elicit distinct transcription responses, highlighting the diverse mechanisms of action and stress responses activated in biofilm-associated *L. monocytogenes* cells. Further research is needed to investigate the temporal dynamics of the *L. monocytogenes* transcriptome following exposure to different sanitizers at sub-lethal level. Understanding how gene expression changes over time will provide deeper insights into the bacterial stress responses and adaptive mechanisms.

7.2 Introduction

Listeria monocytogenes is a foodborne pathogen of concern within multiple industries, including dairy, meat, produce, and pet food. Between July 2023-July 2024 in the United States, a total of 49 product recalls were issued due to *L. monocytogenes* contamination or potential to be contaminated, related to a wide variety of food types; 2 pet food, 12 dairy products, 11 fresh or minimally processed produce, 20 ready-to-eat (RTE) foods, 3 seafood, and 1 related to processed nuts (FDA, 2024). While it is not considered one of the most frequently acquired foodborne illnesses, *Listeriosis* is widely recognized to have a comparatively high risk of hospitalization and mortality (Scharff, 2012).

L. monocytogenes is considered ubiquitous in many environments, and can survive and replicate during refrigeration, causing increased risk associated with foods that are minimally processed prior to consumption (Dhama et al., 2015). Considering the widespread potential for contamination, understanding how sanitizer application can impact *L. monocytogenes* is important to help better inform facility sanitation practices and prevent pathogen spread. Like other bacteria, *L. monocytogenes* has the ability to form biofilms when in contact with surfaces, which can impact sanitizer efficacy when compared to sessile cells (Chavant et al., 2004).

A variety of sanitizers are used within food processing and handling facilities to mitigate bacterial pathogens. Oxidative sanitizers such as chlorine and peracetic acid (PAA) have been shown to be effective at reducing populations of *Listeria* biofilms (Hua et al., 2019). Additionally, organic acids such as lactic acid have also been shown to be effective against *L. monocytogenes* (Yang et al., 2009; Wang et al., 2015). The use of silver ions in combination with organic acids has been demonstrated to be effective at inhibiting bacterial and viral pathogen growth (Feng et al., 2000; Jo et al., 2007; Belluco et al., 2016; Manuel et al., 2017). There is some evidence indicating that exposure to a variety of sanitizers at sublethal conditions can result in changes in gene expression (Kastbjerg et al., 2010; Kokkoni et al., 2021). However, there is a lack of knowledge surrounding how inducing stress conditions via sanitizer exposure impacts *L. monocytogenes* biofilm gene expression. This study aims to identify differences between gene expression from *L. monocytogenes* biofilms after sublethal exposure to four different commercially available sanitizers (chlorine, PAA, silver dihydrogen citrate (SDC), and lactic acid (LA)) used within food handling facilities.

7.3 Materials and Methods

7.3.1 Bacterial strains and inoculum preparation

Three strains of *Listeria monocytogenes* were used for this study: CFSAN136779 (serotype 1/2a) and CFSAN136778 (serotype 1/2b) (both environmental isolates from a 2011 cantaloupe outbreak), and CFSAN136780 (serotype 4b), a clinical isolate from a caramel apple outbreak in 2014-2015. Strains were obtained from frozen stock at the Kansas State University-Food Safety and Microbiology Laboratory collection, and had been stored on CyroCare Bacteria

Preserver beads (Key Scientific, Stamford, TX) and kept at −80 °C until the experiment. Strains were plated on Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA) and passaged prior to biofilm growth. A three-strain bacterial cocktail was prepared as previously described by Manville et al., 2023 to reach an initial population of ~ 1 x 10^8 CFU/ml.

7.3.2 Biofilm growth

Biofilms were grown in 5 cm non-treated, sterile petri dishes. Prior to inoculation, 3 ml of 20 g/L TSB (Tryptic Soy Broth, BD Difco, Sparks, MD) was placed into each petri dish. 300 µl of the three-strain bacterial cocktail was then added. Biofilms were then incubated for 48 hours at 25 ± 2 °C without agitation. Four (4) different independent biological replications were grown for each treatment.

7.3.3 Sanitizer selection, application, and cell recovery

Four sanitizers were selected for use in this study; 10 ppm peracetic acid (PAA) (Pace International, Wapato, WA, USA), 10 ppm chlorine (Diversey, Fort Mill, SC, USA), silver dihydrogen citrate (SDC) with 0.003% silver and 4.85% citric acid (Pure Bioscience, El Cajon, CA, USA), and 0.4% lactic acid (LA) (Corbion, Lenexa, KS, USA). To determine exposure levels, current practices within the fresh produce industry were taken into consideration. PAA is approved for use within the United States at 100 ppm for direct contact with fruits and vegetables, and between 100 – 200 ppm for food contact surfaces (40 CFR part 180). Chlorine bleach solutions are approved for use in fruit or vegetable wash solutions at concentrations less than 2000 ppm if followed by a potable water rinse (21 CFR part 173) and on food processing equipment at levels less than 200 ppm (21 CFR part 178). Two of the sanitizers tested, LA and

SDC, do not have federally mandated exposure levels. With the exception of SDC, which is a pre-mixed solution, sanitizer levels selected were at a tenfold dilution of common industry use. Industry-standard contact time for these sanitizers varies dependent upon commodity; exposure time for this study was determined prior to the start of the experiment and based off surviving population counts after exposure to each sanitizer.

Prior to sanitizer application, all remaining TSB was pipetted from the petri dishes without disruption of the biofilm. Sanitizers were applied by pipetting 1 mL of sanitizer onto the biofilm, then removing after 15 seconds, again without disrupting the biofilm. Control biofilms had 1 mL of sterile DI water applied instead of one of the sanitizers. Immediately after removal of the sanitizer or DI water, 1 mL of Dey Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA, USA) was placed onto the biofilm for 15 seconds. Neutralizing assays were performed prior to starting experiments. Dey Engley broth was then removed, and cells were resuspended in 1 mL of freshly prepared ice cold 15% ethanol. Resuspended cells were transferred into a 15 mL conical tube with an additional 9 mL of ice cold 15% ethanol. After retaining 1 mL for enumeration via serial dilution, the remaining solution was centrifuged at 4°C for 12 minutes at 4300 ×g. Cells were then resuspended in 1-mL of ice cold 15% ethanol in preparation for RNA extraction.

