

Risk characterization of *Escherichia coli* and *Listeria monocytogenes* as pathogens of concern in
produce safety

by

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Abstract

Both *Escherichia coli* and *Listeria monocytogenes* represent a challenge for produce safety. Through the years, the causal agent of several fresh produce foodborne outbreaks has been traced back to irrigation water systems contaminated with *E. coli*. Rising concerns regarding the safety of agricultural water has led to the implementation of governmental preventative regulation and investigations into how irrigation water systems are getting contaminated with *E. coli*. At the same time farm and produce operations offer ideal conditions for *L. monocytogenes*. This organism has become a top threat to the fresh produce industry with numerous routes of direct and indirect contamination due to its persistence and biofilm forming abilities. The produce industry needs to understand how to effectively combat both *E. coli* and *L. monocytogenes* to reduce the chances of fresh produce related foodborne outbreaks. Therefore, the objectives of this study were as follows: 1) characterize *E. coli* from irrigation water in Kansas and Missouri through Whole-Genome Sequencing; 2) analyze the attachment and formation of *L. monocytogenes* biofilms on different surfaces; and 3) evaluate the effectiveness of single and simultaneous applications of UV-C light and sanitizers against *L. monocytogenes* biofilms.

For objective 1: quarterly water samples collected from five farms in Kansas and Missouri were filtered using the EPA Method 1603. Presumptive *E. coli* were then confirmed by polymerase chain reaction (PCR) before undergoing whole-genome sequencing (WGS). Further analysis was conducted *in-silico* to understand serotype, Antimicrobial Resistant (AMR) genes, virulent genes, source of contamination, and phylogenetic relationships. Results indicate the presence of 99 different serotypes, all samples had at least two AMR genes, and four isolates

were found to be Shiga-toxin producing. More than half of the isolates collected (53%) could be traced back to a bovine source of contamination.

Objective 2 included the use of a Center for Disease Control and Prevention (CDC) biofilm reactor to grow multi-strain *L. monocytogenes* biofilms on wood, nylon, and polycarbonate coupons at $20\pm 2^{\circ}\text{C}$. Biofilm formation and attachment was evaluated through a rinse assay while cell hydrophobicity was completed using the standard sessile drop technique. Wood coupons were the most hydrophilic surface ($\sim 35^{\circ}$) and overall cells exhibited significantly greater attachment to nylon ($P < 0.05$).

Finally, objective 3 was aimed to test the effectiveness of sanitizers, after biofilm growth. Individual applications of UV-C light or a chemical sanitizer as well as the simultaneous application of a chemical sanitizer and UV-C light were studied. Following treatment, coupons were neutralized, and remaining cells enumerated. Material (wood, nylon, and polycarbonate) were found to play a significant role in the efficacy of sanitizers ($P < 0.05$) with the most effective treatment (up to 4 log CFU/cm² reduction) being the simultaneous application of UV-C light and 5% silver citric acid ($P < 0.05$).

The results obtained in this research underline the importance of agricultural water sources and environmental conditions when assessing produce safety. It also provides an initial understanding of *L. monocytogenes* biofilms and their response to the use of UV-C light and chemical sanitizers to effectively control this pathogen in produce processing environments.

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Dedication

I would like to dedicate this thesis to my family who have supported me through the ups and downs of this journey; to Kevin Snell for being a strong support system and a constant morale booster; to the friends that became family during my time here; and finally, to Matthew Jacobs for his encouragement and unyielding support.

Chapter 1 - Literature Review

1.1 Food Safety Modernization Act | FSMA

Foodborne illness is an important public health issue that when estimated causes 48 million people in the United States to get sick each year (CDC, 2018). Of those millions affected 128,000 are hospitalized and 3,000 die all of which are the result of a primarily preventable cause (CDC, 2018). Incidents involving contaminated foods have widely changed consumers eating habits leading to a large public push to implement more rigorous and proactive food policies (Strauss, 2011). Regulating the safety of the U.S. food supply falls into the shared responsibilities of the Food and Drug Administration (FDA) and the U. S. Department of Agriculture (USDA) (Johnson R., 2016). The U.S. food system was found to be lacking in the funding, organization, structure, and regulatory means to confidently contest the threats of foodborne illness (Johnson R., 2016). In 2011, to overhaul the nation's established food safety laws President Obama signed into effect the Food Safety Modernization Act (FSMA) (Strauss, 2011).

The FSMA law is encompassed under four main titles:

Title I- Improving Capacity to Prevent Food Safety Problems

Title II- Improving Capacity to Detect and Respond to Food Safety Problems

Title III- Improving the Safety of Imported Food

Title IV- Miscellaneous Provisions

This law reformed the 70-year-old U.S. Federal Food, Drug, and Cosmetics (FD&C) Act shifting the focus of the U.S. food supply chain from responding to food contamination to preventing it (Kheradia et al., 2013). The focus on prevention revolves around microbial sources of contamination rather than physical or chemical hazards due to how uncommon illnesses are

associated with those hazards (Jung et al., 2014). This new law only effects products within FDA regulation, giving the agency the power to mandate recalls, enhance food safety surveillance systems, enhance traceability, increase inspection, and broadened FDA's position on imported goods from foreign companies (Congressional Research Service, 2016). This comprehensive law is vast, and the provisions require a timeline of adjustment for members within the food supply chain. Once fully implemented, FSMA will create a prevention-based food system that could potentially lower the social and economic burden of foodborne illness within the United States. The elements of FSMA can be identified under five main points:

1. Preventive Controls
2. Inspection and Compliance
3. Response
4. Imported Food Safety
5. Enhanced Partnerships

During the development of those main points, seven “foundational” rules were identified for the full implementation of FSMA. Those rules include Preventive Controls for Human Food, Preventive Controls for Animal Food, Produce Safety, Foreign Supplier Verification Programs, Third Party Certification, Sanitary Transportation, and Intentional Adulteration (Congressional Research Service, 2016). In the present research we will only review the Produce Safety Rule (PSR): since it established science-based standards for safe growing, harvesting, picking, handling, and holding of fresh produce for human consumption on foreign and domestic farming operations (FSMA §105(a)).

1.1.1 FSMA Produce Safety Rule| PSR

The food system within the U.S. signifies one of the most important components of the nation's economy affecting each consumer's health, environment, and social well-being (Nesheim et al., 2015). Understanding the role, the food system plays in people's everyday lives provides perspective on the importance of foodborne illness prevention. The PSR finalized in 2016 installed the first-ever regulatory standard for all aspects of the fresh produce industry. This ruling covers domestic and wild animals; employee health and hygiene; biological soil amendments; equipment, tools, buildings, and sanitation; sprout production; and agricultural water quality (FDA, 2018). The goal of this regulation was to increase consumer confidence by implementing preventive measures so fresh fruits and vegetables are produced and handled with consumers' health and safety in mind. Furthermore, the PSR was established based on the causal agents of previous produce outbreaks those agents include irrigation water, soil amendments, wild and domestic animals, worker hygiene, and post-harvest handling and sanitation. Pathogens that can be linked with fresh produce are *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. (Jung et al., 2014). For this review the section on irrigation water within the PSR will be explored in more detail to understand more about the microbial hazards associated with agricultural water.

Agricultural water is defined by the FDA (2022) as “water that is intended to, or likely to, contact the harvestable portion of covered produce or food-contact surfaces”. The established guidelines for water quality are based on the presence of generic *Escherichia coli*, a common indicator for fecal contamination (FDA, 2022). Under the current PSR requirements, a microbial water quality profile (MWQP), surveying a minimum of 20 samples over 2 to 4 years, must be established when the source of surface water irrigation is directly applied to produce growing

systems (other than sprouts) (Havelaar et al., 2017). The results of the survey will then be used to build a rolling dataset for the assessment of MWQP, calculating the geometric mean (GM) that should not exceed 126 CFU/100ml and the statistical threshold value (STV) that should not exceed 410 CFU/100ml, for preharvest agricultural water (FDA, 2022). The GM is the measure of the distribution of water quality by finding the central tendency and STV is the measure of the variability among the distribution of water quality (Perez et al., 2021). If the water does not meet the parameters established, it should not be used by the grower until proper corrective measures have been implemented and the water complies with the established criteria. After the MWQP has been established the grower must continue to do annual surveys throughout the years the source is used as an agricultural water source. At the same time, there is a proposed revision to the rule that if finalized will replace the current microbial quality criteria and testing requirements as previously stated (FDA 2022). Under the proposed revision farms covered by the ruling would be required to complete pre-harvest agricultural water analysis only once on an annual basis (FDA, 2022). Regardless of the final ruling there must be no detectable generic *E. coli* in water that is to be used during and after harvesting that includes water used for hand washing, ice making, and others. (Allende et al., 2018). Mitigating water risk requires the implementation of strict regulation standards. Agricultural water is a key risk factor for produce contamination and a large portion of the PSR.

1.2 *Escherichia coli* in irrigation water systems

Escherichia coli in irrigation water systems is a growing field of investigation aiming to provide consumers with the safest produce available. In recent years numerous foodborne outbreaks involving pathogenic *Escherichia coli*, have been traced back to irrigation water systems (CDC, 2019). In 2018 an outbreak of *E. coli* on romaine lettuce, crossing into multiple

states resulting in 210 infections was traced back to dirty canal water (CDC, 2018). The source of *E. coli* contamination has been linked to manure pollutants commonly introduced to surface water by ruminant mammals (Pachepsky et al., 2011). In 2005, a large outbreak in Sweden implicating lettuce contaminated with verotoxin-producing *E. coli* infecting 135 people was traced back to irrigation water. The stream used to irrigate the lettuce was downstream from a cattle farm where fecal run-off was entering the water (Soderstrom et al., 2008). *E. coli* is commonly found in the intestinal tracts of warm-blooded organisms with a diverse family of pathogenic and non-pathogenic strains (WHO, 2018). Strains of concern linked to food and water outbreaks are usually Shiga toxin-producing *E. coli* (STEC), including *E. coli* O157:H7, which is particularly dangerous to consumers (FDA, 2019). Identifying the strains of *E. coli* commonly found in local water sources as well as understanding the nature of the contamination can be useful to understand which preventative actions are necessary for future produce safety efforts.

Pathogenic strains of *E. coli* can be transmitted through adulterated food or water sources. There are six main virulent *E. coli* pathotypes: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), Shiga toxin-producing (STEC), enteroinvasive (EIEC), and diffusely adherent (DAEC). These virulent types of *E. coli* have been linked to numerous waterborne outbreaks. For example, during the summer of 1975 at Crater Lake national park in Oregon an ETEC strain later identified from the park's water supply caused gastrointestinal illness in 200 staff members and 2000 visitors (Rosenberg, 1977). In the summer of 1998, a large outbreak in Alpine, Wyoming caused 157 illnesses due to a non-chlorinated water supply where STEC was later detected (Olsen, 2002). These are just a few examples of how threatening *E. coli* is to consumers through water. Contaminated drinking water

has historically been proven to be of serious concern now water contaminated with *E. coli* that is applied to fresh produce is of rising concern. In 2018, a STEC outbreak in romaine lettuce was traced back to an agriculture water reservoir on the farm where the produce was grown (CDC, 2019). This shows that water containing *E. coli*, being unsafe to drink, is also unsafe to irrigate fresh fruits and vegetables with. There is now an increasing number of foodborne disease outbreaks associated with fresh produce contaminated through agricultural water.

Agricultural water is typically required for irrigation during the produce growing season. This is especially true in areas where rainfall is scarce, and irrigation is required to maintain high production yields (Truchado et al., 2018). Growers use various sources of surface water including streams, reservoirs, lakes, creeks, ponds, etc. all of which show growing concerns for microbial quality. Today, due to an increase in water scarcity the use of dubious water sources is being heavily relied on (Allende et al., 2015). Based on figures from the U.S. Geological Survey estimates of water use in the U.S., irrigation withdrawals were 40% of the total freshwater withdrawals and surface water accounted for 58% of the total irrigation withdrawals (Lumia et al., 2000). The literature reviewed shows that no two water sources are the same and the assessment of quality varies based on factors such as weather conditions, livestock placement, microbial adherence ability, and others (Truchado et al., 2018; Allende et al., 2015; Pachepsky et al., 2011). All these factors have been studied and confirmed as factors influencing water contamination (Truchado et al., 2018). Monitoring systems for irrigation water have been found to lack consistency or are not very rigorous. Many epidemiological investigations into food poisoning outbreaks implicating irrigation water have been inconclusive with only circumstantial evidence to support the source of the outbreak (Pachepsky et al., 2011). Of these investigations

many were unable to find pathogenic strains of *E. coli* after the outbreaks occurred (Soderstrom et al., 2008; Cooley et al., 2007).

Detecting fecal indicator bacteria is a suitable way to generally understand the safety of an agricultural water source; however, it does not offer specificity. Fecal indicator bacteria provide no details on the contaminating *E. coli* strains host. Nevertheless, they do offer produce farmers a cost-effective method for improving consumers' safety. Federal and state governments are working with local resources to provide funding and information to growers on water quality. These efforts aim to build an infrastructure that provides the safest fruits and vegetables to consumers no matter the farm's size as well as create a system that prioritizes prevention rather than reaction to foodborne outbreaks. To prevent foodborne outbreaks, we still need to better understand where the contamination is occurring, how the contamination entered the water source, and how to link pathogens to agricultural water sources.

1.2.1 The use of generic *Escherichia coli* as microbial indicator

Many strategies for tracking methods of waterborne disease rely heavily on fecal indicator bacteria (FIB) such as generic *Escherichia coli*, total coliforms, fecal coliforms, and enterococci. The use of FIB is ideal and most widely used due to the varying testing formats that are relatively cheap, easy to use, and do not require extensive laboratory equipment (Rochelle-Newall et al., 2015). These indicator bacteria are thought to inhabit the intestinal tract of warm-blooded animals and are introduced to a water system through feces. An ideal fecal indicator will not reproduce outside the host and should have a comparable life span to pathogenic microorganisms. Fecal source tracking is used to exclusively measure fecal contamination in water and is not a method of identifying the contamination source (Field et al., 2007). Finding the presence of FIB in water does not directly indicate the presence of a pathogenic

microorganism; however, it does prove that there is an increased risk of fecal-borne bacteria and viruses.

Generic *E. coli*, although still under assessment by the FDA, has been considered a consistent indicator of fecal contamination and is currently used as the method of microbial assessment under the Public Safety Rule (PSR) (Rodrigues et al., 2019). FDA acknowledges that *E. coli* is beneficial for assessing fecal contaminants in irrigation water as well as assessing the effectiveness of producers' corrective actions to improve surface water quality.

The use of generic *E. coli* as FIB is limited due to its ability to survive and establish populations in natural environments (Field et al., 2007). In recent studies, the effectiveness of fecal indicator bacteria has come into question with research suggesting *E. coli* and enterococci can survive in soil and in cold-blooded animals (Field et al., 2007; Byappanahalli et al., 2020; Ksiazczyk et al., 2021). New research utilizing next generation sequencing has provided more information on the ecology of *E. coli*. *E. coli* indigenous to soil and water sources are being identified and have been found to be genetically different from those isolated from host-specific sources (Luo et al., 2011). The naturalization of *E. coli* populations in environments brings into question the reliability of this bacterium as a fecal indicator (Ishii et al., 2009). Research conducted by Gu et al., 2013, found that populations of fecal coliforms strongly correlated with the occurrence of *E. coli* O157 rather than generic *E. coli*. Despite the limited potential of generic *E. coli* as an indicator, this microorganism is still considered a valid way to identify contamination in a water source. The presence of generic *E. coli* implies fecal contamination, which is a known reservoir of pathogenic microorganisms (Rodrigues et al., 2019).

1.2.2 The use of whole-genome sequencing for characterization and source tracking

Molecular methods have been improved to increase the specificity and sensitivity of researchers' abilities to characterize and source-track bacteria. With rising popularity and accessibility, these methods are expanding the amount of information that can be collected and analyzed from varying isolates. The capability to genotype a microorganism has opened doors for studying the global evolution of pathogens and understanding genetic similarities in microorganisms of interest.

Whole-genome sequencing (WGS) is among some of the most popular applications of next generation sequencing. WGS is a method that orders the bases within a genome of a microorganism, identifying unique DNA fingerprints (CDC, 2016). This tool has a broad scope of applications within the food industry including monitoring current and emerging pathogens, determining bacterial persistence and antimicrobial resistance, and assisting in traceback investigations. Numerous studies have proven the expansive and detailed results that can come from WGS outputs. One study conducted on *E. coli* isolates highlighted the genetic variability between isolates found within the gastrointestinal tracts of warm-blooded animals and the environment (Luo et al., 2011). Iguchi et al. (2009), did an analysis of EPEC *E. coli* O127:H6 strain E2348/69 providing the first opportunity for researchers to fully dissect (within the genomic context) the virulent strategies used during pathogenic adhesion and effacing. Researchers are now continuously learning more about the mechanisms bacteria use to persist, cause human illness, and adapt environmentally.

The high resolving power of WGS has opened the door for a broad range of opportunities regarding genetic analysis and identifying the origin of contamination. The establishment of all-encompassing databases have provided ease for investigators to track the sources of isolates. The

question of whether different isolates can originate from the same source can be found through the analysis of phylogenetic trees, single-nucleotide polymorphisms (SNP), and epidemiological data (Pightling et al., 2018). The limitation of this method of traceback is the dependence on the database of genome sequences. The database supported by international collaborations from researchers and governments alike is GenomeTrakr Network via National Center for Biotechnology Information's (NCBI) Pathogen Detection Portal, which is the most extensive in terms of food safety (Pightling et al., 2018; FDA, 2022). Due to the broad diversity of *E. coli* strains, including over 180 different O-groups and 53 H-groups, library dependent tracking methods could be limited (Jang et al., 2017; Fratamico et al., 2016). Currently, on NCBI, most of the 250,000 *E. coli* genomes uploaded were isolated from clinical settings with only 40,000 isolated environmentally (NCBI, 2022). Therefore, an analysis of serotypes could be used as an alternative method for potential source identification. Serotyping is a traditional method of classification used for *E. coli* isolates with both standardized procedures and *in-silico* methods (Joensen et al., 2015). The O-antigen undergoes uncompromising selection pressure from the host immune system creating specific host-pathogen interactions (Ingle et al., 2016; DeRoy et al., 2011). Urdahl et al. (2003) found distinct association with *E. coli* serotypes and animal species when studying sheep and cattle. Therefore, after serotyping one could link specific *E. coli* serotypes to a potential host. Whole-genome sequencing has made genotypic and phenotypic characterization a rapid method that will become more widely used and standard protocol for labs upon receiving isolates. With continual growth in the understanding of microorganism's diversity, the use of whole-genome sequencing will be an effective tool for expanding researchers' knowledge.

1.3 *Listeria monocytogenes* on fresh produce

Listeria monocytogenes, a recurring problem for many members within the food industry, is now a major causal agent in fresh produce outbreaks. With 21 reported species among the *Listeria* genus, *Listeria monocytogenes* remains to be the pathogenic species of concern (Nwaiwu, 2020). *L. monocytogenes* in the invasive form of listeriosis poses a great risk to human health with high mortality rates to the very young, elderly, immune-compromised, and pregnant (FDA, 2022).

This organism is widely distributed throughout the natural environment being found in water, soil, manure, etc. with numerous routes of contamination within the food supply chain (Garner et al., 2016). Many of these routes remain uncharacterized, with most research efforts being focused on food processing environments (Belias et al., 2022; Leong et al., 2014; Aryal et al., 2019). Historically, *L. monocytogenes* has been a concern in Ready-To-Eat (RTE) foods, dairy products, and meat products with high tolerances and adaptation for refrigeration temperatures, low pH, and high salinity environments (Townsend et al., 2021). This organism is known for its ability to persist in hostile circumstances, which can be linked to its biofilm forming abilities (Srey et al., 2013). Concern over the prevalence of *Listeria* in fresh produce has greatly increased within the industry in recent years as there is no kill step involved with minimally processed foods (Townsend et al., 2021). Research efforts are now looking at detection, prevalence, and distribution within fresh produce facilities to understand the significance of the threat *L. monocytogenes* holds (Sullivan et al., 2020; John et al., 2020; Murugesan et al., 2015).