7.3.4 RNA extraction and sequencing

Total RNA was extracted using the commercially available Total RNA Purification Plus Kit (Norgen, Biotek, ON, CA) following the manufacturer's protocols for Gram-positive bacteria. After extraction, total RNA concentration was determined using a Qubit 4 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and quality checked using a 4150 Tapestation

(Agilent, Waldbronn, Germany). Samples were snap frozen with dry ice, then stored at -80 °C until library preparation and sequencing.

Preparation of cDNA libraries was performed utilizing the Illumina Stranded Total RNA Prep, Ligation with RiboZero Plus kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Pooled libraries were then sequenced on Illumina NextSeq 2000 platform using a P3 kit with 300 cycles. RNA sequence raw data have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSExxxxxx.

7.3.5 RNAseq data analysis

After performing quality checks, read counts for each sample were generated using Salmon v1.10.2 (Patro et al., 2017) with the transcriptome indices built from *Listeria monocytogenes* EGD-e (NCBI:txid169963). The DESeq2 R package version 1.44.0 was used to calculate differential gene expression of *Listeria monocytogenes* in biofilm after sanitizer exposure. Differentially expressed genes (DEGs) were reported with ≥0.5-|log2fold differences| in the transcript abundances between treatments and control, with adjusted P-value or a false discovery rate (FDR) of less than 0.05. All reported DEGs were imported to DAVID Functional Annotation Tools (https://davidbioinformatics.nih.gov/tools.jsp) for gene enrichment analysis. Functional clusters with Benjamini *Q*- values lower than 0.05 were considered significant.

7.4 Results and Discussion

Overall, different sanitizers induced drastically different initial transcriptome response in *L. monocytogenes* in biofilm after exposure to sub-lethal concentration (Figure 1). When comparing sanitizer-exposed *L. monocytogenes* biofilms to untreated biofilms, exposure to SDC,

0.4% LA, 10 ppm PAA and 10 ppm chlorine resulted in six, one, 65, and 100 significant DEGs, respectively, in *L. monocytogenes*. (Table 1). Within the sanitizer-exposed biofilms, DEGs were either downregulated or upregulated. Downregulation of genes indicates decreased gene expression when compared to the untreated biofilms; in contrast, upregulation indicates higher levels of gene expression.

All six DEGs observed in *L. monocytogenes* biofilm cells after 15 seconds exposure to SDC were downregulated, with log2fold changes ranging from -0.95 to -5.01. The two most downregulated genes, lmo2498 and lmo2499 (-3.85 and -5.01 log2fold, respectively) are located in the plasma membrane and have been indicated to be involved in encoding phosphate ABC transporter components and a phosphate ABC transporter substrate-binding protein (PstS), respectively. In other bacteria species, *pstS* has been shown to be involved in phosphate starvation responses (Allenby et al., 2005), and downregulation could indicate a potential disruption in phosphate uptake and metabolism in the cells within the biofilm after exposure to SDC. Phosphate is essential for various cellular processes, including energy production, nucleotide synthesis, and cell signaling. A decrease in phosphate transporters could impair these processes. The downregulation may be part of a broader stress response mechanism, and it might be a strategic response to reduce cellular activity and conserve energy under adverse conditions. Considered a nonorganic sanitizer, SDC utilizes a combination of silver ions and citric acid (Pure Bioscience Inc., 2016). Silver ions disrupt structural and metabolic proteins within sulfur-containing thiol groups, while citric acid has been shown to impact cell membrane integrity and overall cell structure (Zhao et al., 2017). When used together, silver ions and citric acid could have a synergistic effect on phosphate transport in *L. monocytogenes* by disrupting membrane

integrity, inactivating transport proteins, inducing oxidative stress, lowering environmental pH, and chelating essential cofactors.

Of the four sanitizers evaluated in this study, two (chlorine and PAA) are oxidizing agents. Compared to the other two sanitizers, both chlorine and PAA exposure resulted in higher levels of DEGs. After exposure to chlorine, eight genes were significantly downregulated (between -0.81 and -0.53 log2fold changes) and 92 genes were significantly upregulated (log2fold changes between 0.55 and 4.68), for a total of 100 DEGs (Figure 2). One functional annotation cluster was significantly regulated (*Q*-value = 0.0049), containing 13 genes located in the cytosol (lmo0229, lmo0031, lmo0734, lmo0488, lmo0852, lmo0189, lmo0579, lmo0797, lmo1507, gltC, lmo0425, lmo0522, and phoP) involved in the regulation of DNA-templated transcription. *L. monocytogenes* with mutations in gltC have been shown to have reduced biofilm formation ability, in addition to reduced oxidative stress tolerance, (Huang et al., 2013).

Exposing *L. monocytogenes* biofilms to PAA resulted in 65 significant DEGs, three of which were downregulated and 62 upregulated (log2fold ranges of -0.69 to -0.58 and 0.51 to 4.76, respectively) **(**Figure 3). The oxidative stress and damage caused by PAA can cause extensive damage to bacterial membranes and proteins, and disrupt normal metabolic processes. The bacteria may need to activate various repair and stress response pathways to mitigate the cellular damage, and reprogram their metabolism quickly, leading to the differential expression of genes involved in energy production, biosynthesis, and nutrient uptake (Table 1). For example, phosphate ABC transporter function plays an essential role in nutrient transport (Higgins, 2001). The increase in the phosphate ABC transporter function.in biofilm associated *L. monocytogenes* after exposure to PAA indicated the reprogram of bacteria metabolism.

Comparison of DEGs after exposure to the two oxidizing sanitizers tested indicated that limited overlap between the transcriptional response. Only 10% (1/10) of the downregulated DEGs in biofilms exposed to chlorine or PAA were shared (Figure 4). Of the upregulated DEGs, 22% (28/126) were shared (Figure 5). None of the DEGs identified within the functional annotation cluster for chlorine-exposed biofilm were up- or down-regulated within the biofilm treated with PAA; despite both causing oxidative stress, chlorine exposure appears to have a greater impact on DNA-templated transcription regulation.

One potential cause of the differences between DEGs after exposure to two oxidizing sanitizers is the differences in oxidation-reduction potential (ORP) between PAA and chlorine; PAA has been shown to have higher potential than chlorine (Luukkonen & Pehkonen, 2017). ORP can be used as an indication of sanitizer efficacy (Bergendal & Stevens, 2005), and PAA has been shown to have higher efficacy against foodborne pathogens than chlorine (Neo et al., 2013; Su et al., 2022; Krishnan et al., 2023). PAA has been shown to target amino acids, specifically cysteine, methionine, and histidine (Du et al., 2018).

Exposure to LA has been shown to be effective at controlling both sessile and biofilm populations of *L. monocytogenes* (Akbas & Ölmez, 2007; Yang et al., 2009; Tomalok et al., 2022). Previous research has shown that prolonged exposure to oxidative stress caused by sanitizer in sessile *L. mono*cytogenes results in minor differences in DEGs, while acidification due to prolonged LA exposure resulted in high levels of DEGs (Cortes et al., 2020). In our study, 15 seconds exposure of *L. monocytogenes* biofilms to LA only resulted in upregulation of one DEG, while after exposure to either of the oxidizing agents (chlorine and PAA) resulted in 92 and 62 upregulated DEGs, respectively. The higher number of DEGs suggests that PAA and chlorine elicit a strong and immediate stress response from *L. monocytogenes*. This rapid

response indicates that the bacteria are quickly activating a wide range of defense mechanisms to counteract the oxidative and damaging effects of these sanitizers. On the other hand, lactic acid primarily affects bacteria by lowering the pH, which can disrupt enzyme function and membrane integrity, but it is generally less immediately damaging compared to oxidizing agents like PAA and chlorine. This could result in a very minor transcriptional response with fewer DEGs. In addition, lactic acid is a common stressor that bacteria often encounter in various environments, possibly leading to a quick adaptation of *L. monocytogenes*. Biofilms have been shown to be more resistant to sanitizer exposure due to the presence of the EPS (Stewart & Costerton, 2001). The presence of EPS can induce changes in gene expression in biofilm-associated bacteria, including upregulation of stress response genes, efflux pumps, and genes involved in EPS production (Table 1). Using a combination of sanitizers with different modes of action can help overcome the protective effects of EPS. For example, using an oxidizing agent like PAA to degrade the EPS followed by a sanitizer like lactic acid may enhance the overall efficacy.

7.5 Conclusion

Research has shown that exposure to stress can cause *Listeria* to adapt and alter gene expression (Freitag, Port, & Miner, 2009; Bansal et al., 2018**;** Bucur et al., 2018). The data obtained from this study indicates the difference in induction of DEGs by short-term exposure to various sanitizers, highlighting the potent impact of these oxidizing agents like PAA and chlorine on bacterial cells in biofilm. Nevertheless, further research is needed to better understand how prolonged exposure to various sanitizers further impacts transcriptional response in *Listeria monocytogenes* biofilm under sub-lethal conditions. This will provide a more comprehensive understanding of how sanitizer exposure affects stress and adaptive response

Table 7.1. 172 differentially expressed genes (DEGs) identified when comparing sanitizer-exposed *L. monocytogenes* biofilms to untreated biofilms

Sanitizer	Regulation	GeneID	Name	log2FoldChange	padj	Protein function
Chlorine	Downregulated	987930	lmo2345	-0.809	0.020	hypothetical protein
		986448	lmo0973, dltB	-0.626	0.007	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
		986542	PdhD	-0.601	0.005	dihydrolipoamide dehydrogenase
		986393	lmo2845	-0.591	0.018	MFS transporter
		985374	lmo0823	-0.582	0.005	oxidoreductase
		984369	lmo2362	-0.553	0.007	amino acid antiporter
		986860	lmo2529, atpD	-0.541	0.031	ATP synthase F0F1 subunit beta
		985651	menB	-0.532	0.015	naphthoate synthase
	Upregulated	986468	lmo0964	0.551	0.037	hypothetical protein
		987652	lmo0393	0.556	0.003	hypothetical protein
		987867	lmo0425	0.563	0.045	transcriptional antiterminator BglG
		986638	lmo0580	0.567	0.011	hypothetical protein
		984919	lmo2055	0.570	0.020	hypothetical protein
		985257	prs	0.582	0.045	phosphoribosyl pyrophosphate synthetase
		986189	lmo1135	0.595	0.028	hypothetical protein
		987643	lmo0387	0.606	0.031	hypothetical protein
		987345	lmo0260	0.615	0.042	hypothetical protein
		985300	lmo0521	0.619	0.007	6-phospho-beta-glucosidase
		987446	lmo2425	0.673	0.031	glycine cleavage system protein H
		986565	lmo0078	0.674	0.007	phosphoglycerate dehydrogenase
		987410	lmo0274	0.677	0.020	hypothetical protein
		986960	lmo0509, comEB	0.684	0.012	competence protein ComEB
		985623	lmo1691	0.692	0.040	deoxyuridine triphosphate nucleotidohydrolase
		985811	lmo1068	0.707	0.042	hypothetical protein
		986011	lmo1246	0.721	0.031	ATP-dependent RNA helicase

		985175	lmo0445	0.731	0.017	transcripitonal regulator
		986500	lmo0911	0.736	0.007	hypothetical protein
		987496	lmo2403	0.753	0.040	hypothetical protein
		988009	lmo2269	0.774	0.005	hypothetical protein
		985426	lmo0797	0.780	0.031	hypothetical protein
		985312	lmo0928	0.811	0.007	3-methyladenine DNA glycosylase
		987852	lmo0955	0.821	0.045	hypothetical protein
		986318	lmo1042	0.823	0.010	molybdopterin biosynthesis protein MoeA
		987018	lmo0192	0.832	0.016	PurR family transcriptional regulator
		985416	lmo2206, clpB	0.832	0.039	Clp protease subunit B
		986679	lmo1467	0.839	0.010	phosphate starvation-induced protein PhoH
		987443	lmo2426	0.840	0.006	hypothetical protein
		986637	serC	0.852	0.012	phosphoserine aminotransferase
		986982	lmo2792	0.872	0.001	hypothetical protein
		986980	lmo0902	0.875	0.045	GntR family transcriptional regulator
		986669	lmo1748	0.896	0.023	hypothetical protein
		987383	lmo2454	0.913	0.049	hypothetical protein
		984472	lmo0871	0.917	0.039	hypothetical protein
		985633	lmo1684	0.929	0.010	glycerate dehydrogenase
		987816	lmo0413	0.940	0.022	hypothetical protein
		985078	lmo0719	0.952	0.022	hypothetical protein
		987540	lmo2393	0.958	0.003	hypothetical protein
		987756	lmo1507	0.981	0.001	response regulator
		986462	lmo1408	0.999	0.028	hypothetical protein
		986199	lmo0431	1.031	0.044	acetyltransferase
		986608	lmo0095	1.040	0.007	hypothetical protein
		985274	lmo0522	1.040	0.003	transcriptional regulator
		985367	lmo1735, gltC	1.083	0.003	transcription activator of glutamate synthase operon GltC
		987186	lmo0229	1.085	0.003	CtsR family transcriptional regulator