The first *L. monocytogenes* outbreak associated with fresh vegetables occurred in the fall of 1979 in Boston, MA with 23 people hospitalized. The causal product could not be confirmed due to the lack of molecular confirmation techniques at that time (Ho et al., 1986; Garner et al.,

2016). This episode was followed by other small outbreaks from the 1980s up until 2011 when *Listeria* was linked to a cantaloupe outbreak. This nationwide outbreak affected 28 states with an identified 147 patients which included 143 hospitalizations and 33 deaths, making *L. monocytogenes* the agent responsible for one of the largest foodborne disease outbreaks of the decade (McCollum et al., 2013). This outbreak was also unique because it was the first outbreak to implicate a whole cantaloupe, bringing into question how *L. monocytogenes* spread and where/how it could be controlled (Graner et al., 2016). Since 2011, *L. monocytogenes* has been responsible for outbreaks in leafy greens, mushrooms, stone fruits, apples, sprouts, and more (CDC, 2022; Garner et al., 2016; Zhu et al., 2017). Fresh produce has become a high-risk commodity that requires attention to employ effective preventative measures. There is a need to understand how *L. monocytogenes* attaches to surfaces and what preventative strategies are available to aspiring and small-scale farmers.

Farmers not involved in large, commercialized produce production need the attention of researchers to fully prevent *L. monocytogenes* from becoming a major problem. Local food has become a mainstream preference with 68.7 percent of locally grown food being sold directly to consumers (Martinez et al., 2021). This figure is based on an evaluation of marketing practices throughout the U.S. by the USDA Economic Research Service where local foods are defined as products sold directly from the farm to consumers, retail markets, institutions, or intermediate markets within a 400-mile radius of origin or is within the same state of origin (Martinez et al., 2021; Martinez et al., 2010). Equipment used in small scale production tends to be inexpensive and simple due to the high cost of new equipment not being a feasible investment (Pressman A, 2011). Researchers need to understand how to control *L. monocytogenes* on food contact surfaces and equipment for both large and small-size production facilities. Local farmers are

projected to continue growing economically with great promise within the food system (Brainard et al., 2017). Supporting small growers through their journey in food safety is just a tier within the complex web of the nation's food system.

1.3.1 *Listeria* biofilms

Listeria is one of many species of bacteria with the capability of forming biofilms. This complex physiological process causes nearly 60% of foodborne outbreaks (Han et al., 2017). Biofilms create more resistant cells that are difficult to eradicate, resulting in recurrent growth in food processing and handling environments. Biofilms are an assembly of microbial cells held together through extracellular polymeric substance (EPS) that can adhere and grow on biotic and abiotic surfaces (Donlan R.M., 2002; Srey et al., 2013). The developmental stages of a biofilm can be broken down into five general steps: initial attachment, irreversible attachment, propagation, maturation, and dispersion (Srey et al., 2013).

The first step in the process of biofilm formation is initial attachment. This is highly dependent on the environment surrounding the cell, and the physiochemical properties of the bacterial cell and the surface (Srey et al., 2013). The initial attachment is a reversible process that includes planktonic cells responding to stressors in their environment (Muhammad et al., 2020). Environmental factors such as surface charge, hydrophobicity, pH, temperature, shear force, available nutrients, and textural surface differences play key roles in the first phase of biofilm formation (Srey et al., 2013; Colagiorgi et al., 2017). Once the initial attachment has been established, the cells undergo a series of morphological changes shifting from reversible to irreversible attachment (Srey et al., 2013). Once cells have reached this point, they become substantially more difficult to remove. From here the cells begin to propagate with the production of EPS. EPS is a tacky glue-like substance made up of polysaccharides, protein, and

DNA (Costa et al., 2018). The formation of EPS is essential in biofilm formation influencing structure complexity and stability, increasing resistance to antimicrobials, improving cell-to-cell communication, increasing resistance to environmental stressors as well as providing nutrient reserves to viable cells (Srey et al., 2013; Donlan RM., 2002; Colagiorgi et al., 2017; Costa et al., 2018). Henceforth the biofilm begins to mature which is loosely defined by the development of differential layers of microorganisms in a complex structure (Lavery et al., 2015). At this stage, the biofilm is a fully self-produced system that can survive in many harsh conditions. The formation of a biofilm is a cycle, once the cells reach maturation they can disperse and start the cycle all over.

1.4 The use of antimicrobial interventions in the produce industry

There are numerous points within the fruit and vegetable supply chain where microbial contamination can occur. Therefore, to prevent the introduction of pathogens growers have implemented preventive measures as well as intervention strategies into their production processes. This includes the application of good manufacturing practices and improved cleaning/sanitation methods (Gil et al., 2015). The most commonly used strategies for cleaning and sanitation in the produce industry are quaternary ammonium compound (QAC), chlorine, chlorine dioxide, hydrogen peroxide, alcohols, and peroxyacetic acid (Aryal et al., 2019; Skowron et al., 2018). Hua et al, (2019), did a comprehensive study on commonly used commercial sanitizers (QAC, PAA (peracetic acid), chlorine, and chlorine dioxide) against 7-day old *L. monocytogenes* biofilms developed on stainless steel, low-density polyethylene, polyvinyl chloride, polyester, and rubber. This research revealed that QAC and chlorine sanitizers lack efficacy on irregular food contact surfaces. In addition, chlorine-based products have shown little success in inhibiting *L. monocytogenes* biofilms (Dharmarha et al., 2020; Aryal et al., 2019).

Chlorine is the most widely used sanitizer but has proven ineffective when organic and inorganic components intermix within the sanitation solution (Murray et al., 2017). Today agricultural production has been seen to greatly impact the environment with many farmers moving towards more long-term sustainable farming systems (Roesch et al., 2021). Breaking away from traditional chemical sanitizers and exploring sustainable and green opportunities for cleaning and sanitation could be a way to meet consumer's demand for safe produce while investigating their ability to control and prevent biofilms.

1.4.1 Lactic acid

Lactic acid is a multi-faceted organic acid with many uses such as food preservation, flavoring agent, and antibacterial. Lactic acid is derived from lactic acid bacteria (LAB) where upon fermentation, the by-product lactic acid is naturally produced to inhibit competing bacteria by creating an acidic to neutral pH environment (Abedi et al., 2020). Commercial applications have modified the gram-positive bacteria to tolerate more acidic conditions to extract and purify lactic acid (Abedi et al., 2020). Numerous studies have been conducted on the effectiveness of lactic acid as a sanitizer on both gram-positive and negative bacteria. A study by Alakomi et al., (2000) demonstrated the ability of lactic acid to permeate the outer membrane of *E. coli*, *Salmonella*, and *Pseudomonas*. In a study conducted by Wang et al. (2015), lactic acid resulted in higher levels of protein leakage in *Listeria* compared to *Salmonella* and *E. coli*. The lethality of lactic acid bacteria is due to the undissociated molecules that flow through the cell membranes, causing an acidic pH both inside and outside the cell resulting in distortion and damage to DNA and proteins (Stanojevic-Nikolic et al., 2016). Undissociated molecules have been found to penetrate the cell membrane lipid bilayer more easily indicating that “weak” acids are strong microbial inhibitors (Raybaudi-Massilia et al., 2009). Lactic acid as a chemical

sanitizer has proven to be effective against *L. monocytogenes* biofilms when studied on food and food-contact surfaces. Srey et al., (2014) was able to achieve a 3-log reduction of 24-h old *L. monocytogenes* biofilms on cabbage using 2% (v/v) lactic acid. Yang et al., (2009), found lactic acid to be the most effective sanitizer compared to other commercial options against >7-day old *L. monocytogenes* biofilms on high-density polyethylene. In a study by Chen et al. (2019) analyzing the efficacy of lactic acid against *L. innocua* in brussels sprouts a 3-log reduction of cells was achieved after 120s of exposure to 2% lactic acid.

1.4.2 Thymol

Thymol is a monoterpene phenol extracted from plants within the Lamiaceae family with numerous applications in the food industry as an essential oil (Marchese et al., 2016). Thymol has been used as flavoring, a preservative, and as an antibacterial. This essential oil has a broad antimicrobial spectrum (Rota et al., 2008; Olasupo et al., 2003; Gavaric et al., 2015). Thymol appears to permeate the cell membrane with slightly different modes of action between gram-positive and gram-negative bacteria. In gram-positive bacteria, thymol is shown to integrate within the phospholipid bilayer creating physical distortion, establishing passive permeability of the cell membrane (Burt S. 2004). In gram-negative bacteria, thymol disintegrates the outer membrane freeing lipopolysaccharides and increasing permeability of the cytoplasmic membrane (Burt S. 2004). Desai et al., (2012) used thymol at 0.05% concentration on 24-h old *L. monocytogenes* biofilms where nearly a 5-log reduction was observed. Vazquez-Sanchez et al., (2018), found thymol to be the most effective essential oil (EO) at disrupting 24-h old *L. monocytogenes* biofilms as compared to other EOs: *Lippia sidoides* or *Pimenta pseudochariophyllus*. Further studies have looked at thymol used in combination with other

chemicals; few have studied the efficiencies of thymol on *Listeria* biofilms that are over 24-h old.

1.4.3 Silver dihydrogen citrate

Silver has a lengthy history as an antimicrobial in the medical field being used on surgical needles, wound dressings, medical implants, medical equipment and more (Sim et al., 2018). Silver cations penetrate the cells' outer membrane disrupting metabolic pathways, inhibiting cellular respiration, and interrupting DNA replication (Sim et al., 2018). Despite silver's broad applications in the medical field against many microorganisms silver ions are usually highly unstable resulting in a short lifespan requiring constant re-application (Manuel 2017). Nevertheless, silver ions were found to stabilize with citric acid creating silver dihydrogen citrate (SDC), a patented molecule by Pure Bioscience (El Cajon, CA) (Arata A.B., 2005). The addition of citric acid diversified the mode of action against bacteria, compelling organisms to see SDC as a food source allowing the molecule within the cell membrane where the silver ions denature the DNA, stopping cellular replication (Pure Bioscience, Inc., 2022). This compound is non-toxic, colorless, odorless, and capable of being incorporated into solution. Few studies have been conducted on the effects of this molecule on *Listeria* biofilms, but studies on *Listeria* have proven this compound as an effective sanitizer. Masuku et al., (2014) achieved a 4-5 log reduction of *Listeria* cells on aluminum and stainless steel after 2-min of treatment exposure. Further research has yet to explore SDC against *L. monocytogenes* biofilms.

1.4.4 UV irradiation

Ultraviolet irradiation is traditionally used as a non-thermal disinfectant for surface, air, and water sanitization (Gayan et al., 2014). There are three types of UV radiation: UV-A (315-400nm), UV-B (280-315nm), and UV-C (200-280nm) (FDA, 2021). UV-C light is a form of

irradiation with the most lethal range of wavelengths that microorganisms are equally sensitive to after exposure (Dai et al., 2012). Ultraviolet light damages cells' genetic material forming dimers within DNA making it difficult for cells to replicate which results in cell death (Dai et al., 2012). The use of UV-C light has become popular in processing environments due to the lack of physical scrubbing and its inability to damage the textural properties of food products (Khan et al., 2017). The effectiveness of UV-C light against several foodborne pathogens has been demonstrated in many studies (Lim and Harrison, 2016; Sommers et al., 2015; Morey et al., 2010). Bernbom et al. (2011), found that UV-C light was able to kill *L. monocytogenes* 1-h and 7-day old biofilms on stainless steel coupons after 10 minutes of exposure. With wavelengths between 200-280 nm and direct exposure to the food contact surface, germicidal inactivation is a possible alternative for mechanical sanitation strategies (FDA, 2021).

1.4.5 Simultaneous use of antimicrobial strategies

Food safety has now turned to synergistic effects or hurdle technologies to combat pathogens in food processing environments. Synergism takes two or more processes (hurdles) that when applied sequentially or simultaneously create a greater germicidal effect than if those same tools were used separately (Berdejo et al., 2019). This concept can range in application within the food industry from reducing the loss of nutrients, loss of sensory quality, and/or to improve food safety. Combining physical, chemical, and biological techniques when applied with intent has been shown to alter specific factors (pH, temperature, redox potential, water activity, competitive microorganisms, and others) creating stressful environments that are difficult for microorganisms to overcome (Mukhopadhyay and Gorris, 2014; Putnik et al., 2020). Each hurdle process when used correctly is meant to inactivate, eliminate, or inhibit unwanted pathogens. Hurdle technologies can be easily adjusted based on the virulence of a microorganism. It can also be

more economical without compromising product quality, and it fits consumers' desire for minimally processed foods (Mukhopadhyay and Gorris, 2014).

The simultaneous application of UV-C light and a chemical sanitizer could represent a unique strategy to overwhelm microorganisms' ability to endure the application of sanitizers. Many studies have already been analyzing the combined efforts of antimicrobial strategies. Kim et al. (2016), found the combined treatments of UV-C irradiation and sodium hypochlorite to effectively reduce 24-h old *L. monocytogenes* biofilms on eggshells and stainless steel. Park et al., (2018), looked at the combined application of UV-C light and chlorine dioxide on fresh produce to reduce *Salmonella*, *E. coli*, and *Listeria*: in combination, sanitizers were found to be highly effective. Two additional studies found similar results regarding synergistic sanitizer use on food surfaces (Ha et al., 2011; Mukhopadhyay et al., 2015). The improvement of sanitation efficiency on food surfaces using hurdle technologies can be found throughout scientific literature, however, few studies have analyzed the effects on food contact surfaces harboring biofilms. Establishing strong measures for cleaning fresh produce is important, but efficiently sanitizing food contact surfaces will reduce recontamination. The fresh produce industry is directing its focus towards prevention; therefore, the simultaneous applications of antimicrobial strategies on food contact surfaces could be the next step in implementing strong preventative procedures.

References

- Abedi, E., & Hashemi, S. M. B. (2020). Lactic acid production – producing microorganisms and substrates sources-state of art. *Heliyon*, 6(10), e04974. <https://doi.org/10.1016/j.heliyon.2020.e04974>
- Alakomi, H.-L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Applied and Environmental Microbiology*, 66(5), 2001–2005. <https://doi.org/10.1128/AEM.66.5.2001-2005.2000>
- Allende, A., Datta, A. R., Smith, W. A., Adonis, R., MacKay, A., & Adell, A. D. (2018). Implications of new legislation (US FSMA) and guidelines (EC) on the establishment of management systems for agricultural water. *Food Microbiology*, 75, 119–125. <https://doi.org/10.1016/j.fm.2017.10.002>
- Allende, A., & Monaghan, J. (2015). Irrigation Water Quality for Leafy Crops: A Perspective of Risks and Potential Solutions. *International Journal of Environmental Research and Public Health*, 12(7), 7457–7477. <https://doi.org/10.3390/ijerph120707457>
- Arata, A. B. (2005). *Silver Dihydrogen Citrate Composition Comprising a Second Antimicrobial Agent* (Patent No. US 2005/0202066A1). <https://patentimages.storage.googleapis.com/2a/73/53/29aa947ff07c67/US20050202066A1.pdf>
- Aryal, M., & Muriana, P. M. (2019). Efficacy of Commercial Sanitizers Used in Food Processing Facilities for Inactivation of *Listeria monocytogenes*, *E. Coli*

O157:H7, and Salmonella Biofilms. *Foods*, 8(12), 639.

<https://doi.org/10.3390/foods8120639>

Belias, A., Sullivan, G., Wiedmann, M., & Ivanek, R. (2022). Factors that contribute to persistent *Listeria* in food processing facilities and relevant interventions: A rapid review. *Food Control*, 133, 108579.

<https://doi.org/10.1016/j.foodcont.2021.108579>

Berdejo, D., Pagán, E., García-Gonzalo, D., & Pagán, R. (2019). Exploiting the synergism among physical and chemical processes for improving food safety. *Current Opinion in Food Science*, 30, 14–20.

<https://doi.org/10.1016/j.cofs.2018.08.004>

Bernbom, N., Vogel, B. F., & Gram, L. (2011). *Listeria monocytogenes* survival of UV-C radiation is enhanced by presence of sodium chloride, organic food material and by bacterial biofilm formation. *International Journal of Food Microbiology*, 147(1), 69–73. <https://doi.org/10.1016/j.ijfoodmicro.2011.03.009>

Brainard, L., James Bullard, Tropp, D., Moraghan, M. R., Danaher, K., Harris, G., Jablonski, B. B., Hendrickson, M., Vogel, S., Schmit, T., Kagan, A., Merrigan, K. A., Feldstein, S., Lo, J., Spach, C., Phillips, R., Wallace, D., Matteson, G., Gwin, L., ... Wascalus, J. (2017). *Harvesting Opportunity: The Power of Regional Food System Investments to Transform Communities*. Federal Reserve Bank of St. Louis and the Board of Governors of the Federal Reserve System.

https://www.stlouisfed.org/-/media/project/frbstl/stlouisfed/files/pdfs/community-development/harvesting-opportunity/harvesting_opportunity.pdf#page=15

- Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, 94(3), 223–253.
<https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>
- Byappanahalli, M., Fowler, M., Shively, D., & Whitman, R. (2003). Ubiquity and Persistence of *Escherichia coli* in a Midwestern Coastal Stream. *Applied and Environmental Microbiology*, 69(8), 4549–4555.
<https://doi.org/10.1128/AEM.69.8.4549-4555.2003>
- Center for Disease Control and Prevention (CDC). (2016). Whole genome sequencing (WGS). <https://www.cdc.gov/pulsenet/pathogens/wgs.html>.
- Center for Disease Control and Prevention (CDC). (2018). Multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce (final update).
<https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html>
- Center for Disease Control and Prevention (CDC). (2018). Burden of foodborne illness: findings. <https://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html>
- Center for Disease Control and Prevention (CDC). (2019). Outbreak of *E. coli* infection slinked to romaine lettuce. <https://www.cdc.gov/ecoli/2018/o157h7-11-18/index.html>.
- Center for Disease Control and Prevention (CDC). (2022). *Listeria* outbreaks.
<https://www.cdc.gov/listeria/outbreaks/index.html>.
- Chen, L., Zhang, H., Liu, Q., Pang, X., Zhao, X., & Yang, H. (2019). Sanitising efficacy of lactic acid combined with low-concentration sodium hypochlorite on *Listeria innocua* in organic broccoli sprouts. *International Journal of Food Microbiology*, 295, 41–48.
<https://doi.org/10.1016/j.ijfoodmicro.2019.02.014>

- Colagiorgi, A., Bruini, I., Di Ciccio, P. A., Zanardi, E., Ghidini, S., & Ianieri, A. (2017). *Listeria monocytogenes* Biofilms in the Wonderland of Food Industry. *Pathogens*, 6(3), 41. <https://doi.org/10.3390/pathogens6030041>
- Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M. T., Myers, C., Rose, C., Keys, C., Farrar, J., & Mandrell, R. E. (2007). Incidence and Tracking of *Escherichia coli* O157:H7 in a Major Produce Production Region in California. *PLoS ONE*, 2(11), e1159. <https://doi.org/10.1371/journal.pone.0001159>
- Costa, O. Y. A., Raaijmakers, J. M., & Kuramae, E. E. (2018). Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation. *Frontiers in Microbiology*, 9, 1636. <https://doi.org/10.3389/fmicb.2018.01636>
- Dai, T., Vrahas, M. S., Murray, C. K., & Hamblin, M. R. (2012). Ultraviolet C irradiation: An alternative antimicrobial approach to localized infections? *Expert Review of Anti-Infective Therapy*, 10(2), 185–195. <https://doi.org/10.1586/eri.11.166>
- DebRoy, C., Roberts, E., & Fratamico, P. M. (2011). Detection of O antigens in *Escherichia coli*. *Animal Health Research Reviews*, 12(2), 169–185. <https://doi.org/10.1017/S1466252311000193>
- Desai, M. A., Soni, K. A., Nannapaneni, R., Schilling, M. W., & Silva, J. L. (2012). Reduction of *Listeria monocytogenes* Biofilms on Stainless Steel and Polystyrene Surfaces by Essential Oils†. *Journal of Food Protection*, 75(7), 1332–1337. <https://doi.org/10.4315/0362-028X.JFP-11-517>
- Dharmarha, V., Boyer, R. R., Strawn, L. K., Drape, T. A., Eifert, J., Vallotton, A. D., Pruden, A., & Ponder, M. A. (2020). An Assessment of Produce Growers'

- Sanitizer Knowledge and Practices on the Correct Use of Sanitizers. *Food Protection Trends*, 40(3), 140–146.
- Donlan, R. M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, 8(9), 881–890. <https://doi.org/10.3201/eid0809.020063>
- Field, K. G., & Samadpour, M. (2007). Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research*, 41(16), 3517–3538. <https://doi.org/10.1016/j.watres.2007.06.056>
- Food Safety Modernization Act (FSMA), no. H.R. 2751, 111th Cong., 124 3885 (2011). <https://www.fda.gov/food/food-safety-modernization-act-fsma/full-text-food-safety-modernization-act-fsma#SEC105>
- Fratamico, P. M., DebRoy, C., Liu, Y., Needleman, D. S., Baranzoni, G. M., & Feng, P. (2016). Advances in Molecular Serotyping and Subtyping of *Escherichia coli*†. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.00644>
- Garner, D., & Kathariou, S. (2016). Fresh Produce–Associated Listeriosis Outbreaks, Sources of Concern, Teachable Moments, and Insights. *Journal of Food Protection*, 79(2), 337–344. <https://doi.org/10.4315/0362-028X.JFP-15-387>
- Gavaric, N., Mozina, S. S., Kladar, N., & Bozin, B. (2015). Chemical Profile, Antioxidant and Antibacterial Activity of Thyme and Oregano Essential Oils, Thymol and Carvacrol and Their Possible Synergism. *Journal of Essential Oil Bearing Plants*, 18(4), 1013–1021. <https://doi.org/10.1080/0972060X.2014.971069>

Gayán, E., Condón, S., & Álvarez, I. (2014). Biological Aspects in Food Preservation by Ultraviolet Light: A Review. *Food and Bioprocess Technology*, 7(1), 1–20.