		985369	lmo0826	1.092	0.031	transporter
		986865	lmo0579	1.107	0.042	hypothetical protein
		985313	lmo0852	1.131	0.042	TetR family transcriptional regulator
		986596	lmo0954	1.133	0.013	hypothetical protein
		985003	lmo0657	1.142	0.031	hypothetical protein
		985927	lmo1802	1.187	0.045	DNA-binding protein
		987407	lmo2442	1.379	0.027	hypothetical protein
		987904	phoP	1.392	0.045	two-component response phosphate regulator
		987846	lmo0932	1.394	0.031	hypothetical protein
		985863	lmo0734	1.404	0.042	LacI family transcriptional regulator
		986382	lmo2851	1.459	0.010	AraC family transcriptional regulator
		987455	lmo0292	1.463	0.004	heat-shock protein htrA serine protease
		987970	lmo1966	1.469	0.011	hypothetical protein
		985861	pyrR	1.474	0.003	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
		987423	lmo2432	1.487	0.001	hypothetical protein
		985225	lmo0488	1.510	0.031	LysR family transcriptional regulator
		985116	lmo0031	1.526	0.007	LacI family transcription regulator
		984570	lmo2259	1.527	0.028	PTS beta-glucoside transporter subunit IIA
		987134	lmo2690	1.545	0.018	TetR family transcriptional regulator
		987015	lmo0189	1.546	0.001	Veg protein
		987143	lmo2683	1.547	0.039	PTS cellbiose transporter subunit IIB
		986671	lmo1719	1.563	0.041	PTS lichenan transporter subunit IIA
		984799	lmo2339	1.610	0.011	hypothetical protein
		985559	lmo1747	1.623	0.005	ABC transporter ATP-binding protein
		984638	lmo2207	1.663	0.020	hypothetical protein
		985583	lmo0047	1.715	0.003	hypothetical protein
		986759	lmo1423	1.719	0.003	hypothetical protein
		985862	lmo1841	1.733	0.041	hypothetical protein
		987169	lmo2650	1.850	0.011	MFS transporter
		987750	lmo1506	1.895	0.003	transporter

		984639	lmo2203	1.938	0.001	N-acetylmuramoyl-L-alanine amidase
		986476	lmo0942	2.078	0.003	heat shock protein 90
		985146	lmo0599	2.161	0.005	hypothetical protein
		985344	lmo0600	2.195	0.001	hypothetical protein
		985934	lmo1800	2.227	0.012	protein-tyrosine phosphatase
		984830	lmo0601	2.237	0.000	cell surface protein
		984393	lmo2651	2.276	0.011	PTS mannitol transporter subunit IIA
		986981	lmo1484, comEA	2.492	0.000	competence protein ComEA
		986457	lmo1444	2.798	0.000	foldase
		987432	lmo0278	3.179	0.003	sugar ABC transporter ATP-binding protein
		985171	lmo0442	3.297	0.000	hypothetical protein lmo0442
		987897	lmo2498	3.396	0.020	phosphate ABC transporter permease
		987313	lmo2496	3.544	0.031	phosphate ABC transporter ATP-binding protein
		987906	lmo2499	3.859	0.003	phosphate ABC transporter substrate-binding protein
		984698	lmo2147	3.906	0.000	hypothetical protein
		986550	lmo0881	4.678	0.000	hypothetical protein
Lactic Acid	**Upregulated**	984698	lmo2147	3.363	0.000	hypothetical protein
PAA	**Downregulated**	985178	lmo0452	-0.691	0.010	hypothetical protein
		985151	lmo0433, inlA	-0.578	0.001	internalin A
		985374	lmo0823	-0.520	0.030	oxidoreductase
	Upregulated	987652	lmo0393	0.510	0.014	hypothetical protein
		985300	lmo0521	0.547	0.041	6-phospho-beta-glucosidase
		985684	lmo1647	0.613	0.038	1-acylglycerol-3-phosphate O-acyltransferase
		987089	lmo0225, folA	0.668	0.026	dihydroneopterin aldolase
		986982	lmo2792	0.683	0.026	hypothetical protein
		985168	lmo0441	0.734	0.040	D-alanyl-D-alanine carboxypeptidase

		985824	lmo1870	0.781	0.049	alkaline phosphatase
		987852	lmo0955	0.909	0.039	hypothetical protein
		987051	lmo0648	0.973	0.027	hypothetical protein
		987695	lmo1316, cdsA	1.014	0.048	phosphatidate cytidylyltransferase
		986462	lmo1408	1.032	0.041	hypothetical protein
		987990	lmo1941	1.057	0.023	hypothetical protein
		986843	lmo1438	1.104	0.040	penicillin-binding protein
		985569	lmo1746	1.158	0.029	ABC transporter permease
		986199	lmo0431	1.175	0.041	acetyltransferase
		987455	lmo0292	1.190	0.041	heat-shock protein htrA serine protease
		987694	lmo1315	1.253	0.008	UDP pyrophosphate synthase
		986781	lmo0948	1.258	0.032	transcriptional regulator
		985173	lmo0444	1.263	0.041	hypothetical protein
		986964	lmo0178	1.272	0.041	xylose repressor
		985114	lmo1290	1.369	0.041	internalin
		985583	lmo0047	1.407	0.033	hypothetical protein
		984639	lmo2203	1.450	0.040	N-acetylmuramoyl-L-alanine amidase
		985837	lmo2242	1.461	0.000	O6-methylguanine-DNA methyltransferase
		984575	lmo2258	1.468	0.007	hypothetical protein
		985559	lmo1747	1.477	0.023	ABC transporter ATP-binding protein
		987383	lmo2454	1.596	0.000	hypothetical protein
		984864	lmo1983, ilvD	1.600	0.011	dihydroxy-acid dehydratase
		987423	lmo2432	1.655	0.000	hypothetical protein
		984638	lmo2207	1.752	0.022	hypothetical protein
		986035	acpP	1.762	0.041	acyl carrier protein
		986877	lmo1720	1.767	0.026	PTS lichenan transporter subunit IIB
		986846	lmo0160	1.783	0.011	peptidoglycan binding protein
		987750	lmo1506	1.832	0.007	transporter
		987169	lmo2650	1.912	0.017	MFS transporter