<https://doi.org/10.1007/s11947-013-1168-7>

Gil, M. I., Selma, M. V., Suslow, T., Jacxsens, L., Uyttendaele, M., & Allende, A. (2015). Pre- and Postharvest Preventive Measures and Intervention Strategies to Control Microbial Food Safety Hazards of Fresh Leafy Vegetables. *Critical Reviews in Food Science and Nutrition*, 55(4), 453–468.

<https://doi.org/10.1080/10408398.2012.657808>

Gu, G., Luo, Z., Cevallos-Cevallos, J. M., Adams, P., Vellidis, G., Wright, A., & van Bruggen, A. H. C. (2013). Factors affecting the occurrence of *Escherichia coli* O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed. *Canadian Journal of Microbiology*, 59(3), 175–182.

<https://doi.org/10.1139/cjm-2012-0599>

Ha, J.-H., Lee, D.-U., Auh, J.-H., & Ha, S.-D. (2011). Synergistic Effects of Combined Disinfecting Treatments Using Sanitizers and UV to Reduce Levels of *Bacillus cereus* in Oyster Mushroom. *Journal of the Korean Society for Applied Biological Chemistry*, 54(2). <https://doi.org/10.3839/jksabc.2011.042>

Han, Q., Song, X., Zhang, Z., Fu, J., Wang, X., Malakar, P. K., Liu, H., Pan, Y., & Zhao, Y. (2017). Removal of Foodborne Pathogen Biofilms by Acidic Electrolyzed Water. *Frontiers in Microbiology*, 8, 988.

<https://doi.org/10.3389/fmicb.2017.00988>

Havelaar, A. H., Vazquez, K. M., Topalcengiz, Z., Muñoz-Carpena, R., & Danyluk, M. D. (2017). Evaluating the U.S. Food Safety Modernization Act Produce Safety

- Rule Standard for Microbial Quality of Agricultural Water for Growing Produce. *Journal of Food Protection*, 80(11), 1832–1841. <https://doi.org/10.4315/0362-028X.JFP-17-122>
- Ho, J. L., Shands, K. N., Friedland, G., Eckind, P., & Fraser, D. W. (1986). An Outbreak of Type 4b *Listeria monocytogenes* Infection Involving Patients From Eight Boston Hospitals. *Archives of Internal Medicine*, 146(3), 520. <https://doi.org/10.1001/archinte.1986.00360150134016>
- Hua, Z., Korany, A. M., El-Shinawy, S. H., & Zhu, M.-J. (2019). Comparative Evaluation of Different Sanitizers Against *Listeria monocytogenes* Biofilms on Major Food-Contact Surfaces. *Frontiers in Microbiology*, 10, 2462. <https://doi.org/10.3389/fmicb.2019.02462>
- Iguchi, A., Thomson, N. R., Ogura, Y., Saunders, D., Ooka, T., Henderson, I. R., Harris, D., Asadulghani, M., Kurokawa, K., Dean, P., Kenny, B., Quail, M. A., Thurston, S., Dougan, G., Hayashi, T., Parkhill, J., & Frankel, G. (2009). Complete Genome Sequence and Comparative Genome Analysis of Enteropathogenic *Escherichia coli* O127:H6 Strain E2348/69. *Journal of Bacteriology*, 191(1), 347–354. <https://doi.org/10.1128/JB.01238-08>
- Ingle, D. J., Valcanis, M., Kuzevski, A., Tauschek, M., Inouye, M., Stinear, T., Levine, M. M., Robins-Browne, R. M., & Holt, K. E. (2016). In silico serotyping of *E. coli* from short read data identifies limited novel O-loci but extensive diversity of O:H serotype combinations within and between pathogenic lineages. *Microbial Genomics*, 2(7). <https://doi.org/10.1099/mgen.0.000064>

- Congressional Research Service. (2016). Implementation of the FDA Food Safety Modernization Act (*FSMA, P.L. 111-353*) (No. R43724; pp. 1–30).
https://www.everycrsreport.com/files/20161216_R43724_0dbf28baf5500c2b9955e545ed4c42c774b49f79.pdf
- Interagency Food Safety Analytics Collaboration (IFSAC). (2020). Foodborne illness source attribution estimates for 2018 for Salmonella, Escherichia coli O157, Listeria monocytogenes, and Campylobacter using multi-year outbreak surveillance data, United States. GA and D.C.: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Food and Drug Administration, U.S. Department of Agriculture's Food Safety and Inspection Service. <https://www.cdc.gov/foodsafety/ifsac/pdf/P19-2018-report-TriAgency-508.pdf>
- Ishii, S., & Sadowsky, M. J. (2009). Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental Microbiology*, 11(4), 733–740. <https://doi.org/10.1111/j.1462-2920.2008.01856.x>
- Jang, J., Hur, H.-G., Sadowsky, M. J., Byappanahalli, M. N., Yan, T., & Ishii, S. (2017). Environmental *Escherichia coli*: Ecology and public health implications-a review. *Journal of Applied Microbiology*, 123(3), 570–581.
<https://doi.org/10.1111/jam.13468>
- Joensen, K. G., Tetzschner, A. M. M., Iguchi, A., Aarestrup, F. M., & Scheutz, F. (2015). Rapid and Easy *In Silico* Serotyping of Escherichia coli Isolates by Use of Whole-Genome Sequencing Data. *Journal of Clinical Microbiology*, 53(8), 2410–2426.
<https://doi.org/10.1128/JCM.00008-15>

- John, J., Joy, W.-C., & Jovana, K. (2020). Prevalence of *Listeria* spp. In produce handling and processing facilities in the Pacific Northwest. *Food Microbiology*, 90, 103468. <https://doi.org/10.1016/j.fm.2020.103468>
- Johnson, R. (2016). *The Federal Food Safety System: A Primer* (No. RS22600). Congressional Research Service. <http://nationalaglawcenter.org/wp-content/uploads/assets/crs/RS22600.pdf>
- Jung, Y., Jang, H., & Matthews, K. R. (2014). Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. *Microbial Biotechnology*, 7(6), 517–527. <https://doi.org/10.1111/1751-7915.12178>
- Khan, I., Tango, C. N., Miskeen, S., Lee, B. H., & Oh, D.-H. (2017). Hurdle technology: A novel approach for enhanced food quality and safety – A review. *Food Control*, 73, 1426–1444. <https://doi.org/10.1016/j.foodcont.2016.11.010>
- Kheradia, A., & Warriner, K. (2013). Understanding the Food Safety Modernization Act and the role of quality practitioners in the management of food safety and quality systems. *The TQM Journal*, 25(4), 347–370. <https://doi.org/10.1108/17542731311314854>
- Kim, M., Park, S. Y., & Ha, S.-D. (2016). Synergistic effect of a combination of ultraviolet–C irradiation and sodium hypochlorite to reduce *Listeria monocytogenes* biofilms on stainless steel and eggshell surfaces. *Food Control*, 70, 103–109. <https://doi.org/10.1016/j.foodcont.2016.05.003>
- Książczyk, M., Dudek, B., Kuczkowski, M., O’Hara, R., Korzekwa, K., Wzorek, A., Korzeniowska-Kowal, A., Upton, M., Junka, A., Wieliczko, A., Ratajszczak, R., & Bugla-Płoskońska, G. (2021). The Phylogenetic Structure of Reptile, Avian and

- Uropathogenic *Escherichia coli* with Particular Reference to Extraintestinal Pathotypes. *International Journal of Molecular Sciences*, 22(3), 1192.
<https://doi.org/10.3390/ijms22031192>
- Lavery, G., Gorman, S. P., & Gilmore, B. F. (2015). Biofilms and implant-associated infections. In *Biomaterials and Medical Device—Associated Infections* (pp. 19–45). Elsevier. <https://doi.org/10.1533/9780857097224.1.19>
- Leong, D., Alvarez-Ordaz, A., & Jordan, K. (2014). Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. *Frontiers in Microbiology*, 5.
<https://doi.org/10.3389/fmicb.2014.00436>
- Lim, W., & Harrison, M. A. (2016). Effectiveness of UV light as a means to reduce *Salmonella* contamination on tomatoes and food contact surfaces. *Food Control*, 66, 166–173. <https://doi.org/10.1016/j.foodcont.2016.01.043>
- Lumia, D.S., Linsey, K. S., Barber, N.L. (2000). Estimated use of water in the United States in 2000. *U.S. Geological Survey*. <https://pubs.usgs.gov/fs/2005/3051/>
- Luo, C., Walk, S. T., Gordon, D. M., Feldgarden, M., Tiedje, J. M., & Konstantinidis, K. T. (2011). Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. *Proceedings of the National Academy of Sciences*, 108(17), 7200–7205.
<https://doi.org/10.1073/pnas.1015622108>
- Manuel, C. S., Moore, M. D., & Jaykus, L. -A. (2017). Efficacy of a disinfectant containing silver dihydrogen citrate against GI .6 and GII .4 human norovirus.

Journal of Applied Microbiology, 122(1), 78–86.

<https://doi.org/10.1111/jam.13331>

- Marchese, A., Orhan, I. E., Daglia, M., Barbieri, R., Di Lorenzo, A., Nabavi, S. F., Gortzi, O., Izadi, M., & Nabavi, S. M. (2016). Antibacterial and antifungal activities of thymol: A brief review of the literature. *Food Chemistry*, 210, 402–414. <https://doi.org/10.1016/j.foodchem.2016.04.111>
- Martinez, S., Hand, M., Da Pra, M., Pollack, S., Ralston, K., Smith, T., Vogel, S., Clark, S., Lohr, L., Low, S., & Newman, C. (2010). *Local food systems concepts, impacts, and issues* (Economic Research Report No. 97). United States Department of Agriculture. https://www.ers.usda.gov/webdocs/publications/46393/7054_err97_1_.pdf
- Martinez, S.W. & Timothy P. (2021). Marketing Practices and Financial Performance of Local Food Producers: A Comparison of Beginning and Experienced Farmers, EIB-225, U.S. Department of Agriculture, Economic Research Service.
- Masuku, S. M., Babu, D., Martin, E. M., O'Bryan, C. A., Crandall, P. G., & Ricke, S. C. (2014). Lethality of moist heat and silver dihydrogen citrate sanitizer combinations on *Listeria* spp. Adhered to components of a deli meat slicer. *Food Control*, 44, 227–232. <https://doi.org/10.1016/j.foodcont.2014.03.030>
- McCollum, J. T., Cronquist, A. B., Silk, B. J., Jackson, K. A., O'Connor, K. A., Cosgrove, S., Gossack, J. P., Parachini, S. S., Jain, N. S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N. W., Gorny, J. R., Mogen, K. M., Spires, C., Teitell, P., ... Mahon, B. E. (2013). Multistate Outbreak of Listeriosis Associated with Cantaloupe. *New England Journal of Medicine*, 369(10), 944–953. <https://doi.org/10.1056/NEJMoa1215837>

- Morey, A., McKee, S. R., Dickson, J. S., & Singh, M. (2010). Efficacy of Ultraviolet Light Exposure Against Survival of *Listeria monocytogenes* on Conveyor Belts. *Foodborne Pathogens and Disease*, 7(6), 737–740.
<https://doi.org/10.1089/fpd.2009.0464>
- Muhammad, M. H., Idris, A. L., Fan, X., Guo, Y., Yu, Y., Jin, X., Qiu, J., Guan, X., & Huang, T. (2020). Beyond Risk: Bacterial Biofilms and Their Regulating Approaches. *Frontiers in Microbiology*, 11, 928.
<https://doi.org/10.3389/fmicb.2020.00928>
- Mukhopadhyay, S., & Gorris, L. G. M. (2014). Hurdle Technology*. In *Encyclopedia of Food Microbiology* (pp. 221–227). Elsevier. <https://doi.org/10.1016/B978-0-12-384730-0.00166-X>
- Mukhopadhyay, S., Ukuku, D. O., & Juneja, V. K. (2015). Effects of integrated treatment of nonthermal UV-C light and different antimicrobial wash on *Salmonella enterica* on plum tomatoes. *Food Control*, 56, 147–154.
<https://doi.org/10.1016/j.foodcont.2015.03.020>
- Murray, K., Wu, F., Shi, J., Jun Xue, S., & Warriner, K. (2017). Challenges in the microbiological food safety of fresh produce: Limitations of post-harvest washing and the need for alternative interventions. *Food Quality and Safety*, 1(4), 289–301. <https://doi.org/10.1093/fqsafe/fyx027>
- Murugesan, L., Kucerova, Z., Knabel, S. J., & LaBORDE, L. F. (2015). Predominance and Distribution of a Persistent *Listeria monocytogenes* Clone in a Commercial Fresh Mushroom Processing Environment. *Journal of Food Protection*, 78(11), 1988–1998. <https://doi.org/10.4315/0362-028X.JFP-15-195>

- National Library of Medicine: National Center for Biotechnology Information (NCBI). (2022). Pathogen detection. <https://www.ncbi.nlm.nih.gov/pathogens/>
- Nesheim, M. C., Oria, M., Yih, P. T., National Research Council (U.S.), & Institute of Medicine (U.S.) (Eds.). (2015). *A framework for assessing the effects of the food system*. National Academies Press.
- Nwaiwu, O. (2020). What are the recognized species of the genus *Listeria*? *Access Microbiology*, 2(9). <https://doi.org/10.1099/acmi.0.000153>
- Olasupo, N. A., Fitzgerald, D. J., Gasson, M. J., & Narbad, A. (2003). Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. *Letters in Applied Microbiology*, 37(6), 448–451. <https://doi.org/10.1046/j.1472-765X.2003.01427.x>
- Olsen, S. J. (2002). A Waterborne Outbreak of *Escherichia coli* O157:H7 Infections and Hemolytic Uremic Syndrome: Implications for Rural Water Systems. *Emerging Infectious Diseases*, 8(4), 370–375. <https://doi.org/10.3201/eid0804.000218>
- Pachepsky, Y., Shelton, D. R., McLain, J. E. T., Patel, J., & Mandrell, R. E. (2011). Irrigation Waters as a Source of Pathogenic Microorganisms in Produce: A Review. In *Advances in Agronomy* (Vol. 113, pp. 75–141). Elsevier. <https://doi.org/10.1016/B978-0-12-386473-4.00002-6>
- Park, S.-H., Kang, J.-W., & Kang, D.-H. (2018). Inactivation of foodborne pathogens on fresh produce by combined treatment with UV-C radiation and chlorine dioxide gas, and mechanisms of synergistic inactivation. *Food Control*, 92, 331–340. <https://doi.org/10.1016/j.foodcont.2018.04.059>

- Perez, A. P., Weaver, R., Fryer, J., Cureau, N. (2021). Microbial water quality profile (MWQP): how to interpret your lab results. <https://www.uaex.uada.edu/farm-ranch/crops-commercial-horticulture/horticulture/arkansas-produce-safety/MWQP%20Lab%20Results.pdf>
- Pightling, A. W., Pettengill, J. B., Luo, Y., Baugher, J. D., Rand, H., & Strain, E. (2018). Interpreting Whole-Genome Sequence Analyses of Foodborne Bacteria for Regulatory Applications and Outbreak Investigations. *Frontiers in Microbiology*, 9, 1482. <https://doi.org/10.3389/fmicb.2018.01482>
- Putnik, P., Pavlič, B., Šojić, B., Zavadlav, S., Žuntar, I., Kao, L., Kitonić, D., & Kovačević, D. B. (2020). Innovative Hurdle Technologies for the Preservation of Functional Fruit Juices. *Foods*, 9(6), 699. <https://doi.org/10.3390/foods9060699>
- Pressman A. (2011). Equipment and tools for small-scale intensive crop production. NCAT. <https://attra.ncat.org/htmlpub/equipment-and-tools-for-small-scale-intensive-crop-production/>
- Pure Bioscience, Inc. (2022). Our Technology. <https://purebio.com/technology/>
- Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., Soliva-Fortuny, R., & Martín-Belloso, O. (2009). Control of Pathogenic and Spoilage Microorganisms in Fresh-cut Fruits and Fruit Juices by Traditional and Alternative Natural Antimicrobials. *Comprehensive Reviews in Food Science and Food Safety*, 8(3), 157–180. <https://doi.org/10.1111/j.1541-4337.2009.00076.x>
- Rochelle-Newall, E., Nguyen, T. M. H., Le, T. P. Q., Sengtaheuanghoung, O., & Ribolzi, O. (2015). A short review of fecal indicator bacteria in tropical aquatic

- ecosystems: Knowledge gaps and future directions. *Frontiers in Microbiology*, 6.
<https://doi.org/10.3389/fmicb.2015.00308>
- Rodrigues, C., da Silva, A. L. B. R., & Dunn, L. L. (2019). Factors Impacting the Prevalence of Foodborne Pathogens in Agricultural Water Sources in the Southeastern United States. *Water*, 12(1), 51. <https://doi.org/10.3390/w12010051>
- Roesch, A., Nyfeler-Brunner, A., & Gaillard, G. (2021). Sustainability assessment of farms using SALCA sustain methodology. *Sustainable Production and Consumption*, 27, 1392–1405. <https://doi.org/10.1016/j.spc.2021.02.022>
- Rosenberg, M. L. (1977). Epidemic Diarrhea at Crater Lake from Enterotoxigenic *Escherichia coli*: A Large Waterborne Outbreak. *Annals of Internal Medicine*, 86(6), 714. <https://doi.org/10.7326/0003-4819-86-6-714>
- Rota, M. C., Herrera, A., Martínez, R. M., Sotomayor, J. A., & Jordán, M. J. (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control*, 19(7), 681–687.
<https://doi.org/10.1016/j.foodcont.2007.07.007>
- Sim, W., Barnard, R., Blaskovich, M. A. T., & Ziora, Z. (2018). Antimicrobial Silver in Medicinal and Consumer Applications: A Patent Review of the Past Decade (2007–2017). *Antibiotics*, 7(4), 93. <https://doi.org/10.3390/antibiotics7040093>
- Skowron, K., Hulisz, K., Gryń, G., Olszewska, H., Wiktorczyk, N., & Paluszak, Z. (2018). Comparison of selected disinfectants efficiency against *Listeria monocytogenes* biofilm formed on various surfaces. *International Microbiology*, 21(1–2), 23–33. <https://doi.org/10.1007/s10123-018-0002-5>

- Söderström, A., Österberg, P., Lindqvist, A., Jönsson, B., Lindberg, A., Blide Ulander, S., Welinder-Olsson, C., Löfdahl, S., Kaijser, B., De Jong, B., Kühlmann-Berenzon, S., Boqvist, S., Eriksson, E., Szanto, E., Andersson, S., Allestam, G., Hedenström, I., Ledet Muller, L., & Andersson, Y. (2008). A Large *Escherichia coli* O157 Outbreak in Sweden Associated with Locally Produced Lettuce. *Foodborne Pathogens and Disease*, 5(3), 339–349.
<https://doi.org/10.1089/fpd.2007.0065>
- Sommers, C. H., & Sheen, S. (2015). Inactivation of avirulent *Yersinia pestis* on food and food contact surfaces by ultraviolet light and freezing. *Food Microbiology*, 50, 1–4. <https://doi.org/10.1016/j.fm.2015.02.008>
- Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control*, 31(2), 572–585.
<https://doi.org/10.1016/j.foodcont.2012.12.001>
- Srey, S., Park, S. Y., Jahid, I. K., & Ha, S.-D. (2014). Reduction effect of the selected chemical and physical treatments to reduce *L. monocytogenes* biofilms formed on lettuce and cabbage. *Food Research International*, 62, 484–491.
<https://doi.org/10.1016/j.foodres.2014.03.067>
- Stanojević-Nikolić, S., Dimić, G., Mojović, L., Pejin, J., Djukić-Vuković, A., & Kocić-Tanackov, S. (2016). Antimicrobial Activity of Lactic Acid Against Pathogen and Spoilage Microorganisms: Antimicrobial Activity of Lactic Acid. *Journal of Food Processing and Preservation*, 40(5), 990–998. <https://doi.org/10.1111/jfpp.12679>

- Strauss, D. M. (2011). An Analysis of the FDA Food Safety Modernization Act: Protection for consumers and Boon for Business. *Food and Drug Law Journal*, 66(3), 353–376.
- Sullivan, G., & Wiedmann, M. (2020). Detection and Prevalence of *Listeria* in U.S. Produce Packinghouses and Fresh-Cut Facilities. *Journal of Food Protection*, 83(10), 1656–1666. <https://doi.org/10.4315/JFP-20-094>
- Townsend, A., Strawn, L. K., Chapman, B. J., & Dunn, L. L. (2021). A Systematic Review of *Listeria* Species and *Listeria monocytogenes* Prevalence, Persistence, and Diversity throughout the Fresh Produce Supply Chain. *Foods*, 10(6), 1427. <https://doi.org/10.3390/foods10061427>
- Truchado, P., Hernandez, N., Gil, M. I., Ivanek, R., & Allende, A. (2018). Correlation between *E. coli* levels and the presence of foodborne pathogens in surface irrigation water: Establishment of a sampling program. *Water Research*, 128, 226–233. <https://doi.org/10.1016/j.watres.2017.10.041>
- Urdahl, A. M., Beutin, L., Skjerve, E., Zimmermann, S., & Wasteson, Y. (2003). Animal host associated differences in Shiga toxin-producing *Escherichia coli* isolated from sheep and cattle on the same farm. *Journal of Applied Microbiology*, 95(1), 92–101. <https://doi.org/10.1046/j.1365-2672.2003.01964.x>
- U. S. Food and Drug Administration (FDA). (2018). What the produce safety rule means for consumers. <https://www.fda.gov/food/food-safety-modernization-act-fsma/what-produce-safety-rule-means-consumers#:~:text=The%20Produce%20Safety%20Rule%20establishes,wide%20diversity%20of%20produce%20farms.>

- U.S. Food and Drug Administration (FDA). (2019). *Escherichia coli* (*E. coli*).
<https://www.fda.gov/food/foodborne-pathogens/escherichia-coli-e-coli>.
- U. S. Food and Drug Administration (FDA). (2021). UV lights and lamps: ultraviolet-C radiation, disinfection, and coronavirus. <https://www.fda.gov/medical-devices/coronavirus-covid-19-and-medical-devices/uv-lights-and-lamps-ultraviolet-c-radiation-disinfection-and-coronavirus>
- U.S. Food and Drug Administration (FDA). (2022). FSMA final rule on produce safety. <https://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-final-rule-produce-safety>
- U.S. Food and Drug Administration (FDA). (2022). FSMA proposed rule on agriculture water. <https://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-proposed-rule-agricultural-water>
- U.S. Food and Drug Administration (FDA). (2022). GenomeTrakr Network.
<https://www.fda.gov/food/whole-genome-sequencing-wgs-program/genometrakr-network>
- U.S. Food and Drug Administration (FDA). (2022). Listeria (listeriosis).
<https://www.fda.gov/food/foodborne-pathogens/listeria-listeriosis>
- Vázquez-Sánchez, D., Galvão, J. A., Ambrosio, C. M. S., Gloria, E. M., & Oetterer, M. (2018). Single and binary applications of essential oils effectively control *Listeria monocytogenes* biofilms. *Industrial Crops and Products*, 121, 452–460.
<https://doi.org/10.1016/j.indcrop.2018.05.045>
- Wang, C., Chang, T., Yang, H., & Cui, M. (2015). Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella Enteritidis*,

Escherichia coli and Listeria monocytogenes. *Food Control*, 47, 231–236.

<https://doi.org/10.1016/j.foodcont.2014.06.034>

World Health Organization (WHO). (2018). *E. coli*. <https://www.who.int/news-room/fact-sheets/detail/e-coli>.

Yang, H., Kendall, P. A., Medeiros, L. C., & Sofos, J. N. (2009). Efficacy of Sanitizing Agents against Listeria monocytogenes Biofilms on High-Density Polyethylene Cutting Board Surfaces. *Journal of Food Protection*, 72(5), 990–998.

<https://doi.org/10.4315/0362-028X-72.5.990>

Zhu, Q., Gooneratne, R., & Hussain, M. (2017). Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. *Foods*, 6(3), 21.

<https://doi.org/10.3390/foods6030021>

7 CFR § 205.203

Chapter 2 - Research Importance

Escherichia coli and *L. monocytogenes* are both important subjects of concern to the safety of fresh produce. There have been a rising number of investigations into irrigation water as the causal agent of *E. coli* outbreaks associated with raw produce. This has led to strict regulations regarding water quality. Nevertheless, research focusing on the microbial quality of irrigation water is limited with many efforts focusing on recreational, drinking, and wastewater. A detailed understanding of the *E. coli* that threaten irrigation waters may help in developing risk management strategies to reduce the risk of *E. coli* contaminating water and consequently taint fresh produce.

L. monocytogenes has become a leading threat to the produce industry through its ubiquitous and persistent biofilm forming abilities. Knowledge on how this organism attaches and forms complex biofilm structures on a variety of surfaces used during the harvesting and handling of produce is still elusive. Many researchers have analyzed the behavior of *L. monocytogenes* biofilms on common food contact surfaces; however, none have focused on the irregular surfaces found on farms used for pre- and post- harvest handling. Furthermore, efforts to understand how to eradicate mature *L. monocytogenes* biofilms on these irregular surfaces using sustainable methods is also very limited.

For these reasons, the objectives of this research were to:

- Characterize *E. coli* isolated from produce irrigation water systems in Kansas and Missouri through whole-genome sequencing. Further analysis was conducted *in-silico* to understand serotype, antimicrobial resistant (AMR) genes, virulent genes, seasonality, and identify the potential source of contamination.

- Understand the attachment and formation characteristics of a 96-h multi-strain *L. monocytogenes* biofilms grown on wood, nylon, and polycarbonate.
- Evaluate the efficacy of chemical sanitizers (lactic acid, silver dihydrogen citrate, thymol) and UV-C light alone and simultaneously against *L. monocytogenes* biofilms grown on wood, nylon, and polycarbonate.

Chapter 3 - Characterization of *Escherichia coli* Isolates from Produce Irrigation Water in Kansas and Missouri by Whole-Genome Sequencing

Abstract

Contaminated irrigation water has been a reported source of pathogenic *Escherichia coli* in recent produce-related outbreaks. The purpose of this study was to characterize *E. coli* isolates from produce irrigation water sources using whole-genome sequencing (WGS). Ground and surface water samples were collected quarterly from five farms in Missouri and Kansas over a one-year period. Presumptive *E. coli* was isolated after each sample was filtered using EPA Method 1603. A total of 530 isolates were analyzed by polymerase chain reaction (PCR) with 191 confirmed as *E. coli*. WGS was carried out using an Illumina MiSeq system. *De novo* genome assemblies were obtained with the Shovill pipeline version 0.9. NCBI Pathogen Detection was used to identify antimicrobial resistant (AMR) genes. Confirmed *E. coli* counts were higher during spring and summer as compared to winter. A diverse serotype pool was observed from the collected samples. Over 53% of isolates could be linked to bovine as an initial contamination source. AMR analysis showed that 100% of the strains carried at least two antimicrobial resistant genes. Recognizing the diversity of *E. coli* serotypes may help guide agricultural water assessments as proposed in the new Food Safety Modernization Act Produce Safety Rule agricultural water rule.

3.1 Introduction

Escherichia coli is a bacterial species commonly found in the intestinal tracts of humans and animals (WHO, 2018). Not all *E. coli* strains are pathogenic. Nevertheless, over the years numerous foodborne outbreaks linked to pathogenic *Escherichia coli*, have been traced back to irrigation water systems (CDC, 2019). The most common strains of concern for consumers are Shiga Toxin-producing *E. coli* (STEC) which can result in severe gastrointestinal diseases upon ingestion (FDA, 2019). Other strains of concern that have been associated with waterborne outbreaks include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) (Hunter PR, 2003). This pathogen has been found to survive in a variety of environments with a high evolutionary capacity (van Elsas et al., 2011). With the increasing awareness of the survivability of *E. coli* in water and the advancements in molecular methodologies, research efforts are seeking to understand pathogen transmission routes into agricultural water systems and the impact on public health.

The Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) established guidelines for produce farmers to assess the quality of agricultural water sources since water sources have been found to be one of the most important pathways of produce contamination (FDA, 2022). Since generic *E. coli* is recognized as an indicator of fecal contamination (Rodrigues et al., 2019), its presence and quantity can help guide producers' actions to improve water quality. Finding *E. coli* in water does not directly indicate the presence of pathogenic microorganisms; it does however prove that there is an increased risk of fecal-borne bacteria present (Rodrigues et al., 2019).

Recent multistate produce outbreaks have highlighted the need for implementing controls and analysis programs in accordance with new regulatory frameworks (FDA, 2022). It is important for produce growers to understand where potential sources of contamination might be entering into their agriculture water system so that they can implement control measures to reduce the likelihood of pathogenic organisms contaminating their produce. The importance of using rapid methods to trace contaminations in the food supply chain, as compared to more traditional and time-consuming tests will improve outbreak investigations (Brown et al., 2019). Genomic techniques, such as Whole Genome Sequencing (WGS), allow for rapid identification of pathogens and have led to the development of databases (e.g. GenomeTrakr) with epidemiological information available to assist microbial monitoring, surveillance, and source identification across the food industry. Therefore, the main objective of this study was to characterize *E. coli* isolates from produce irrigation water sources in Kansas and Missouri using WGS and to trace isolates back to the source that likely contaminated the water.

3.2 Materials and Methods

3.2.1 Sample collection

Based on a previous study (Haley et al., 2022), where produce farms in Missouri and Kansas received free water analysis for generic *E. coli*, five water sources with high MPN (Most Probable Number) values of generic *E. coli* were selected for further investigation. The water sources on these five farms were sampled quarterly over a one-year period during the fall, winter, spring, and summer of 2020-2021. The sources of water sampled included ground (below the Earth's surface) and surface water (exposed to the environment). All ground water samples were collected from a well, while the surface water samples were collected from ponds and a cistern from rainwater catchment. Each pond water source had a 500ml composite sample of five

100ml samples randomly collected around the circumference of the pond. For the cistern, 300ml composite samples were collected with three 100ml samples taken at varying depths of the cistern. Well samples were collected through an attached water pump with two 100ml samples taken after allowing water to run for 30s making a 200ml composite sample. Samples were collected in a sanitary manner in sterilized 120ml polyethylene bottles containing sodium thiosulfate (IDEXX, Westbrook, Maine). For the surface water sampling, collection bottles were fixed to a 3.5-m-long pole in a way that allowed the bottle to enter the water inverted just below the surface of the water, be rotated to collect the water sample, and turned upright to be removed from the water (Fig 3.1). Upon collection, retrieved bottles were capped and put on ice to be transported for further processing within 12 hours of the sampling period.

3.2.2 *Escherichia coli* isolation (EPA Method 1603)

The EPA Method 1603 was followed for water collection, filtration, and colony isolation (EPA, 2014). Briefly, 100ml was taken from the composite sample to be filtered through a 0.45µm membrane filter. Sample dilutions were completed for spring and summer samples to 1/10 and 1/100 respectively, while no dilution was performed for fall and winter samples. This was due to the overwhelming quantity of isolates collected through filtration during the spring and summer seasons, making colony isolation difficult. After filtration, membrane filters were placed on modified membrane-Thermotolerant *Escherichia coli* Agar (mTEC, BD Difco, Sparks, MD). Presumptive *E. coli* colonies (magenta and/or red) were counted and reported as *E. coli* CFU/100ml. Presumptive positive colonies were streaked for isolation on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD). From each TSA plate, a single colony was selected and transferred to Tryptic Soy Broth (BD Difco, Sparks, MD). Cells were preserved using CryoCare

bacteria preservers (Key Scientific Products, INC, Stamford, Texas) and stored at -80°C until further use.

3.2.3 Identification and confirmation of *Escherichia coli* by PCR

A modified PCR protocol was used based on the study by Walker et al. (2017). The primers targeting genes *clpB*, *uidA*, and *ybbW* (Biosearch Technologies, Petaluma, California) targeting *E. coli* spp. isolates are listed in Table 3.1 with positive samples having amplicons of 449, 454, and/or 447 bp. All primer stocks were prepared in 1X Tris-EDTA buffer (Integrated DNA Technologies, Coralville, Iowa) to achieve a 100µM stock concentration. Equal volumes and concentrations of primers were mixed with the addition of nuclease free water to reach a working solution of 10µM. Each reaction contained a combined total volume of 20µl, which includes 1µl of the three-target primer mix at 10µM solution concentration, 10µl iQ Multiplex Powermix (Biorad, Hercules, California), 5µl of template DNA, and 4µl of nuclease-free water. The parameters for the thermal cycler were set as follows: denaturation at 94°C for 5 minutes, followed by 29 cycles of 94 °C for 30s, 62°C for 30s and 68°C for 75s, and a final extension step of 7 min at 68°C. All reactions were completed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Singapore).

To separate amplified DNA fragments, PCR products were run on a 2200 TapeStation (Agilent, Waldronn, Germany). Positive *E. coli* samples showed tight bands with an amplicon at ~450bp and to further confirm the presence of *E. coli*, positive samples were streaked on MacConkey Agar (BD Difco, Sparks, MD).

3.2.4 DNA preparation

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 (Hilden, Germany). DNA concentrations were measured with a Qubit Fluorometer 3.0 using the dsDNA HS assay kit, according to the manufacturer's instructions (Thermo Fisher). DNA extracts were stored at -20°C until WGS analysis.

3.2.5 WGS characterization

The Nextera XT Library Preparation kit (Illumina, San Diego, CA) 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 0.9 (<https://github.com/tseemann/shovill>), available in the GalaxyTrakr pipeline (<http://galaxytrakr.org/>) (Afgan et al., 2018). Default parameters were used in all analyses (Zhang et al., 2015). The NCBI Prokaryotic Genomes Automatic Annotation pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) was used to annotate draft genomes of each isolate. Characterization was performed using an *in-silico* Multilocus Sequence Typing (MLST) for both *E. coli*, based on genome sequences of seven housekeeping genes and core genome MLST analysis using Enterobase v1.1.2 (<https://enterobase.warwick.ac.uk/>).

3.2.6 Isolate characterization

The genetic relatedness of the isolates sequenced were compared with sequences of more than 200,000 *E. coli* isolates from various environmental and clinical sources available in the National Center for Biotechnology Information (NCBI) Pathogen Detection database. Through this database the AMR profiles of each isolate were identified through the AMRFinderPlus 3.10 tool (<https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/>). Further characterization of *E. coli* isolates was conducted *in-silico* through the Center for Genomic Epidemiology (CGE) services. Virulence factors were predicted through the alignment of draft

genome assemblies per isolate against the database through VirulenceFinder 2.0 (v2.0.3). Virulent factors that passed the default threshold of $\geq 90\%$ nucleotide identity and $\geq 60\%$ coverage was accepted as present in the isolate. Rapid serotype identification was completed using SerotypeFinder 2.0 (v2.0.1) with accepted serotype genes falling within the default threshold of $\geq 85\%$ nucleotide identity and $\geq 60\%$ coverage per isolate. A phylogenetic tree was constructed based on the core genome MLST as defined by Ridom SeqSphere (v.8.4) (Ridom GmbH, Germany) where genomes were grouped based on serotype identification with distance centered on the 7 *E. coli* Warwick genes. A retrospective literature study was used to further group isolates centered around animal reservoirs associated with specific serotypes. Articles obtained as suitable for this study were peer-reviewed original research articles

Accession numbers. The sequences of all isolates used in this research are available under the BioProject 357722 and 832234 on NCBI (<https://www.ncbi.nlm.nih.gov/bioproject/357722>) (<https://www.ncbi.nlm.nih.gov/bioproject/832234>).

3.3 Results and Discussion

3.3.1 Prevalence of presumptive *E. coli* in sampled water sources

A total of 570 presumptive generic *E. coli* isolates were collected during the one-year sampling period from 9 different irrigation water sources and 191 samples were confirmed *E. coli* through PCR. Most *E. coli* isolates were sampled during the summer (47%), followed by the spring (42%), fall (6%), and winter (5%) (Fig. 3.2). As expected, a seasonal effect was observed: in warmer months a higher number of microorganisms were present in the different water sources (spring and summer with temperatures reaching over 20°C) as compared to the winter sampling period, where the recorded temperatures were as low as 0°C.

The seasonal impacts on *E. coli* concentrations in water are important to understand when implementing methods that prevent contaminants from entering the food supply. Confounding views on the seasonal variation of *E. coli* concentrations in water sources can be found throughout scientific literature. Limitations such as a lack of monitoring uniformity, climatic zone differences, climate change, and sampling size make it difficult to generalize the seasonal patterns of *E. coli* (Kostyla et al., 2015). Nevertheless, in the present study we observed that the summer season held the highest concentration of *E. coli* isolates. Byappanahalli et al. (2003), found high concentrations of *E. coli* in fresh water near Lake Michigan during warmer sampling periods with concentrations peaking during the late summer. This is also supported by research from Oliver and Page (2016) as well as Vivar and Fuentes (2016) where warmer seasons showed increased numbers in excreted bacterium from animals leading to water contamination. This, when in combination with weather patterns and high temperatures, tends to favor high reproductive and strong survivability environments for *E. coli*. Higher temperatures have been shown to increase microbial concentrations in water, yet the relationship of other meteorological factors greatly influences microbial load (Ward et al., 2015).