		986476	lmo0942	1.971	0.009	heat shock protein 90
		987842	lmo0418	1.975	0.041	hypothetical protein
		984633	lmo2210	1.979	0.008	hypothetical protein
		986981	lmo1484, comEA	2.007	0.005	competence protein ComEA
		985344	lmo0600	2.062	0.002	hypothetical protein
		986457	lmo1444	2.076	0.006	foldase
		985021	lmo0676, fliP	2.090	0.007	flagellar biosynthesis protein FliP
		984811	lmo2199	2.095	0.000	hypothetical protein
		985608	lmo1229	2.100	0.027	hypothetical protein
		985934	lmo1800	2.168	0.032	protein-tyrosine phosphatase
		984830	lmo0601	2.237	0.000	cell surface protein
		985171	lmo0442	2.295	0.007	hypothetical protein
		984874	ulaA	2.570	0.006	PTS ascorbate transporter subunit IIC
		984870	lmo1974	2.620	0.000	GntR family transcriptional regulator
		984873	lmo1972	2.834	0.049	PTS pentitol transporter subunit IIB
		986513	lmo0996	2.860	0.011	methylated-DNA-protein-cysteine methyltransferase
		987968	lmo1973	2.957	0.010	PTS sugar transporter subunit IIA
		987458	lmo0295	3.167	0.000	FMN-containing NADPH-linked nitro/flavin reductase
		987895	lmo2494	3.235	0.003	PhoU family transcriptional regulator
		987314	lmo2495	3.240	0.007	phosphate ABC transporter ATP-binding protein
		987906	lmo2499	3.362	0.018	phosphate ABC transporter substrate-binding protein
		987432	lmo0278	3.690	0.000	sugar ABC transporter ATP-binding protein
		986550	lmo0881	3.901	0.000	hypothetical protein
		987312	lmo2497	4.128	0.010	phosphate ABC transporter permease
		984698	lmo2147	4.154	0.000	hypothetical protein
		987897	lmo2498	4.181	0.004	phosphate ABC transporter permease

		987313	lmo2496	4.757	0.003	phosphate ABC transporter ATP-binding protein
SDC	**Downregulated**	987906	lmo2499	-5.078	0.000	phosphate ABC transporter substrate-binding protein
		987897	lmo2498	-3.845	0.045	phosphate ABC transporter permease
		985840	lmo1854	-3.348	0.003	hypothetical protein
		986930	lmo0901	-2.213	0.000	PTS cellbiose transporter subunit IIC
		984669	lmo2175, fabG	-1.225	0.000	3-ketoacyl-ACP reductase
		984589	lmo0574	-0.949	0.007	beta-glucosidase

Figure 7.1. PCoA chart displaying resemblances between gene expression of untreated *L. monocytogenes* biofilm compared to *L. monocytogenes* biofilms after exposure to 10 ppm chlorine, 10 ppm peracetic acid (PAA), 0.4% lactic acid (LA), or silver dihydrogen citrate (SDC)

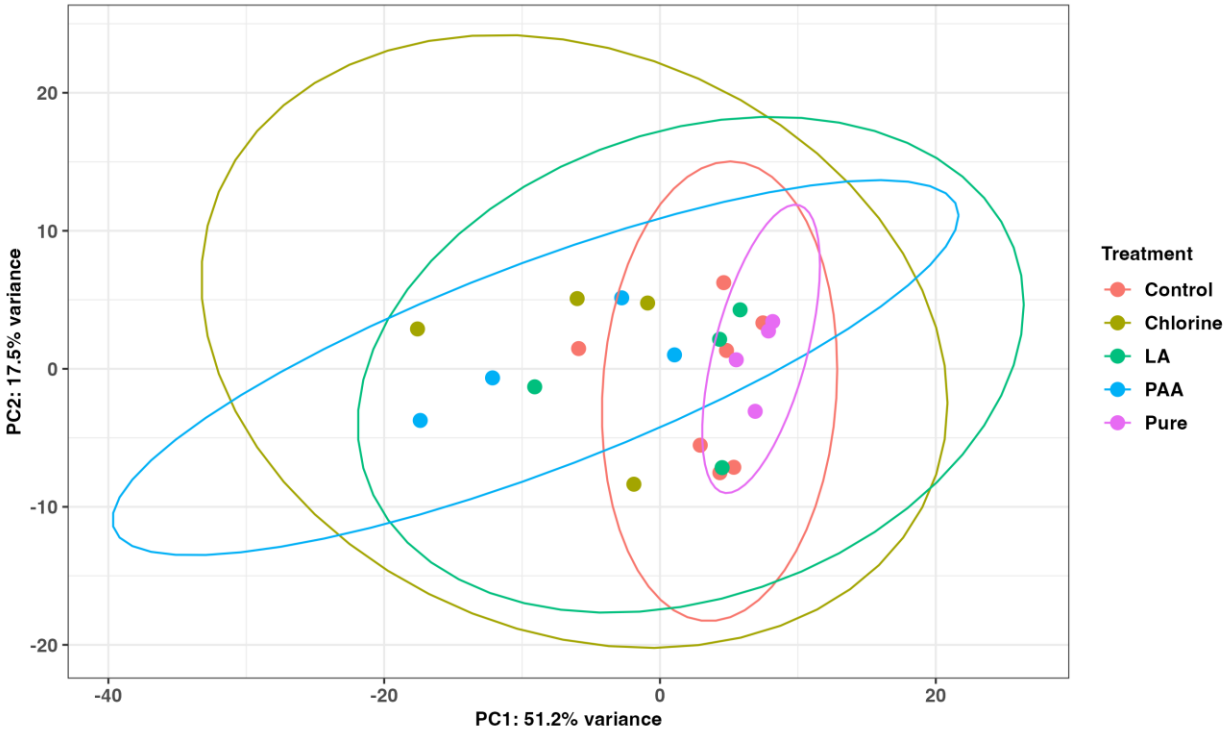


Figure 7.2. Volcano plot of differentially expressed genes (DEGs) for *L. monocytogenes* biofilms after exposure to 10 ppm chlorine compared to unexposed biofilms.