No difference between ground and surface water sources could be observed. Provided the limited number of ground water sources collected within the parameters of this study it is difficult to understand if surface and ground water sources carry equal risks when characterizing *E. coli*. It can however be noted that the cistern and well were observed to be safeguarded water sources inaccessible to domestic and wild animals, yet still resulted in *E. coli* contamination. The cistern sampled had a large stone lid covering the reservoirs opening but was not consistently covered and was housed in a shed near the main entrance of the building where people were frequently passing. Cisterns can be contaminated with *E. coli* through organic debris or

associated animal feces whether that be through roof runoff or dust kicked up by foot traffic. This is supported by Levesque et al. (2008), who found that soil and dust carried by wind can be washed into freshwater containers after analyzing rainwater collection tanks in Bermuda with 66% of the 102 tanks sampled containing *E. coli*. Flood and heavy rains have also been known to wash debris and fecal pollutants into rain catchment systems and cisterns (CDC, 2022). It is important to note that maintaining the water quality of a cistern can be difficult with numerous routes of microbial contamination and should be regularly monitored (CDC, 2022). The well was the only ground water source and had a covered opening with a hand pump to collect water. Even though the well was not exposed to the environment large quantities of *E. coli* could be found. Andrade et al. (2018), addresses three ways *E. coli* could contaminate well water: 1) directly through well opening, 2) water recharge/deep drainage (geological pathways including shifts in soil), 3) direct migration (contaminated groundwater mixing with non-contaminated ground water. Any of these factors could explain the transfer of *E. coli* contaminants into the well sampled during this study. A study by Sasakova et al. (2018) found contaminated surface water could eventually lead to the contamination of ground water sources and that an aquifer environment could facilitate pathogen survival. Many factors can facilitate the microbial quality of ground water sources, so it is important to note that these sources are still susceptible to contamination.

3.3.2 Isolates characterization

A total of 99 different serotypes of *E. coli* could be identified and results are reported in Table 3.2. Reports of *E. coli* in water have exposed high discrepancies of serotypes with both pathogenic and non-pathogenic isolates. Our study presented with 99 different serotypes; Maloo et al. (2017) and Ramteke et al. (2007) noted the remarkable diversity found among the serotypes

collected from both recreational water and drinking water. The most prevalent serotype identified in our study was *E. coli* O65:H38 with 12 identified isolates all of which were collected during the spring. Other isolates include O43:H2 (n=9), O8:H8 (n=9), O1:H7 (n=8), :H21 (n=7), O23:H16 (n=7), O74:H8 (n=5), -:H2 (n=5), O8:H19 (n=4), -:H7 (n=4), O22:H8 (n=4), O8:H7 (n=4), O175:H16 (n=4), O22:H7 (n=3), O141:H16 (n=3), -:H16 (n=3), -:H2 (n=3), O9:H30 (n=3), -:H42 (n=3), -:H21 (n=3), O73:H45 (n=2), O6:H49 (n=2), O178:H7 (n=2), O17:H45 (n=2), O8:H28 (n=2), O88:H21 (n=2), -:H10 (n=2), -:H8 (n=2), -:H34 (n=2), -:H38 (n=2), -:H20 (n=1), -:H11 (n=1), -:H26 (n=1), -:H31 (n=1), -:H51 (n=1), -:H9 (n=1), O53:H20 (n=1), O1:H19 (n=1), O106:H31 (n=1), O107:H27 (n=1), O113:H5 (n=1), O12:H16 (n=1), O120:H10 (n=1), O133:H29 (n=1), O136:H12 (n=1), O138:H28 (n=1), O140:H36 (n=1), O144:H7 (n=1), O146:H28 (n=1), O147:H21 (n=1), O147:H7 (n=1), O148:H28 (n=1), O148:H8 (n=1), O149:H2 (n=1), O149:H45 (n=1), O15:H18 (n=1), O150:H45 (n=1), O155/O8:H20 (n=1), O159:H21 (n=1), O156:H12 (n=1), O17:H18 (n=1), O174:H4 (n=1), O176:H4 (n=1), O180:H10 (n=1), O21:H12 (n=1), O22:H21 (n=1), O26:H11 (n=1), O3:H21 (n=1), O3:H8 (n=1), O30:H25 (n=1), O35:H21 (n=1), O39:H34 (n=1), O39:H7 (n=1), O4:H10 (n=1), O45:H8 (n=1), O59:H28 (n=1), O7:H16 (n=1), O75:H31 (n=1), O8:H10 (n=1), O81:H1 (n=1), O81:H27 (n=1), O84:H2 (n=1), O88:H7 (n=1), O89:H4 (n=1), O9:H10 (n=1), O9:H12 (n=1), O9:H14 (n=1), O96:H20 (n=1), OX13:H10 (n=1), -:H12 (n=1), -:H16 (n=1), -:H19 (n=1), -:H2/H47 (n=1), -:H26 (n=1), -:H29 (n=1), -:H32 (n=1), -:H46 (n=1), and -:H7 (n=1). Of the total *E. coli* isolates only 2 were found to be a part of the “Big 6” including O26 (SAMN28816604) and O45 (SAMN23828792) (Bertoldi et al., 2017). Each, however, were sourced from two different ponds during different seasons: O26 was isolated during the spring, whereas *E. coli* O45 was isolated in the summer.

In Table 3.2 isolates are grouped by phylogenetic relatedness as well as probable contamination source. Literature supporting serotype-based identification shows isolates deriving from more than one potential host. Bovine were found to be the most common source of likely contamination, encompassing 53% of the *E. coli* serotypes. Other isolates could likely be traced back to human (38%), ovine (17%), poultry (11%), porcine (8%), and deer (2%) sources.

Naturally, a part of a ruminant's microbiota it has been suggested that wildlife are asymptomatic reservoirs that further disseminate *E. coli* strains into or near water sources (Fischer et al., 2001; Stein and Katz, 2017). Cattle are considered the primary hosts of *E. coli* corresponding with the information collected in this study where over half the isolates could be traced back to bovine origination. A study by Osman et al., (2012), found domestic calves, sheep, and goats to harbor many of the same *E. coli* serovars as confirmed in this research. This finding supports the hypothesis that there could be a direct or indirect animal-to-human or animal-to-animal transmission of *E. coli* creating overlap in serotype profiles (Johnson et al, 2001). Observations collected during the sampling periods further supports the likely causes of contamination linked through serotype identification. All pond sources were collected on farms with domesticated animals including ovine, porcine, bovine, dogs, and poultry that could be observed residing near or within a nearby field. All ponds, excluding Pond A, were surrounded by a fence, and many were inaccessible to domesticated animals. Yet, *E. coli* collected from the pond sources could be linked to animals that were observed to be residing on the farms. This indicates that *E. coli* is being spread regardless of the confinements put on the water source. For example, a deer running through a cow field could carry *E. coli* from that field on its hooves and eventually contaminate a water source. *E. coli* can be carried on animals' fur, hooves, paws, skin, feathers, and more spreading *E. coli* to the areas where they roam (CDC, 2019). *E. coli*

contamination in agricultural water as observed is difficult to track with numerous direct and indirect routes of transmission.

At least two antimicrobial resistant genes were expressed in all the isolates collected as reported in Table 3.2. Over 15 different AMR genes were reported through *in-silico* analysis covering many of the antibiotic classes. Each antibiotic class was grouped based on chemical structure (Werth BJ, 2022). The most prevalent AMR gene was *blaEC*, expressing resistance to beta-lactam, spanning across 98% of the samples. The next most prevalent Amr gene was *mdtM* (94%), a gene expressing a multi-drug resistant protein including resistance to nucleoside, phenicol, lincosamides, and fluoroquinolone antibiotics through an efflux pump complex (Alcock et al., 2020). Fifty-seven % of the isolates had the AMR gene *acrF* (57%) encoding for an efflux pump resistance mechanism to fluoroquinolone (Alcock et al., 2020). Finally, the other most frequent genes expressed resistance to tetracyclines (*tetA* (14%) and *tetB* (10%)) and aminoglycosides (*aph(3'')*- Ib (15%) and *aph(6)*-Id (15%)). Coinciding with the results of our study, research by Liao et al. (2021) found all collected *E. coli* isolates from an aquafarm to have at least two drug resistant genes indicating a multi-drug resistant sampling pool. One study suggests bodies of water are ideal places for bacterial AMR evolution to occur naturally due to dense bacterial populations and pollution forcing bacteria to adapt (Lupo et al., 2012). Water sources are opportunistic environments for bacterial AMR gene acquisitions (Liao et al., 2021). Furthermore, Masse et al. (2014), found residual antibiotics in the feces of livestock. A few of the most prevalent antibiotics used in livestock include tetracyclines, fluoroquinolone, and beta-lactams all of which appear as AMR genes within the collected isolates.

Virulent genes are also shown in Table 3.2 with over 45 different genes identified through *in-silico* analysis. Four isolates, all serotyped to be O22:H8 (SAMN23828800,

SAMN23828816, SAMN23828817, and SAMN23894239), could be confirmed as Shiga-toxin producing (*stx2*), one of the most puissant toxins that is associated with the manifestation of hemolytic uremic syndrome (Melton-Celsa AR, 2014). This toxin when ingested can cause severe illness to death and is a threat to public health. This is pressing information because many STEC virulent markers are on mobile genetic elements and can be easily transferred to other isolates (Franz et al., 2007). These Shiga-toxin producing isolates were each collected from a pond source during summer.

E. coli isolates collected from water have shown high rates of virulent factors as reported in numerous surveillance studies including an investigation of the U.S. Salish Sea, by Vingino et al. (2021) and a study of the U.S. Great Lakes by Hamelin et al. (2006). In this present study, the most frequently expressed virulent gene found was *gad* (73%), a highly specific gene to the *E. coli* species commonly used as a prescreening marker for pathogenic groups (Grant et al., 2001). This gene allows isolates to tolerate highly acidic conditions when enduring routes through the gastrointestinal tract by the decarboxylation of glutamate where cells can repel incoming protons (Bergholz et al., 2007). The virulent gene *ipfA* was found in 64% of the isolates, while the gene *iss* was found in 23%. These two genes, generally found in EHEC or EPEC pathotypes, encode for fimbriae that help cellular adhesion and increase serum survival, respectively (Valat et al., 2012). Virulent gene *terC* was found in more than half of the isolates (51%) and represents the key protein needed for tellurite resistance (Turkovicova et al., 2016). Of the total samples, only 7 *E. coli* isolates did not show any virulent genes.

Overall *E. coli* has a great degree of assortment among virulence genes with rapid adaptation resulting in capricious pathogenicity (Kuhnert et al., 2000). Understanding virulent gene profiles may contribute to knowledge on emergent *E. coli* pathotypes

3.4 Conclusion

This study highlights the importance of agricultural water sources and seasonality when assessing produce safety. Cisterns and wells as observed in this study can harbor high concentrations of *E. coli* despite those sources not being exposed to the surrounding environments. Produce growers should consider this when selecting a water source to irrigate fresh produce. Furthermore, our research shows the need for a more expansive assessment of produce irrigation water within Kansas and Missouri to understand the true diversity of *E. coli* in the waters within those states and the importance of characterizing *E. coli* serotypes to help guide agricultural water assessments as proposed in the new agricultural water rule. It can also be concluded that there are numerous direct and indirect routes that *E. coli* can use to contaminate both ground and surface water sources. Produce growers may be able to use the information collected in this study to make informed decisions on how to better prevent *E. coli* contamination routes. As shown in this study *E. coli* can be carried to a water source without an animal host having access to the water source.

3.7 Acknowledgments

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3.6 Figures and Tables



Figure 3.1 Depiction of method used to collect samples from water sources.

Table 3.1 Primer design for *E. coli* PCR identification and confirmation.

Target	Sequence (5'-3')
<i>clpB</i>	FORWARD: CATACGAATGCTGGATGCTG REVERSE: TTTGAAGAACGTTTAAAAGGCG
<i>uidA</i>	FORWARD: ACCACGGTGATATCGTCCAC REVERSE: TACAAGAAAGCCGGGCAAT
<i>ybbW</i>	FORWARD: AATCTGGCCGGGATTTTT REVERSE: 5TGGCTCCGGCAATAATACAT

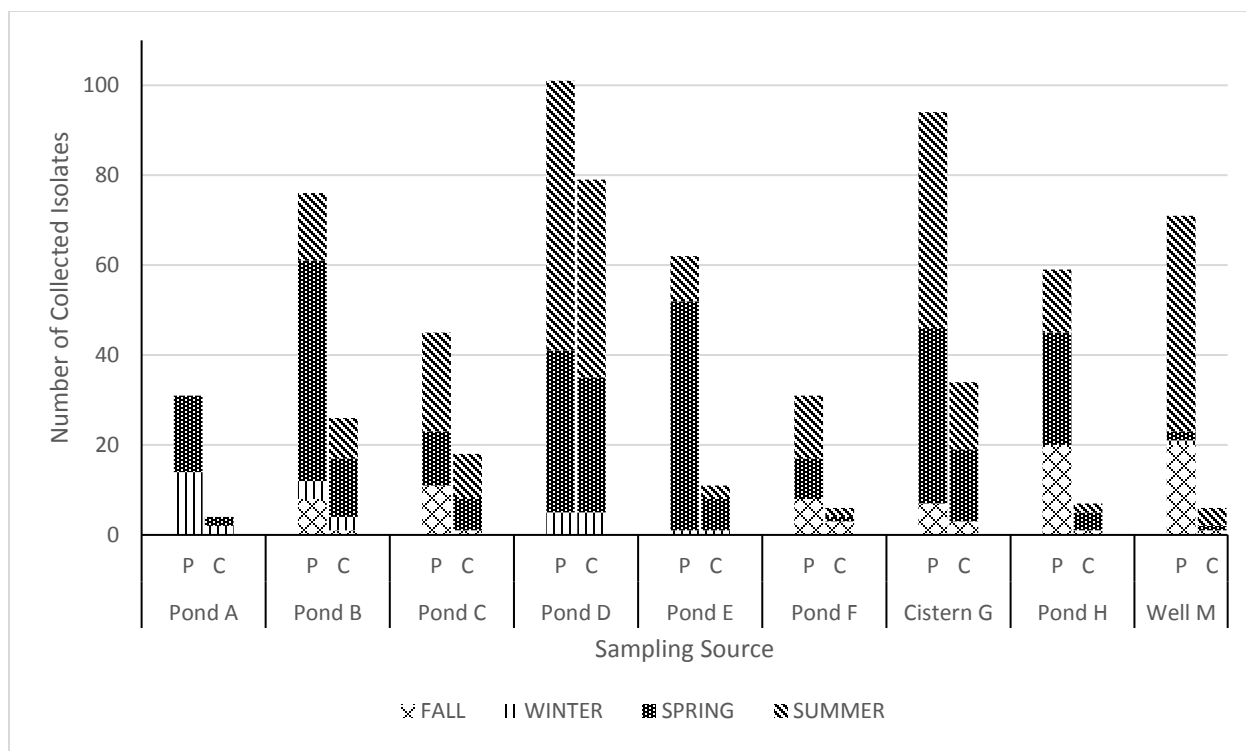


Figure 3.2 Total number of presumptive (P) and confirmed (C) *E. coli* isolates collected quarterly (fall, winter, spring, summer) over a one-year period (2020-2021) shown per water source.

Table 3.2 Most frequent antimicrobial (AMR) and virulent genes found from *E. coli* isolates and potential source identification of isolates based on a literature review of where *E. coli* serotypes can be found.

Biosample ID	Location	Water Source	Serotype	AMR							VIRULENCE							SOURCE TRACKING							
				<i>arcF</i>	<i>blaEC</i>	<i>mdtM</i>	<i>aph(3'')-Ib</i>	<i>aph(6)-Id</i>	<i>tet(A)</i>	<i>tet(B)</i>	<i>gad</i>	<i>lplA</i>	<i>terC</i>	<i>ompT</i>	<i>iss</i>	<i>traT</i>	<i>stx2</i>	Human	Bovine	Ovine	Caprine	Porcine	Poultry	Domestic Pet	Deer
SAMN28571028	MO	Pond D	O74:H8	+	+	+					+		+	+	+	+			+						
SAMN23828784	MO	Pond B	O8:H8	+	+	+					+		+	+	+				+	+					
SAMN23828785	MO	Pond B	O8:H8	+	+	+							+	+	+				+	+					
SAMN23828788	MO	Pond B	O8:H8	+	+	+							+		+				+	+					
SAMN23828789	MO	Pond B	O8:H8	+	+	+							+		+				+	+					
SAMN23828790	MO	Pond B	O8:H8	+	+	+							+	+	+				+	+					
SAMN23828791	MO	Pond B	O8:H8	+	+	+							+	+	+				+	+					
SAMN23828811	MO	Pond D	O74:H8	+	+	+					+		+	+	+	+			+						
SAMN23828820	MO	Pond D	O74:H8	+	+	+					+		+	+	+	+			+						
SAMN23894232	MO	Pond B	O8:H8	+	+	+							+	+	+					+					
SAMN23894240	MO	Pond D	O74:H8	+	+	+							+	+	+	+			+						
SAMN23894243	MO	Pond D	O74:H8	+	+	+							+	+	+	+			+						
SAMN23894250	MO	Pond E	O8:H8	+	+	+							+	+	+					+					
SAMN28544968	MO	Pond C	:H16	+	+	+												+	+			+	+		
SAMN28816569	MO	Pond B	O175:H16		+	+						+								+					
SAMN28816586	MO	Pond B	O175:H16		+	+						+								+					
SAMN28061266	KS	Cistern G	:H16	+	+	+							+					+	+			+	+		
SAMN28816574	MO	Pond B	O175:H16		+	+						+								+					
SAMN28061261	KS	Cistern G	:H16	+	+	+							+					+	+			+	+		
SAMN23828823	MO	Pond D	O175:H16		+	+						+													
SAMN23894246	MO	Pond D	O140:H36	+	+	+					+	+													
SAMN23894234	MO	Pond C	:H7	+	+	+					+	+	+	+	+			+	+						
SAMN28571031	MO	Pond D	O8:H7	+	+	+					+	+	+					+							
SAMN23894238	MO	Pond D	O8:H7	+	+	+					+	+	+					+							
SAMN23828800	MO	Pond D	O22:H8	+	+	+					+	+		+			+		+					+	
SAMN23828816	MO	Pond D	O22:H8	+	+	+					+	+		+			+		+		+			+	
SAMN23828817	MO	Pond D	O22:H8	+	+	+					+	+		+			+		+		+			+	
SAMN23894239	MO	Pond D	O22:H8	+	+	+					+	+		+			+		+		+			+	
SAMN28816588	MO	Pond E	O88:H7	+	+	+				+	+	+	+	+		+			+						

[illegible]

SAMN28571030	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN28571026	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN28816613	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN28571033	MO	Pond D	:H38	+	+	+					+	+	+				+		+
SAMN28571036	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN28571039	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN28571035	MO	Pond D	O65:H38	+	+	+				+	+	+	+				+		
SAMN23894235	MO	Pond D	:H21	+	+	+											+	+	
SAMN23894252	KS	Pond F	O8:H7	+	+	+				+	+	+		+			+		
SAMN28816600	KS	Pond H	O8:H19	+	+	+	+	+	+	+		+	+				+	+	+
SAMN28816578	MO	Pond E	:H21	+	+	+				+	+	+					+	+	
SAMN28545097	MO	Pond D	O1:H7	+	+	+				+	+				+		+	+	
SAMN28545096	MO	Pond D	O1:H7	+	+	+			+	+	+			+	+		+	+	
SAMN28816558	MO	Pond D	O1:H7	+	+	+			+	+	+			+	+		+	+	
SAMN28544966	MO	Pond D	O1:H7	+	+	+			+	+	+			+	+		+	+	
SAMN28545098	MO	Pond D	O1:H7	+	+	+			+	+	+			+	+		+	+	
SAMN28571027	MO	Pond D	O1:H7	+	+	+				+	+			+	+		+	+	
SAMN28571037	MO	Pond D	O1:H7	+	+	+				+	+			+			+	+	
SAMN28571029	MO	Pond D	O1:H7	+	+	+			+	+	+			+	+		+	+	
SAMN23828792	MO	Pond C	O45:H8	+	+	+				+	+			+	+		+		
SAMN23828809	MO	Pond D	O3:H8	+	+	+				+	+		+		+				+
SAMN23894228	MO	Pond B	O149:H45		+	+				+	+		+		+		+		
SAMN23894236	MO	Pond D	:H9	+	+	+				+	+			+			+	+	
SAMN28061271	KS	Cistern G	:H7	+	+	+				+	+	+	+				+	+	
SAMN28816576	MO	Well M	:H21	+	+	+				+	+			+			+	+	
SAMN23894221	KS	Cistern G	:H21	+	+	+					+		+	+			+	+	
SAMN23894222	KS	Cistern G	:H21	+	+	+					+		+	+			+	+	
SAMN23894225	MO	Pond A	:H21	+	+	+				+	+		+	+			+	+	
SAMN23828793	MO	Pond C	:H21	+	+	+				+	+		+	+			+	+	
SAMN28816605	KS	Pond H	:H2/H47	+	+	+				+	+						+	+	
SAMN23894229	MO	Pond B	:H7	+	+	+			+	+	+	+	+	+	+		+	+	
SAMN28816615	MO	Pond E	:H10	+	+	+					+			+			+	+	+
SAMN28816557	MO	Pond E	:H10	+	+	+				+	+			+			+	+	+
SAMN23828822	MO	Pond D	:H11/H54	+	+	+			+	+							+	+	
SAMN23828787	MO	Pond B	O120:H10	+	+	+				+	+		+				+	+	
SAMN23828810	MO	Pond D	O4:H10	+	+	+				+	+						+		
SAMN28544842	KS	Cistern G	O23:H16	+	+	+	+	+	+	+									+

SAMN28061098	KS	Cistern G	:H16	+	+	+	+	+	+	+											+	+																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
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SAMN28816591	MO	Pond A	O96:H20		+	+				+	+	+							+
SAMN28544965	MO	Pond D	O150:H45	+	+	+				+									
SAMN28816604	MO	Pond E	O26:H11	+	+	+			+	+	+	+	+					+	
SAMN28571032	MO	Pond D	O1:H19	+	+	+					+	+						+	
SAMN23894261	KS	Cistern G	:H31	+	+	+			+	+	+							+	+
SAMN23828786	MO	Pond B	:H19			+	+			+	+	+	+	+					+
SAMN23894233	MO	Pond C	O9:H14	+	+	+				+	+							+	
SAMN28816596	MO	Pond B	:H42	+	+	+						+						+	+
SAMN28816611	MO	Pond B	:H42	+	+	+				+		+						+	+
SAMN28816548	MO	Pond B	:H42	+	+	+				+		+						+	+
SAMN24300381	KS	Pond F	O149:H2	+			+			+	+			+	+			+	+
SAMN28061102	MO	Pond E	O84:H2	+	+	+				+	+			+				+	
SAMN23894263	KS	Cistern G	O174:H4	+	+	+					+								+
SAMN28544970	KS	Cistern G	:H29			+	+			+		+						+	
SAMN23894254	KS	Cistern G	O176:H4	+	+	+		+	+			+						+	
SAMN28816584	MO	Pond B	:H32	+	+	+				+		+						+	
SAMN28816553	MO	Pond E	O106:H31			+	+			+		+						+	
SAMN28061108	KS	Cistern G	O21:H12	+	+	+		+	+			+						+	
SAMN28816577	KS	Cistern G	:H12	+	+	+				+		+						+	+
SAMN23828797	MO	Pond C	O180:H10	+	+	+				+		+						+	
SAMN23894253	KS	Pond F	O81:H27	+	+	+				+		+						+	
SAMN23894258	KS	Cistern G	OX13:H10	+	+	+		+	+					+				+	
SAMN24300389	KS	Cistern G	O89:H4	+	+	+				+		+						+	
SAMN23828795	MO	Pond C	O107:H27	+	+	+				+		+						+	
SAMN23894264	KS	Cistern G	:H51	+	+					+								+	
SAMN23894245	MO	Pond D	:H20	+	+	+		+	+	+								+	
SAMN24300390	MO	Pond C	O9:H30			+	+												+
SAMN28061105	MO	Pond D	:H46	+	+	+		+	+	+			+					+	
SAMN28545095	MO	Pond D	:H34	+	+	+						+						+	+
SAMN23894241	MO	Pond D	O75:H31	+			+												+
SAMN24300376	MO	Pond D	O133:H29	+	+								+					+	
SAMN23828825	MO	Pond D	O138:H28	+	+														
SAMN28816598	MO	Pond D	O17:H45	+	+	+												+	
SAMN23828801	MO	Pond D	O73:H45	+	+	+												+	
SAMN23894242	MO	Pond D	O73:H45	+	+	+												+	
SAMN23894215	MO	Pond B	O146:H28	+	+							+	+	+					+
SAMN23894226	MO	Pond A	O81:H1	+	+							+	+						

SAMN28816580	MO	Pond E	O113:H5	+	+					+							+		
SAMN23828802	MO	Pond D	O17:H18	+	+	+				+	+						+		
SAMN23894218	KS	Pond F	O15:H18	+	+					+	+						+		
SAMN24300385	KS	Cistern G	:H45	+	+	+		+			+	+	+				+		
SAMN28816568	MO	Well M	O8:H10	+	+						+						+		+
SAMN28816556	MO	Well M	O148:H8		+	+											+		
SAMN28544969	MO	Pond C	:H2	+	+	+				+	+	+					+	+	
SAMN28545101	MO	Pond C	:H2	+	+	+				+	+	+					+	+	
SAMN28544967	MO	Pond C	:H2	+	+	+				+	+	+			+		+	+	
SAMN28544971	MO	Pond C	:H2	+	+	+				+	+	+					+	+	
SAMN24300378	KS	Pond H	:H2	+	+	+				+	+	+					+	+	
SAMN23828812	MO	Pond D	O39:H7	+	+	+				+	+		+				+	+	
SAMN28544840	MO	Pond C	:H8	+	+	+						+					+	+	
SAMN28545099	MO	Pond C	:H8	+	+	+						+					+	+	

References

- Acosta-Dibarrat, J., Enriquez-Gómez, E., Talavera-Rojas, M., Soriano-Vargas, E., Navarro, A., & Morales-Espinosa, R. (2021). Characterization of commensal *Escherichia coli* isolates from slaughtered sheep in Mexico. *The Journal of Infection in Developing Countries*, 15(11), 1755–1760.
<https://doi.org/10.3855/jidc.14001>
- Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., & Blankenberg, D. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research*, 46(W1), W537–W544.
<https://doi.org/10.1093/nar/gky379>
- Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen AV, Cheng AA, Liu S, Min SY, Miroshnichenko A, Tran HK, Werfalli RE, Nasir JA, Oloni M, Speicher DJ, Florescu A, Singh B, Faltyn M, Hernandez-Koutoucheva A, Sharma AN, Bordeleau E, Pawlowski AC, Zubyk HL, Dooley D, Griffiths E, Maguire F, Winsor GL, Beiko RG, Brinkman FSL, Hsiao WWL, Domselaar GV, McArthur AG. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 8;48(D1):D517-D525. doi: 10.1093/nar/gkz935
- Andrade, L., O'Dwyer, J., O'Neill, E., & Hynds, P. (2018). Surface water flooding, groundwater contamination, and enteric disease in developed countries: A scoping

- review of connections and consequences. *Environmental Pollution*, 236, 540–549.
<https://doi.org/10.1016/j.envpol.2018.01.104>
- Awosile, B., Reyes-Velez, J., Cuesta-Astroz, Y., Rodríguez-Lecompte, J. C., Saab, M. E., Heider, L. C., Keefe, G., Sánchez, J., & McClure, J. T. (2020). Short communication: Whole-genome sequence analysis of 4 fecal blaCMY-2-producing *Escherichia coli* isolates from Holstein dairy calves. *Journal of Dairy Science*, 103(1), 877–883. <https://doi.org/10.3168/jds.2019-16560>
- Barlow, R. S., Gobius, K. S., & Desmarchelier, P. M. (2006). Shiga toxin-producing *Escherichia coli* in ground beef and lamb cuts: Results of a one-year study. *International Journal of Food Microbiology*, 111(1), 1–5.
<https://doi.org/10.1016/j.ijfoodmicro.2006.04.039>
- Bentancor, A., Rumi, M. V., Carbonari, C., Gerhardt, E., Larzábal, M., Vilte, D. A., Pistone-Creydt, V., Chinen, I., Ibarra, C., Cataldi, A., & Mercado, E. C. (2012). Profile of Shiga toxin-producing *Escherichia coli* strains isolated from dogs and cats and genetic relationships with isolates from cattle, meat and humans. *Veterinary Microbiology*, 156(3–4), 336–342.
<https://doi.org/10.1016/j.vetmic.2011.10.030>
- Berenger, B. M., Chui, L., Ferrato, C., Lloyd, T., Li, V., & Pillai, D. R. (2022). Performance of four commercial real-time PCR assays for the detection of bacterial enteric pathogens in clinical samples. *International Journal of Infectious Diseases*, 114, 195–201. <https://doi.org/10.1016/j.ijid.2021.10.035>
- Bergholz, T. M., Tarr, C. L., Christensen, L. M., Betting, D. J., & Whittam, T. S. (2007). Recent Gene Conversions between Duplicated Glutamate Decarboxylase Genes

- (gadA and gadB) in Pathogenic *Escherichia coli*. *Molecular Biology and Evolution*, 24(10), 2323–2333. <https://doi.org/10.1093/molbev/msm163>
- Bertoldi, B., Richardson, S., Schneider, R. G., Kurdmongkoltham, P., Schneider, K. R. (2017). Preventing Foodborne Illness: E. coli The Big Six. <https://www.nifa.usda.gov/sites/default/files/resource/Preventing-Foodborne-Illness-E-coli-the-big-six.pdf>
- Bettelheim, K. A. (1978). The sources of ‘OH’ serotypes of *Escherichia coli*. *Journal of Hygiene*, 80(1), 83–113. <https://doi.org/10.1017/S0022172400053420>
- Bettelheim, K. A., Kuzevski, A., Gilbert, R. A., Krause, D. O., & McSweeney, C. S. (2005). The diversity of *Escherichia coli* serotypes and biotypes in cattle faeces. *Journal of Applied Microbiology*, 98(3), 699–709. <https://doi.org/10.1111/j.1365-2672.2004.02501.x>
- Bosilevac, J. M., & Koohmaraie, M. (2011). Prevalence and Characterization of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolates from Commercial Ground Beef in the United States. *Applied and Environmental Microbiology*, 77(6), 2103–2112. <https://doi.org/10.1128/AEM.02833-10>
- Brown, E., Dessai, U., McGarry, S., & Gerner-Smidt, P. (2019). Use of Whole-Genome Sequencing for Food Safety and Public Health in the United States. *Foodborne Pathogens and Disease*, 16(7), 441–450. <https://doi.org/10.1089/fpd.2019.2662>
- Byappanahalli, M., Fowler, M., Shively, D., & Whitman, R. (2003). Ubiquity and Persistence of *Escherichia coli* in a Midwestern Coastal Stream. *Applied and Environmental Microbiology*, 69(8), 4549–4555. <https://doi.org/10.1128/AEM.69.8.4549-4555.2003>

Center for Disease Control and Prevention (CDC). (2019). *E. coli* infection.

<https://www.cdc.gov/healthypets/diseases/ecoli.html>

Center for Disease Control and Prevention (CDC). (2019). Outbreak of *E. coli* linked to

romaine lettuce. <https://www.cdc.gov/ecoli/2018/o157h7-11-18/index.html>

Center for Disease Control and Prevention (CDC). (2022). Cistern and other rain catchment systems.

<https://www.cdc.gov/healthywater/emergency/drinking/disinfection-cisterns.html#:~:text=Floods%20and%20heavy%20rains%20can,your%20drinking%20water%20is%20contaminated.>

Díaz-Jiménez, D., García-Meniño, I., Fernández, J., García, V., & Mora, A. (2020).

Chicken and turkey meat: Consumer exposure to multidrug-resistant Enterobacteriaceae including mcr-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131. *International Journal of Food Microbiology*, 331, 108750. <https://doi.org/10.1016/j.ijfoodmicro.2020.108750>

Domesle, K. J., Young, S. R., & Ge, B. (2021). Rapid Screening for *Salmonella* in Raw Pet Food by Loop-Mediated Isothermal Amplification. *Journal of Food Protection*, 84(3), 399–407. <https://doi.org/10.4315/JFP-20-365>

Enriquez-Gómez, E., Talavera-Rojas, M., Soriano-Vargas, E., Navarro-Ocaña, A., Vega-Sánchez, V., Oca, S. A.-M. de, & Acosta-Dibarrat, J. (2017). Serotypes, virulence genes profiles and antimicrobial resistance patterns of *Escherichia coli* recovered from feces of healthy lambs in Mexico. *Small Ruminant Research*, 153, 41–47. <https://doi.org/10.1016/j.smallrumres.2017.05.003>

- Fischer, J. R., Zhao, T., Doyle, M. P., Goldberg, M. R., Brown, C. A., Sewell, C. T., Kavanaugh, D. M., & Bauman, C. D. (2001). Experimental and Field Studies of *Escherichia coli* O157:H7 in White-Tailed Deer. *Applied and Environmental Microbiology*, 67(3), 1218–1224. <https://doi.org/10.1128/AEM.67.3.1218-1224.2001>
- Flament-Simon, S.-C., de Toro, M., García, V., Blanco, J. E., Blanco, M., Alonso, M. P., Goicoa, A., Díaz-González, J., Nicolas-Chanoine, M.-H., & Blanco, J. (2020). Molecular Characteristics of Extraintestinal Pathogenic *E. coli* (ExPEC), Uropathogenic *E. coli* (UPEC), and Multidrug Resistant *E. coli* Isolated from Healthy Dogs in Spain. Whole Genome Sequencing of Canine ST372 Isolates and Comparison with Human Isolates Causing Extraintestinal Infections. *Microorganisms*, 8(11), 1712. <https://doi.org/10.3390/microorganisms8111712>
- Franz, E., Klerks, M. M., De Vos, O. J., Termorshuizen, A. J., & van Bruggen, A. H. C. (2007). Prevalence of Shiga Toxin-Producing *Escherichia coli* *stx*₁, *stx*₂, *eaeA*, and *rfbE* Genes and Survival of *E. coli* O157:H7 in Manure from Organic and Low-Input Conventional Dairy Farms. *Applied and Environmental Microbiology*, 73(7), 2180–2190. <https://doi.org/10.1128/AEM.01950-06>
- Fukuyama, M., Yokoyama, R., Sakata, S., Furuhashi, K., Oonaka, K., Hara, M., Satoh, Y., Tabuchi, K., Itoh, T., Kai, A., & Matsuda, M. (1999). Study on the Verotoxin-producing *Escherichia coli*. *Journal of the Japanese Association for Infectious Diseases*, 73(11), 1140–1144. <https://doi.org/10.11150/kansenshogakuzasshi1970.73.1140>

- Gannon, V. P., D'Souza, S., Graham, T., King, R. K., Rahn, K., & Read, S. (1997). Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *Journal of Clinical Microbiology*, 35(3), 656–662. <https://doi.org/10.1128/jcm.35.3.656-662.1997>
- Grant, M. A., Weagant, S. D., & Feng, P. (2001). Glutamate Decarboxylase Genes as a Prescreening Marker for Detection of Pathogenic *Escherichia coli* Groups. *Applied and Environmental Microbiology*, 67(7), 3110–3114. <https://doi.org/10.1128/AEM.67.7.3110-3114.2001>
- Haley, O. C., Nwadike, L., Zhao, Y., Maher, J. M., Gragg, S. E., Trinetta, V., & Bhullar, M. (2022). Comparative Assessment of the Microbial Quality of Agricultural Water on Kansas and Missouri Fresh Produce Farms. *Food Protection Trends*, 42(3), 186–193. <https://doi.org/10.4315/FPT-21-033>
- Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T. A., Bekal, S., Fairbrother, J. M., Harel, J., Maynard, C., Masson, L., & Brousseau, R. (2006). A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Greater Lakes recreational waters. *Applied and Environmental Microbiology*, 72(6), 4200–4206. <https://doi.org/10.1128/AEM.00137-06>
- Hu, Y., Zhang, Q. ., & Meitzler, J. C. (1999). Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. *Journal of Applied Microbiology*, 87(6), 867–876. <https://doi.org/10.1046/j.1365-2672.1999.00938.x>

- Hunter, P. R. (2003). Drinking water and diarrhoeal disease due to *Escherichia coli*. *Journal of Water and Health*, 1(2), 65–72. <https://doi.org/10.2166/wh.2003.0008>
- Hussein, H. S. (2007). Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products^{1,2}. *Journal of Animal Science*, 85(suppl_13), E63–E72. <https://doi.org/10.2527/jas.2006-421>
- Johnson, J. R., Delavari, P., Stell, A. L., Whittam, T. S., Carlino, U., & Russo, T. A. (2001). Molecular Comparison of Extraintestinal *Escherichia coli* Isolates of the Same Electrophoretic Lineages from Humans and Domestic Animals. *The Journal of Infectious Diseases*, 183(1), 154–159. <https://doi.org/10.1086/317662>
- Johnson, R. P., Clarke, R. C., Wilson, J. B., Read, S. C., Rahn, K., Renwick, S. A., Sandhu, K. A., Alves, D., Karmali, M. A., Lior, H., McEwen, S. A., Spika, J. S., & Gyles, C. L. (1996). Growing Concerns and Recent Outbreaks Involving Non-O157:H7 Serotypes of Verotoxigenic *Escherichia coli*. *Journal of Food Protection*, 59(10), 1112–1122. <https://doi.org/10.4315/0362-028X-59.10.1112>
- Karama, M., Mainga, A. O., Cenci-Goga, B. T., Malahlela, M., El-Ashram, S., & Kalake, A. (2019). Molecular profiling and antimicrobial resistance of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O121, O145 and O157 isolates from cattle on cow-calf operations in South Africa. *Scientific Reports*, 9(1), 11930. <https://doi.org/10.1038/s41598-019-47948-1>
- Kostyla, C., Bain, R., Cronk, R., & Bartram, J. (2015). Seasonal variation of fecal contamination in drinking water sources in developing countries: A systematic review. *Science of The Total Environment*, 514, 333–343. <https://doi.org/10.1016/j.scitotenv.2015.01.018>