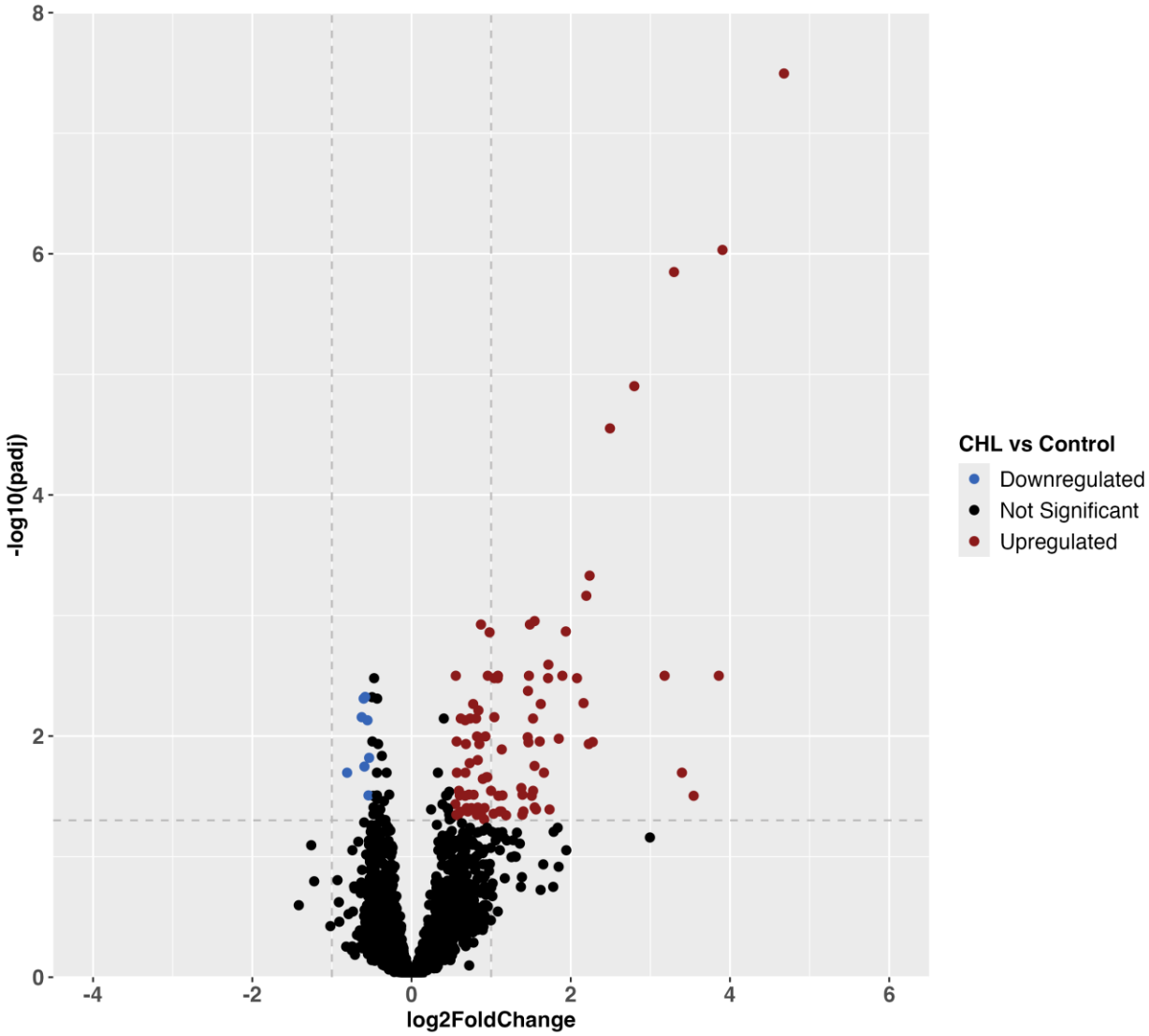


Figure 7.3. Volcano plot of differentially expressed genes (DEGs) for *L. monocytogenes* biofilms after exposure to 10 ppm peracetic acid (PAA) compared to unexposed biofilms.