- Krause, G., Zimmermann, S., & Beutin, L. (2005). Investigation of domestic animals and pets as a reservoir for intimin- (eae) gene positive *Escherichia coli* types. *Veterinary Microbiology*, 106(1–2), 87–95.
<https://doi.org/10.1016/j.vetmic.2004.11.012>
- Kudva, I. T., Hatfield, P. G., & Hovde, C. J. (1997). Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep. *Journal of Clinical Microbiology*, 35(4), 892–899.
<https://doi.org/10.1128/jcm.35.4.892-899.1997>
- Kuhnert, P., Boerlin, P., & Frey, J. (2000). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *FEMS Microbiology Reviews*, 24(1), 107–117. <https://doi.org/10.1111/j.1574-6976.2000.tb00535.x>
- Leotta, G. A., Deza, N., Origlia, J., Toma, C., Chinen, I., Miliwebsky, E., Iyoda, S., Sosa-Estani, S., & Rivas, M. (2006). Detection and characterization of Shiga toxin-producing *Escherichia coli* in captive non-domestic mammals. *Veterinary Microbiology*, 118(1–2), 151–157. <https://doi.org/10.1016/j.vetmic.2006.07.006>
- Lévesque, B., Pereg, D., Watkinson, E., Maguire, J. S., Bissonnette, L., Gingras, S., Rouja, P., Bergeron, M. G., & Dewailly, É. (2008). Assessment of microbiological quality of drinking water from household tanks in Bermuda. *Canadian Journal of Microbiology*, 54(6), 495–500. <https://doi.org/10.1139/W08-038>
- Liao, C.-Y., Balasubramanian, B., Peng, J.-J., Tao, S.-R., Liu, W.-C., & Ma, Y. (2021). Antimicrobial Resistance of *Escherichia coli* From Aquaculture Farms and Their

- Environment in Zhanjiang, China. *Frontiers in Veterinary Science*, 8, 806653.
<https://doi.org/10.3389/fvets.2021.806653>
- Lupo, A., Coyne, S., & Berendonk, T. U. (2012). Origin and Evolution of Antibiotic Resistance: The Common Mechanisms of Emergence and Spread in Water Bodies. *Frontiers in Microbiology*, 3. <https://doi.org/10.3389/fmicb.2012.00018>
- Malahlela, M. N., Cenci-Goga, B. T., Marufu, M. C., Fonkui, T. Y., Grispoldi, L., Etter, E., Kalake, A., & Karama, M. (2022). Occurrence, Serotypes and Virulence Characteristics of Shiga-Toxin-Producing *Escherichia coli* Isolates from Goats on Communal Rangeland in South Africa. *Toxins*, 14(5), 353.
<https://doi.org/10.3390/toxins14050353>
- Maloo, A., Fulke, A. B., Mulani, N., Sukumaran, S., & Ram, A. (2017). Pathogenic multiple antimicrobial resistant *Escherichia coli* serotypes in recreational waters of Mumbai, India: A potential public health risk. *Environmental Science and Pollution Research*, 24(12), 11504–11517. <https://doi.org/10.1007/s11356-017-8760-8>
- Manges, A. R. (2016). *Escherichia coli* and urinary tract infections: The role of poultry-meat. *Clinical Microbiology and Infection*, 22(2), 122–129.
<https://doi.org/10.1016/j.cmi.2015.11.010>
- Maria Ferreira Cavalcanti, A., Tavanelli Hernandez, R., Harummyy Takagi, E., Ernestina Cabílio Guth, B., de Lima Ori, É., Regina Schicariol Pinheiro, S., Sueli de Andrade, T., Louzada Oliveira, S., Cecilia Cergole-Novella, M., Rodrigues Francisco, G., & dos Santos, L. F. (2020). Virulence Profiling and Molecular

- Typing of Shiga Toxin-Producing *E. coli* (STEC) from Human Sources in Brazil. *Microorganisms*, 8(2), 171. <https://doi.org/10.3390/microorganisms8020171>
- Massé, D., Saady, N., & Gilbert, Y. (2014). Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. *Animals*, 4(2), 146–163. <https://doi.org/10.3390/ani4020146>
- Melton-Celsa, A. R. (2014). Shiga Toxin (Stx) Classification, Structure, and Function. *Microbiology Spectrum*, 2(4), 2.4.06. <https://doi.org/10.1128/microbiolspec.EHEC-0024-2013>
- Nielsen, E. M., Skov, M. N., Madsen, J. J., Lodal, J., Jespersen, J. B., & Baggesen, D. L. (2004). Verocytotoxin-Producing *Escherichia coli* in Wild Birds and Rodents in Close Proximity to Farms. *Applied and Environmental Microbiology*, 70(11), 6944–6947. <https://doi.org/10.1128/AEM.70.11.6944-6947.2004>
- Oliver, D. M., & Page, T. (2016). Effects of seasonal meteorological variables on *E. coli* persistence in livestock faeces and implications for environmental and human health. *Scientific Reports*, 6(1), 37101. <https://doi.org/10.1038/srep37101>
- Osman, K. M., Mustafa, A. M., Elhariri, M., & Abdelhamed, G. S. (2013). The Distribution of *Escherichia coli* Serovars, Virulence Genes, Gene Association and Combinations and Virulence Genes Encoding Serotypes in Pathogenic *E. coli* Recovered from Diarrhoeic Calves, Sheep and Goat: The Distribution of *E. coli* Serovars. *Transboundary and Emerging Diseases*, 60(1), 69–78. <https://doi.org/10.1111/j.1865-1682.2012.01319.x>
- Parma, A. E. (2000). [No title found]. *European Journal of Epidemiology*, 16(8), 757–762. <https://doi.org/10.1023/A:1026746016896>

- Ramteke, P. W., & Tewari, S. (2007). Serogroups of *Escherichia coli* from Drinking Water. *Environmental Monitoring and Assessment*, 130(1–3), 215–220.
<https://doi.org/10.1007/s10661-006-9390-7>
- Rehman, M. A., Rempel, H., Carrillo, C. D., Ziebell, K., Allen, K., Manges, A. R., Topp, E., & Diarra, M. S. (2022). Virulence Genotype and Phenotype of Multiple Antimicrobial-Resistant *Escherichia coli* Isolates from Broilers Assessed from a “One-Health” Perspective. *Journal of Food Protection*, 85(2), 336–354.
<https://doi.org/10.4315/JFP-21-273>
- Rey, J. (2003). Serotypes, phage types and virulence genes of Shiga-producing *Escherichia coli* isolated from sheep in Spain. *Veterinary Microbiology*, 94(1), 47–56. [https://doi.org/10.1016/S0378-1135\(03\)00064-6](https://doi.org/10.1016/S0378-1135(03)00064-6)
- Reyes-Rodriguez, N. E., Barba-León, J., Navarro-Ocaña, A., Vega-Sanchez, V., Gómez De Anda, F. R., Talavera-Gonzalez, J. M., & Talavera-Rojas, M. (2020). Serotipos y aislamientos de *Escherichia coli* productora del subtipo Stx2 de la toxina Shiga provenientes de canales y heces de ganado bovino. *Revista Mexicana de Ciencias Pecuarias*, 11(4), 1030–1044.
<https://doi.org/10.22319/rmcp.v11i4.5049>
- Rodrigues, C., da Silva, A. L. B. R., & Dunn, L. L. (2019). Factors Impacting the Prevalence of Foodborne Pathogens in Agricultural Water Sources in the Southeastern United States. *Water*, 12(1), 51. <https://doi.org/10.3390/w12010051>
- Sasakova, N., Gregova, G., Takacova, D., Mojzisova, J., Papajova, I., Venglovsky, J., Szaboova, T., & Kovacova, S. (2018). Pollution of Surface and Ground Water by Sources Related to

- Agricultural Activities. *Frontiers in Sustainable Food Systems*, 2, 42.
<https://doi.org/10.3389/fsufs.2018.00042>
- Segura, A., Auffret, P., Klopp, C., Bertin, Y., & Forano, E. (2017). Draft genome sequence and characterization of commensal *Escherichia coli* strain BG1 isolated from bovine gastro-intestinal tract. *Standards in Genomic Sciences*, 12(1), 61.
<https://doi.org/10.1186/s40793-017-0272-0>
- Stein, R. A., & Katz, D. E. (2017). *Escherichia coli*, cattle and the propagation of disease. *FEMS Microbiology Letters*, 364(6). <https://doi.org/10.1093/femsle/fnx050>
- Sun, H., Wan, Y., Du, P., Liu, D., Li, R., Zhang, P., Wu, Y., Fanning, S., Wang, Y., & Bai, L. (2021). Investigation of tigecycline resistant *Escherichia coli* from raw meat reveals potential transmission among food-producing animals. *Food Control*, 121, 107633. <https://doi.org/10.1016/j.foodcont.2020.107633>
- Tanabe, R. H. S., Vieira, M. A., Mariano, N. A. B., Dias, R. C. B., da Silva, R. V., Castro, C. M., dos Santos, L. F., Camargo, C. H., Yamatogi, R. S., Rall, V. L. M., & Hernandez, R. T. (2019). Identification and characterization of atypical enteropathogenic and Shiga toxin-producing *Escherichia coli* isolated from ground beef and poultry breast purchased in Botucatu, Brazil. *Brazilian Journal of Microbiology*, 50(4), 1099–1103. <https://doi.org/10.1007/s42770-019-00101-6>
- Turkovicova, L., Smidak, R., Jung, G., Turna, J., Lubec, G., & Aradska, J. (2016). Proteomic analysis of the TerC interactome: Novel links to tellurite resistance and pathogenicity. *Journal of Proteomics*, 136, 167–173.
<https://doi.org/10.1016/j.jprot.2016.01.003>

- U. S. Environmental Protection Agency (EPA). (2014). Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC).
https://www.epa.gov/sites/default/files/2015-08/documents/method_1603_2009.pdf
- U.S. Food and Drug Administration (FDA). (2019). *Escherichia coli* (*E. coli*).
<https://www.fda.gov/food/foodborne-pathogens/escherichia-coli-e-coli>
- U.S. Food and Drug Administration (FDA). (2022). FSMA proposed rule of agricultural water. <https://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-proposed-rule-agricultural-water>
- U.S. Food and Drug Administration (FDA). (2022). Outbreak investigation of *E. coli* O157:H7- spinach (November 2021). <https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-e-coli-o157h7-spinach-november-2021>
- Valat, C., Auvray, F., Forest, K., Métayer, V., Gay, E., Peytavin de Garam, C., Madec, J.-Y., & Haenni, M. (2012). Phylogenetic Grouping and Virulence Potential of Extended-Spectrum- β -Lactamase-Producing *Escherichia coli* Strains in Cattle. *Applied and Environmental Microbiology*, 78(13), 4677–4682.
<https://doi.org/10.1128/AEM.00351-12>
- van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *The ISME Journal*, 5(2), 173–183. <https://doi.org/10.1038/ismej.2010.80>
- Vettorato, M. P., de Castro, A. F. P., Cergole-Novella, M. C., Camargo, F. L. L., Irino, K., & Guth, B. E. C. (2009). Shiga toxin-producing *Escherichia coli* and atypical

- enteropathogenic *Escherichia coli* strains isolated from healthy sheep of different populations in São Paulo, Brazil. *Letters in Applied Microbiology*, 49(1), 53–59.
<https://doi.org/10.1111/j.1472-765X.2009.02617.x>
- Vingino, A., Roberts, M. C., Wainstein, M., West, J., Norman, S. A., Lambourn, D., Lahti, J., Ruiz, R., D'Angeli, M., Weissman, S. J., & Rabinowitz, P. (2021). Surveillance for antibiotic-resistant *E. coli* in salish sea ecosystem. 10(10), 1201.
<https://doi.org/10.3390/antibiotics10101201>
- Vivar, M., & Fuentes, M. (2016). Using solar disinfected water: On the bacterial regrowth over 1-week of water usage including direct intake after sun exposure and long-term dark storage. *Solar Energy*, 131, 138–148.
<https://doi.org/10.1016/j.solener.2016.02.044>
- Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J. E., Dahbi, G., Mora, A., López, C., González, E. A., & Blanco, J. (2007). Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhoea in Slovakia. *The Veterinary Journal*, 174(1), 176–187.
<https://doi.org/10.1016/j.tvjl.2006.05.019>
- Walker, D. I., McQuillan, J., Taiwo, M., Parks, R., Stenton, C. A., Morgan, H., Mowlem, M. C., & Lees, D. N. (2017). A highly specific *Escherichia coli* qPCR and its comparison with existing methods for environmental waters. *Water Research*, 126, 101–110. <https://doi.org/10.1016/j.watres.2017.08.032>
- Ward, M., Dhingra, R., Remais, J. V., Chang, H. H., Johnston, L. M., Jaykus, L.-A., & Leon, J. (2015). Associations between Weather and Microbial Load on Fresh

Produce Prior to Harvest. *Journal of Food Protection*, 78(4), 849–854.

<https://doi.org/10.4315/0362-028X.JFP-14-381>

Werth, B. J. (2022). Overview of antibiotics. *Merck Manual*.

<https://www.merckmanuals.com/home/infections/antibiotics/overview-of-antibiotics>

World Health Organization (WHO). 2018. E. coli. <https://www.who.int/news-room/fact-sheets/detail/e-coli>

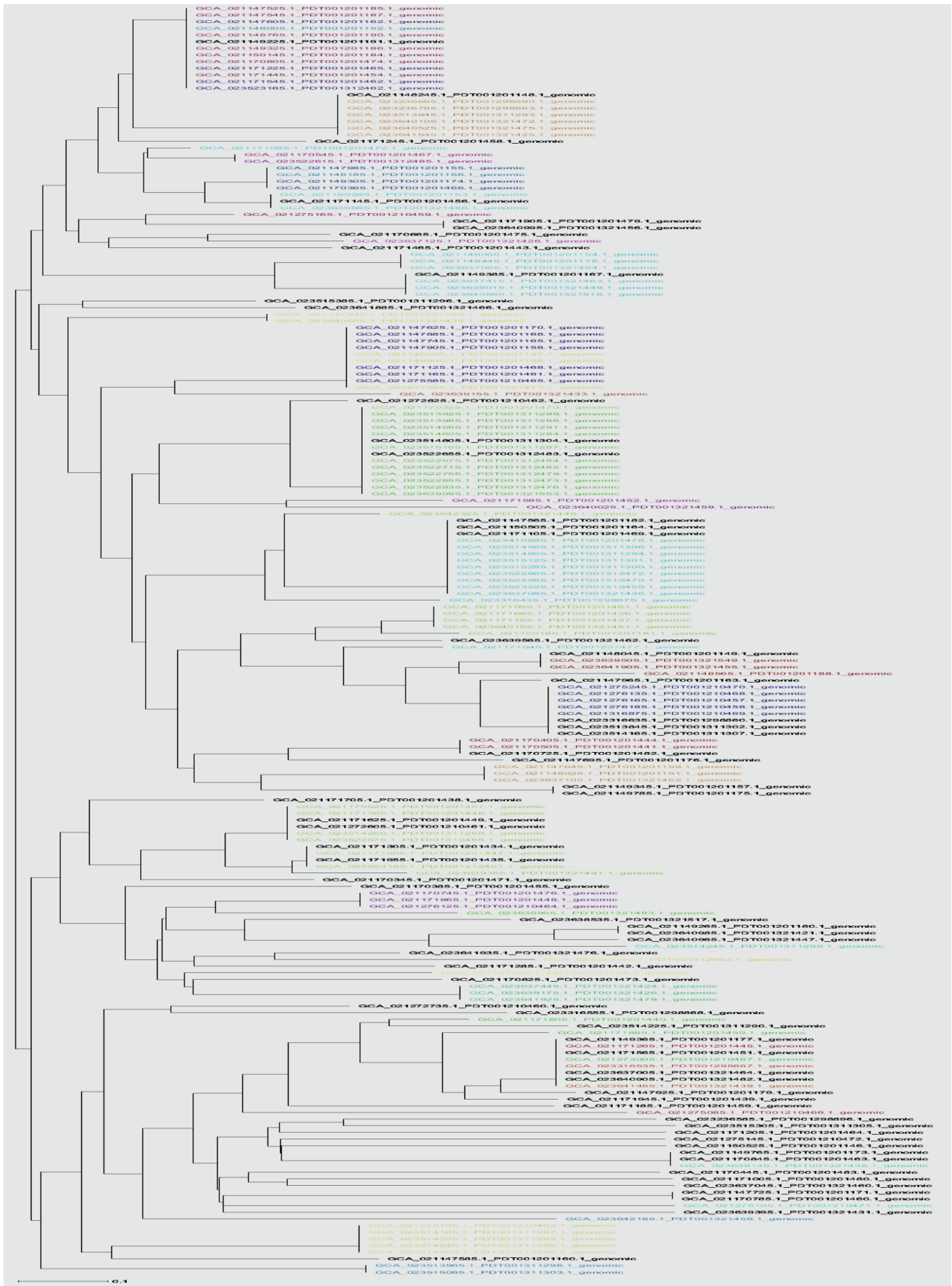
Younis, G., Awad, A., & Mohamed, N. (2017). Phenotypic and genotypic characterization of antimicrobial susceptibility of avian pathogenic *Escherichia coli* isolated from broiler chickens. *Veterinary World*, 10(10), 1167–1172.

<https://doi.org/10.14202/vetworld.2017.1167-1172>

Zhang, S., Yin, Y., Jones, M. B., Zhang, Z., Deatherage Kaiser, B. L., Dinsmore, B. A., Fitzgerald, C., Fields, P. I., & Deng, X. (2015). Salmonella Serotype Determination Utilizing High-Throughput Genome Sequencing Data. *Journal of Clinical Microbiology*, 53(5), 1685–1692. <https://doi.org/10.1128/JCM.00323-15>

Zhi, S., Banting, G. S., Ruecker, N. J., & Neumann, N. F. (2017). Stress resistance in naturalised waste water *E. coli* strains. *Journal of Environmental Engineering and Science*, 12(2), 42–50. <https://doi.org/10.1680/jenes.16.00021>

Appendix A - Supplemental Information



Chapter 4 - Evaluation of *Listeria monocytogenes* Biofilms

Attachment and Formation on Different Surfaces Using a CDC

Biofilm Reactor

Abstract

Listeria monocytogenes can adapt, persist, and form biofilms representing a challenge for food safety. Biofilm attachment and formation can be influenced by the characteristics of the environment, substrate properties and microbial motility. The purpose of this study was to evaluate the ability of *L. monocytogenes* to attach and form biofilms on different surfaces (wood, nylon, and polycarbonate) representative of the materials used during produce harvesting and storing. Multi-strain *L. monocytogenes* biofilms were grown in a CDC Biofilm reactor at $20 \pm 2^\circ\text{C}$ up to 96-h and characterized for the following: attachment strength by remaining cells after rinsing; hydrophobicity and interfacial tension by contact angle measurements using a sessile drop technique by an optical drop tensiometer; structure and bacterial viability by Laser Scanning Confocal Microscopy. All experiments were done in triplicate. Material, incubation, and solvent significantly affected the hydrophobicity and wetting properties of *L. monocytogenes* biofilms ($P < 0.05$). Increased biofilm growth resulted in an increase in hydrophobicity. Biofilms presented a significantly stronger attachment to nylon, as compared to wood and polycarbonate coupons ($P < 0.05$), probably due to material characteristics (e.g. porosity). On the other hand, the highest contact angle and the lowest interfacial tension was observed in polycarbonate coupons. The data presented in this study contributes to understanding the abilities of *Listeria* biofilms to grow on different surfaces relevant to food premises.

4.1 Introduction

Listeria monocytogenes, the pathogen responsible for listeriosis, is the third leading cause of death from foodborne illnesses each year in the United States (CDC, 2022). *L. monocytogenes* is ubiquitously found in natural environments and food, where upon ingestion, it can cause severe disease to immunocompromised people. This bacterium is known in the food industry for its ability to persist and form biofilms in diverse environmental conditions. A biofilm is defined as a community of aggregated microorganisms that adhere together through the production of extracellular polymeric substances (EPS) (da Silva et al., 2013). Biofilm formation can be divided into five steps: (1) attachment to a surface; (2) propagation and formation of EPS; (3) development of more complex structure; (4) maturation; and (5) cell dispersion (da Silva et al., 2013). Hydrophobicity plays a prominent role throughout the developmental stages of a biofilm, since it promotes interactions between cells and surfaces, influencing attachment and retention (Faria et al., 2020). Previous studies suggested that bacterial attachment to surfaces involves van der Waals forces, electrostatic forces, and hydrophobic interactions (Silva et al., 2008; Cerca et al., 2005; Chmielewski et al., 2003). When a planktonic cell approaches a surface, the van der Waals forces attract the microorganism to the substrate wall where a net negative charge can repel the bacterium.