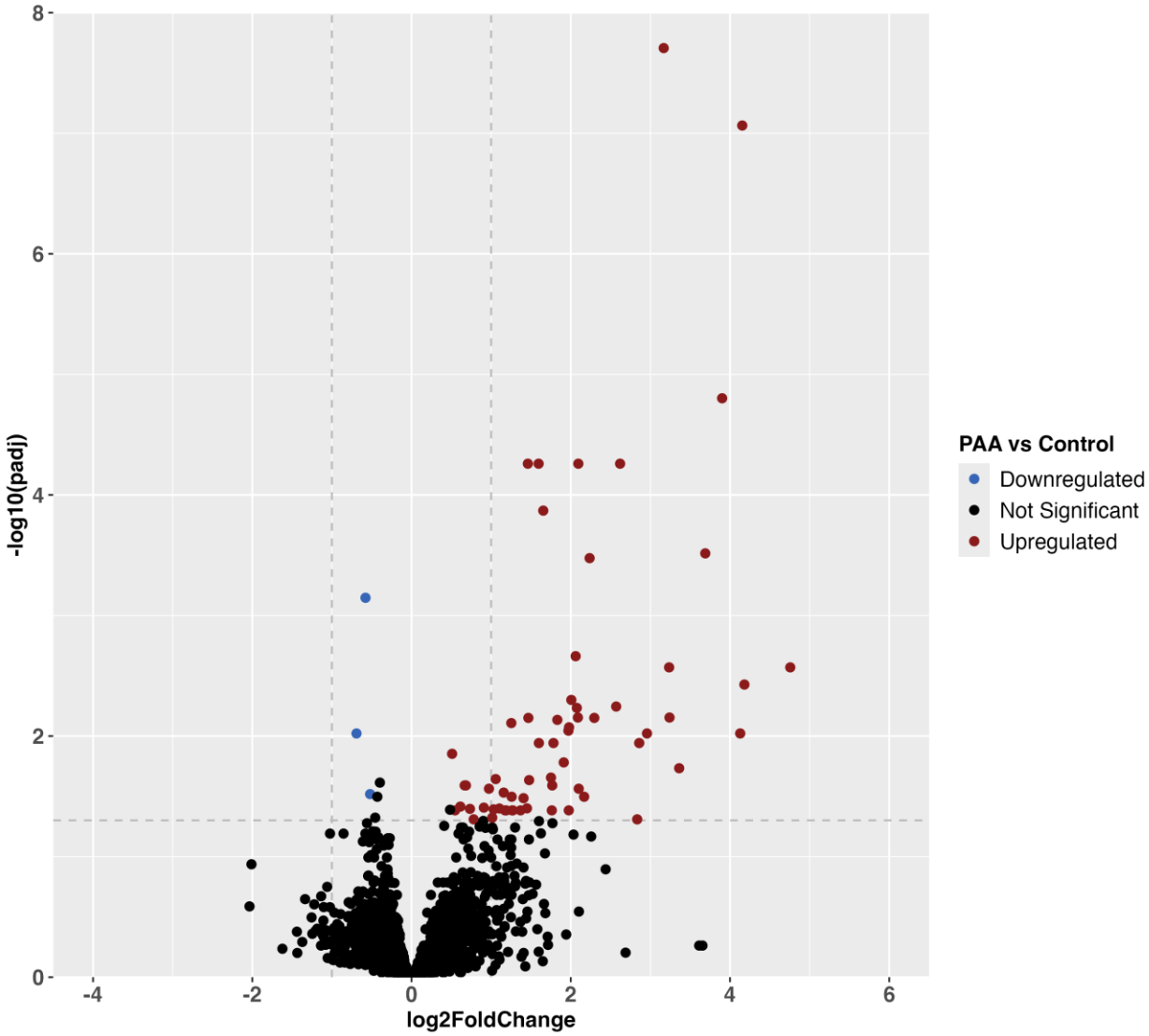


Figure 7.4. Number of unique and shared downregulated differentially expressed genes (DEGs) for *L. monocytogenes* biofilms after exposure to either 10 ppm peracetic acid or 10 ppm chlorine.

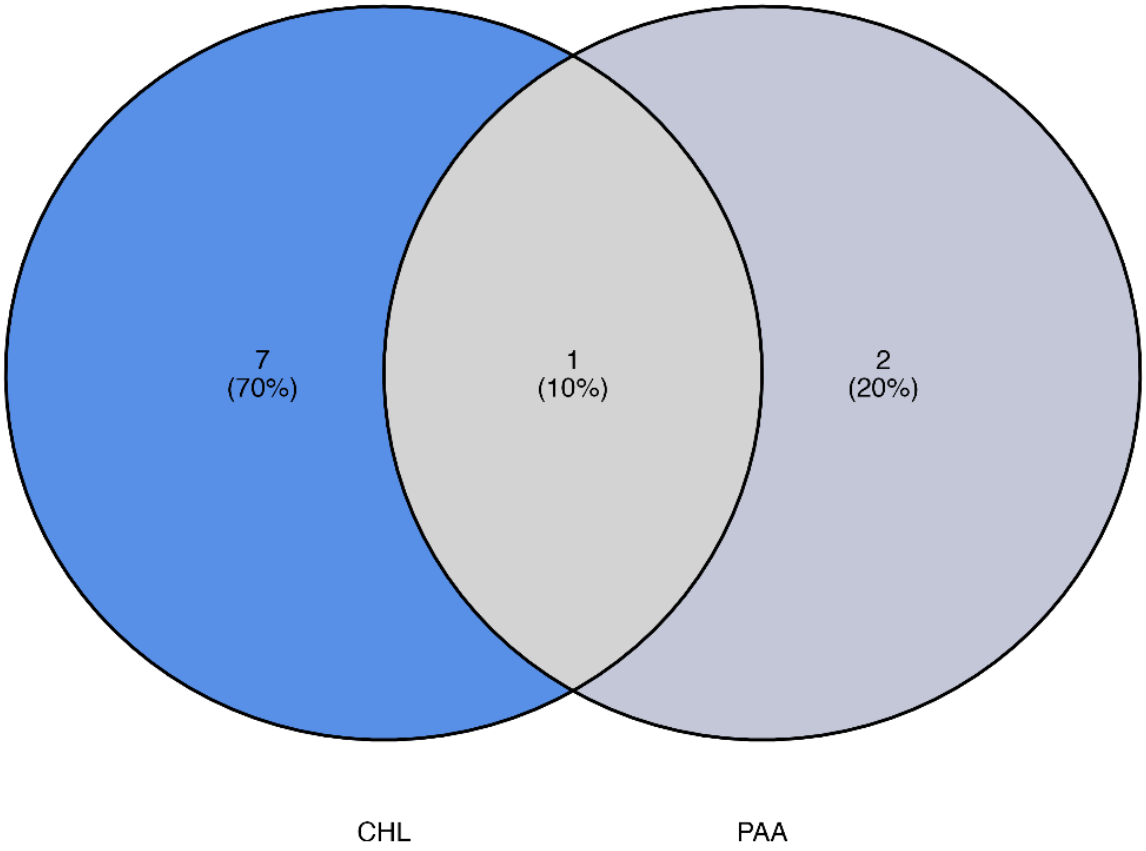
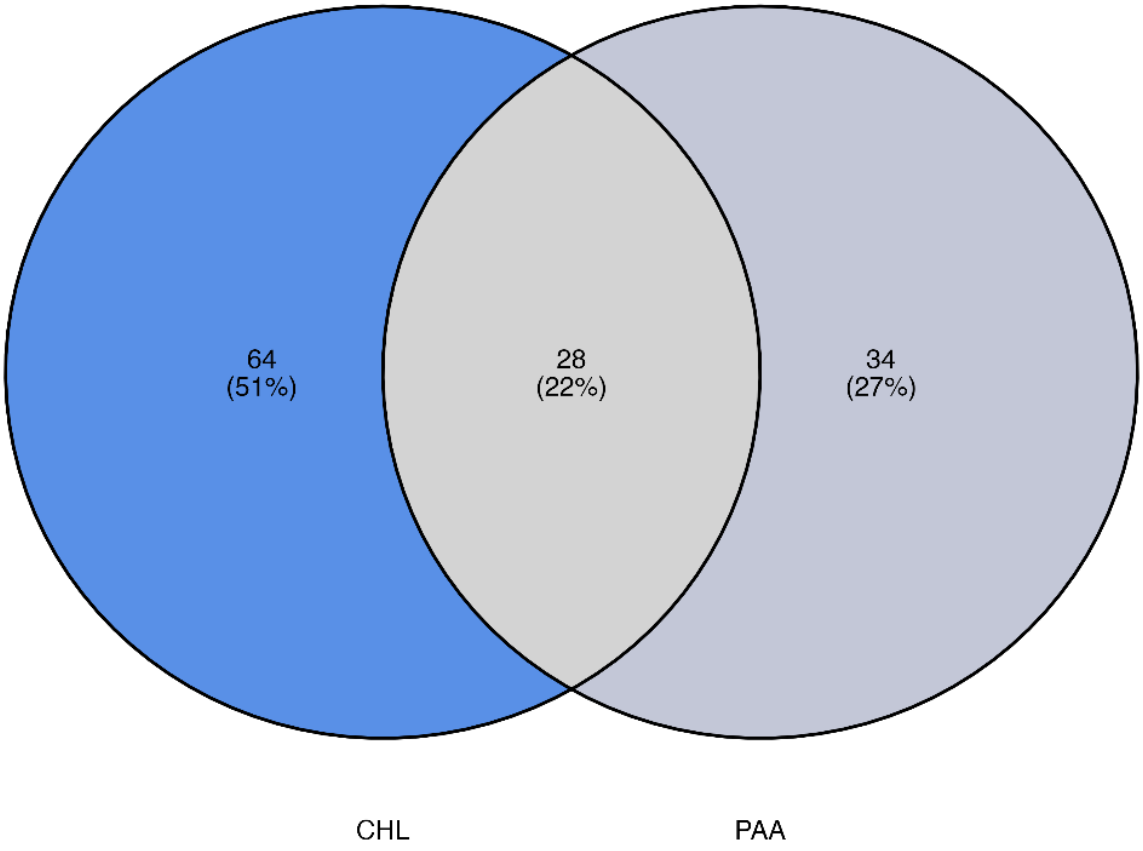


Figure 7.5. Number of unique and shared upregulated differentially expressed genes (DEGs) for *L. monocytogenes* biofilms after exposure to either 10 ppm peracetic acid or 10 ppm chlorine.



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Chapter 8 - Conclusions

Within recent years, there has been growing concern surrounding the presence of foodborne pathogens associated with fresh produce, especially within the fruit industry. Fruit is typically not highly processed prior to making its way into end-consumers' homes, so any potential route of exposure to foodborne pathogens throughout the supply chain needs to be considered. In order to gain a better understanding on how bacterial populations can enter into and survive within the food supply chain, a series of studies were conducted. The contents of this dissertation highlight the different factors that should be considered when working in facilities that handle minimally or non-processed foodstuffs that are part of the food supply chain.

One often-overlooked area of the overall food supply chain is animal food production and manufacturing. Facilities are often hesitant to allow for sampling, so little information is currently available on what bacteria can be frequently found. Feed mills often handle a wide variety of raw ingredients, so understanding what resident bacteria are present and where they are located within the facilities can lead to a better understanding of how microorganisms can enter into the food supply. Sampling swine feed mills located in the Midwest indicated that a minority of bacteria are actually found within finished feed; most were identified via environmental samples taken from non-feed contact surfaces, feed contact surfaces, or transient surfaces. Specifically, this study highlights the abundance of antimicrobial resistance genes (ARG) and metal tolerance genes (MTG) were found. All but one isolate was found to have at least one ARG, and a majority contained at least one MTG as well. Understanding what bacteria are intrinsically present, as well as what resistance genes are circulating within residential populations of bacteria is an important piece of the puzzle when characterizing risk within the field to fork continuum.

Another valuable insight to help minimize risk of foodborne pathogen presence within the food supply is understanding how different sanitizers impact bacterial populations, (specifically after formation of biofilms) on surfaces that are commonly used within tree fruit harvesting process. The tree fruit harvesting process and subsequent storage facilities are recognized to be ideal for bacterial growth, so characterization of sanitizer impact on the surfaces used is necessary to better inform sanitation practices on-farm.

The studies within this dissertation identified that, when tested on *E. coli* and *L. monocytogenes*, significant variations in sanitizer efficacy were noted. Efficacy was also impacted by surface type or exposure time; specifically, high density polyethylene (HDPE) surfaces consistently showed lower recovered bacterial populations when compared to more porous surfaces such as wood. Exposure to chlorine dioxide gas (ClO_2) resulted in the most consistent management of bacterial population, but may still struggle on bacteria found on wood or when used in commercial environments. The variability noted within sanitizer application underscores the importance of tailoring sanitation practices to specific materials and environmental conditions found within harvesting and storage operation, as well as the need for facilities to consider the unique characteristics of their operation when developing sanitation protocols.

In addition to understanding how environmental and physical properties of surfaces can impact sanitizer efficacy, there is a need for ongoing research to better understand how sanitizer exposure can impact bacterial populations. The study within this dissertation examining transcriptome expression identified that *L. monocytogenes* biofilms were found to have differing levels of gene expression after exposure to different types of sanitizers; further research is necessary to characterize the differences in gene expression after prolonged exposure to different

types of sanitizers in order to better understand how sanitizer-induced stress response impacts gene regulation.

The overall results of this research underscore the need to have a more comprehensive understanding of how bacteria and foodborne pathogens are able to not only enter into the food supply, but also survive once introduced. Further research is needed to not only better understand how the types of surfaces, environmental conditions, as well as production practices found within facilities that are handling and storing raw or minimally processed goods impacts sanitation efforts. Additional research is needed to understand how different types of stress can impact what the genetic makeup of these bacterial communities are, as well as how exposure to sublethal conditions can further influence gene expression.