Materials wood, nylon, and polycarbonate are used in the produce industry during the harvesting and handling of fresh fruits and vegetables (Erkan and Dogan, 2019). It has been observed that equipment can be a transmission route for pathogens to contaminate fresh commodities (Stein and Chirila, 2017). In 2014, an outbreak of *L. monocytogenes* was linked to Washington state caramel apples where the equipment was the cause of cross-contamination (Ruiz-Llacsahuanga et al., 2021). Materials such as stainless steel, nylon, polyethylene, vinyl,

and more were found to harbor *L. monocytogenes* cells (Ruiz-Llacsahuanga et al., 2021). Therefore, it is imperative to understand the attachment and formation of *L. monocytogenes* biofilms to different surfaces used in fresh processing environments. Both material and bacterial properties are key factors to biofilm attachment and formation (Silva et al., 2008; Tiexeira et al., 2008; Kushwaha et al., 2009). Recent studies analyzed the physical characteristics of *L. monocytogenes* biofilms in a wide array of contexts including the following: biofilm studies on niche environments and surface topography (Ferreira et al., 2014; Chmielewski et al., 2003); the effects of temperature and contact time on biofilms (Chavant et al., 2002; Lee et al., 2017; Lunden et al. 2000); and strains that are persistent and nonpersistent (Lunden et al., 2000). These studies do not consider the role that surface adherence characteristics play during the growth of multi-strain *L. monocytogenes* biofilms. Therefore, the aim of this research was to study the effect of surface properties such as wettability and hydrophobicity on the attachment and formation of *L. monocytogenes* biofilms on different surfaces.

4.2 Materials and Methods

4.2.1 Bacterial strains and inoculum preparation

L. monocytogenes, L2624 (serotype 1/2b) and L2626 (serotype 1/2a), isolated from the 2001 U.S. cantaloupe outbreak, and J2230 (serotype 4b) from a clinical sample were used in this study (Mendez et al., 2020). Each strain was stored on CyroCare Bacteria Preserver beads (Key Scientific, Stamford, TX) and kept at -80°C until experiment. Bacterial cocktail was obtained as previously described (Mendez et al., 2020) to reach an initial population of $\sim 1 \times 10^8$ CFU/ml.

4.2.2 Biofilm growth

Biofilms were grown in a Center for Disease Control and Prevention Biofilm reactor (BioSurface Technologies, Bozeman, Montana) for up to 96 hours, following a protocol

developed in our laboratory with some modifications (Mendez et al., 2020). Briefly, 1ml of *L. monocytogenes* cocktail was used to inoculate the reactor and the batch phase was maintained for 24-h, followed by 72-h of continuous flow of sterile TSB (Tryptic Soy Broth, BD Difco, Sparks, MD) nutrient at 8 ml/min. Throughout the process, coupons were removed at different time intervals (2, 24, and 96-h) for analyses. Three different materials were used for this study: wood, polycarbonate, and nylon. Polycarbonate coupons of 1.27 cm in diameter were purchased from BioSurface Technologies Corporation (Bozeman, MT). Plywood of unfinished basswood, obtained from a local store, was cut into coupons of 1.27cm in diameter. Nylon fabric, purchased from a local store, was cut into 2cm x 2cm squares to fit within the rods of the reactor. All biofilm growth areas were 1.27cm in diameter with populations forming on both sides of the coupon. The surface materials were selected for their frequent use among small-scale produce growers. Based on a survey previously conducted within our laboratory with local produce growers, wood and polycarbonate were favored materials for the storing and harvesting of produce. Nylon fabric was selected to symbolize harvesting bags used in fruit picking.

4.2.3 Biofilm attachment to different surfaces

The method described by Cerca et al. (2005) was followed to evaluate the attachment of bacterial cells to surfaces. Briefly *L. monocytogenes* cell suspension was allowed to attach on the different coupons for 2, 24 (initial attachment) and 96-h with an initial population of $\sim 1 \times 10^7$ CFU/cm². At each sampling time, in a three-part wash series, the coupons were gently removed from the reactor system and transferred to a 100 ml glass beaker containing water for 10s. Subsequently coupons were placed in another beaker for 10s, followed by a third transfer to allow cell detachment. The washing steps were gently performed to remove any cells suspended in liquid on the substrate surface while minimizing the disruption of attached cells. The coupons

were then dried for 30-minutes before enumeration. Bacterial enumeration required coupons to be placed in 10ml of PBS (Phosphate Buffer Saline, VWR, Solon, OH) where samples were sonicated for 30s followed by 30s of vortexing. This occurred in a three-part series ensuring complete detachment of cells from coupons (ASTM, 2019). Positive controls represent the enumeration of mature 96-h biofilms with no rinse step meaning after 96-h of maturation coupons were removed from the reactor and enumerated. To ensure sterile material before inoculation, negative controls were evaluated by placing the different coupons in TSB without bacterial inoculation.

4.2.4 Biofilm hydrophobicity assay

The hydrophobicity experiments were conducted using three strains of *Listeria innocua* (ATCC 33090, ATCC 33091, ATCC 51742) as surrogates for *L. monocytogenes*, since the tensiometer used in this study is in a BSL1 laboratory.

Hydrophobicity of the biofilms were analyzed by measuring the contact angle and interfacial tension of a sessile droplet (10 μ L of deionized water and formamide) on a biofilm using following the assay described by Cerca et al. (2005). The contact angle for each coupon material and incubation time were automatically measured by the optical drop tensiometer (Attention Theta Lite C311, Nanoscience Instruments, Phoenix, Arizona, USA). Camera adjustments (stage level, lens zoom and focus alterations) were completed using a metal ball (diameter: 4mm) and zoom angle ($\sim 143^\circ$) was obtained. A sessile droplet was created from a U-shaped Luer tip of the Hamilton syringe. The level of the coupon surface was adjusted until the solid surface was visible on the bottom part of the screen. The syringe was lowered until it is just visible at the top of the screen. The viscosity and density values of heavy (water or formamide) and light (air) phases were defined in a recipe sheet. Ten (10) μ L of water/formamide was

automatically dropped from the syringe. Images and angles were recorded for 30s.

Measurements were recorded at three different times (2, 24, and 96-h) for each material after cells were dried for 1-h. For this analysis, two different controls were used: control 1 included dry coupons with no inoculation of cells and control 2 represented materials soaked in TSB for 96-h that were then dried for 1-h with no inoculation of cells. This was completed to understand substrate properties before the introduction of biofilms. Measurements were taken in triplicate at two different locations on the coupon. Contact angle values were calculated using Young-Laplace equation by OneAttension software. Interfacial tension values were calculated based on the following Young's equation (Hebbar et al., 2017).

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\Theta$$

where γ_{sv} is the surface free energy, γ_{sl} is the interfacial tension, γ_{lv} is the surface tension of the droplet and Θ is the contact angle between solid surface and the droplet.

4.2.5 Laser Scanning Confocal Microscopy (LSCM)

Biofilms on the different coupons were analyzed under Laser Scanning Confocal Microscopy (LSCM) (LSM-5 Pascal associated with a Zeiss Axioplan 2) located in the Microscopy Facility of the Biology Department at Kansas State University. Images were captured at three different sampling times (2, 24, and 96-h). Microbial cells were stained to differentiate between living and dead with SYTO 9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR). All images were processed using ImageJ program (NIH, U.S.A).

4.2.6 Statistical analysis

Plating was done in duplicate with all experiments being repeated 3 times. Hydrophobicity experiments were also performed in triplicate. Physical angle results were

expressed by using mean and standard deviation. All the assays were compared using ANOVA Tukey test in SAS Studio for statistical significance (Cary, North Carolina). Differences were denoted as small letters and roman numerals on the top of the standard deviation of the results ($P < 0.05$).

4.3 Results

4.3.1 Attachment of *Listeria monocytogenes*

Biofilm attachment assay results are shown in Table 4.1. Overall, as incubation time increased, cell count increased for all the materials studied. Nylon and wood grew steadily at 2 and 24-h: both developing attachment values $>5 \log \text{CFU/cm}^2$ after 2-h and further increasing by $3 \log \text{CFU/cm}^2$ after 24-h of incubation. Polycarbonate displayed a significantly lower attachment count at each time interval when compared to wood and nylon ($P < 0.05$). After 96-h of development, a significant difference among materials could be discerned ($P < 0.05$): a $1.5 \log \text{CFU/cm}^2$ increase was observed for biofilms growing on nylon, while only a slight change in cell number was reported for wood and polycarbonate ($<1 \log \text{CFU/cm}^2$).

4.3.2 Physiochemical analysis of substrate and biofilm surface

Contact angle measurements and derived interfacial tension values are reported in Figure 4.2 and Table 4.2, respectively. Deionized water and formamide were selected to analyze solvents with different polarities on biofilms. Formamide has lower polarity compared to water (Abu-Lail and Camesano, 2006) The type of solvent used in the experiment had a significant effect ($P < 0.05$): lower values were reported when formamide was used.

Material also showed a significant effect on contact angle measurements ($P < 0.05$). When comparing material surfaces, the lowest value observed was between the wood and formamide ($32^\circ \pm 0.06$), while the highest was found between polycarbonate and water ($72^\circ \pm 0.08$) which

were significantly different values ($P < 0.05$) (Figure 4.2). All material surfaces had contact angle values less than 90° as represented by both control 1 and 2 (Figure 4.2). Similar results were reported also for biofilms: all biofilm surfaces were found to be hydrophilic in nature ($< 90^\circ$). Biofilms grown on wood presented the most hydrophilic surface properties at each incubation period when compared to the other materials, with values of 40° , 43° and 56° . Biofilms grown on polycarbonate coupons had the least hydrophilic surface properties for each incubation period, with values of 46° , 60° , and 72° . Biofilm age directly influenced contact angle: the longer the incubation time, the greater the increase in contact angle values.

Interfacial tension values were calculated based on Young's equation (Figure 4.1). They (Table 4.2) were inversely proportional to the contact angle values (Figure 4.2). The lowest interfacial tension was calculated between polycarbonate surface and water (19.62 ± 0.7) whereas the highest was reported between wood surface and formamide (59.51 ± 0.8).

4.3.3 Laser Scanning Confocal Microscopy (LSCM)

Biofilm images are reported in Figure 4.3. As presented, each material has distinct properties: wood had irregularly spaced pores of different size and shape, nylon showed a threaded surface with uneven dips, while polycarbonate had small grooves running parallel across the material. For all materials tested, it was observed that biofilm masses augmented as the incubation time increased. Wood (Fig 4.3A) demonstrated strong initial growth with numerous observable green viable cells. After 96-h of growth, a thick mass of biofilm cells can be observed. Nylon instead (Fig. 4.3B) showed very few initial cells, but after 24-h, biofilms could be easily observed. After 96-h of incubation complex masses of viable biofilms are easily observed. At 2-h, polycarbonate (Fig 4.3C) showed numerous planktonic cells that started forming large aggregate communities after 24-h, at maturation large complex structures can be seen with varying depths.

4.4 Discussion

Listeria monocytogenes is a ubiquitous foodborne pathogen that is widely recognized for its ability to adhere and form biofilms on a variety of surfaces. Biofilm formation on equipment and materials leads to the constant circulation of foodborne pathogens in and around production areas (Abebe G.M., 2020). Understanding the properties of the surface in which biofilm formation occurs is paramount in controlling contamination and post-contamination (Abebe G.M., 2020). Bacterial attachment is the first step in the process of biofilm formation before forming aggregate communities that develop into mature organized structures that are difficult to eradicate.

In this study we evaluated the attachment properties and development abilities of a multi-strain *L. monocytogenes* biofilm on different surfaces (wood, nylon, and polycarbonate). The results indicated that *L. monocytogenes* enhanced hydrophilic characteristics since contact angle [$^{\circ}$] is recorded as less than 90° for all biofilm samples (Silva et al., 2008). The contact angles of inoculated materials were higher than that of the control groups, highlighting the increase in hydrophobicity with the development of biofilms. A previous study has suggested that the hydrophobic properties of a substrate contribute to a cells ability to attach to a surface (Cerca et al., 2005); in our study all the substrates were found to be hydrophilic in nature, confirming greater cellular attachment. A study conducted by Chavant et al. (2002), analyzed the physiochemical properties of *L. monocytogenes* on stainless steel (hydrophilic) and polytetrafluoroethylene (hydrophobic) discovering that biofilm attachment was stronger on the hydrophilic substrate. Hemmatian et al., (2021), reported that a more hydrophilic surface could result in an increase of cellular adherence. Studies by Abdallah et al. (2014), Khelissa et al. (2017), and Bonsaglia et al. (2014) also found cellular attachment to be greater on hydrophilic

surfaces. In our research, we reported that cell surface hydrophobicity increased with time, as previously observed in those studies. The LSCM images further depict the physical development of *L. monocytogenes* biofilms over the 96-h incubation period. Studies have found an increase in the hydrophobicity of bacterial biofilms to indicate an increase in cell counts and biofilm maturity (De-la-Pinta et al., 2019; Doijad et al., 2015). This explains the steady increase in viable cells, the higher contact angle and the higher hydrophobicity values found on surfaces as biofilms age.

The contact angles measured on biofilm surfaces revealed that the formation of biofilms significantly altered the surface energy of coupons, thus, the interfacial tension between sessile drop and the coupon surfaces. This demonstrated the adhesion capability of wood, nylon, and polycarbonate substrates with *L. monocytogenes* (Briandet et al., 2001). According to the results, biofilm coated coupons exhibited lower interfacial tension than sterile coupons soaked in TSB and dry sterile control coupons. It might be explained with the decline in the van der Waals component and the variation in the Lewis acid-base (electron donor and acceptor) components of the surface free energy of the biofilm material surfaces. Based on Young's equation, surface free energy is directly proportional with interfacial tension (Zhao et al., 2005). Therefore, lower surface free energy represents lower interfacial tension value.

In addition to this, contact angles between sessile drop and biofilms revealed that formamide and water droplets showed differences in contact angle, surface energy and interfacial tension. Lower contact angle values might be interpreted that formamide was energetically more favorable than water to spread and wick into the textured biofilm surface (Epstein et al., 2011). Previous studies also proved that the affinities of solvents with different polarities might show the variation of charges on the cellular surfaces (Satpute et al., 2008; Zammuto et al., 2022).

Moreover, contact angle values were lower in wood surfaces than nylon and polycarbonate. It was expected that the surface of wood would be more wettable than the other two surfaces due to porous and fibrous structural tissues (Connors T., 2015).

The LSCM images clearly depicted the physical characteristics of the surfaces used in our experiments. The more porous nature of nylon and wood provided openings for microbial cells to attach and establish a niche, as confirmed by the images where the depth of viable cells varies but also from the quantitative values obtained during the adhesion assay. Surface texture is one of the major contributing factors in bacterial attachment and higher surface roughness leads to higher bacterial attachment (Wang et al., 2009). This could explain why the polycarbonate surface had less bacterial attachment at each time interval after a mechanical rinse when compared to nylon and wood. In a study analyzing the relationship between material pore structure and bacterial adhesion, Hemmatian et al (2021) found that spatial compactness attenuates bacterial cell depth limiting cell retention. In our study we observed that both nylon and wood substrates have irregularly porous surfaces allowing biofilms to settle in niche environments making cells difficult to remove with an external stressor.

Interestingly, there was no correlation between the number of attached microbial cells and surface hydrophobicity. The smooth polycarbonate surface which displayed the highest hydrophobicity gave the lowest microbial count in each time interval. In support of our findings, a previous study explained that *L. monocytogenes* may show relatively strong adhesion to surfaces that display more regular topography regardless of the amount of microbial attachment (Silva et al., 2008). In another study, it has been mentioned that *L. monocytogenes* might attach to smoother surfaces such as PVC or glass, thus, representing enhanced hydrophobicity

(Takahashi et al., 2010). This explained the higher contact angle and hydrophobicity values on polycarbonate representing less surface roughness.

Although some studies suggested a positive correlation between surface material roughness and hydrophobicity, there are controversial references to this statement in the literature. Some findings revealed that roughness may not always induce the level of biofilm formation and surface hydrophobicity (De-la-Pinta et al., 2019). Variations in surface topography, the degree of surface heights symmetry about the mean plane, number of valleys, and peaks on the surface may differently affect the adhesion scenario (De-la-Pinta et al., 2019; Xu and Siedlecki, 2014). Reduced wettability with a higher contact angle may reduce the availability of the contact area and result in less bacterial adhesion (De-la-Pinta et al., 2019). Moreover, hydrophobicity or interfacial tension between biofilm surface and the sessile droplet could be influenced by the nature of the solvent, temperature of the solid coupon surface, and even the existence of air bubbles on the surface of the material and species of microorganisms (Ashokkumar et al., 2012; Peltonen et al., 2004; Wassmann et al., 2017).

In addition to this, Tolker-Nielsen and Molin (2000) stated that every microbial biofilm presents unique characteristics related to the bacterial species, the number of strains in the cocktail culture, and the physicochemical features of the biofilm surfaces. In our study *L. monocytogenes* grown under the same conditions on three different materials comprised of different LSCM images, contact angle measurements, and attachment enumeration values. Biofilms are complex structures with a wide range of mechanisms at play that promote microbial life and durability. Controlling and preventing these microbial constructions starts with understanding what goes into their development.

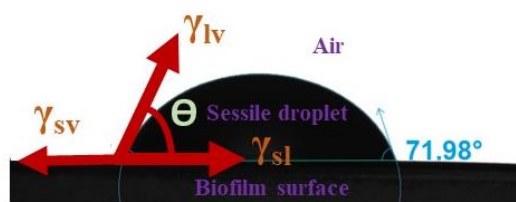
4.5 Conclusion

In conclusion, *L. monocytogenes* has been shown to attach more readily to hydrophilic surfaces such as nylon and wood, and characteristics such as hydrophobicity shape attachment abilities. The material influenced biofilm characteristics (e.g contact angle, interfacial tension, and attachment values) and biofilm cells displayed a stronger attachment to nylon followed by wood and polycarbonate. Produce growers may use this information to make informed decisions on how to effectively clean and sanitize their harvesting equipment. This research shows the need for specific mitigation strategies to combat *L. monocytogenes* biofilms on different surfaces due to the physical differences observed throughout biofilm growth. Further research is still needed to fully understand the role that the food contact surface material plays in the development of biofilms. Nevertheless, this research shows the importance of understanding biofilm surface characteristics and the extreme versatility of the mechanisms behind bacterial cellular attachment. Based on these results, future research can expand these findings to potentially establish systems of biofilm prevention and mitigation in food processing environments for those materials that are difficult to clean.

4.6 Figures and Tables

Table 4.1 Attachment of *L. monocytogenes* (Log CFU/cm² ± SD) to wood, nylon, and polycarbonate after 2, 24, and 96-h of incubation at 20±2°C. Control represents a mature 96-h biofilm with no rinse step, see text for details. Small letter indicates Tukey's multiple comparison test.

	Wood	Nylon	Polycarbonate
2h	5.53± 0.60 ^f	5.70± 0.06 ^f	0.90± 0.00 ^g
24h	8.19± 0.15 ^{cd}	8.20± 0.09 ^{cd}	6.63± 0.19 ^f
96h	8.92± 0.11 ^{abc}	9.62± 0.09 ^a	6.89± 0.20 ^e
Control	8.94± 0.03 ^{bc}	9.73± 0.21 ^{ab}	7.67± 0.07 ^{de}



Young's Equation State for Contact Angle Calculation

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\Theta$$

γ_{sv} : **surface free energy** of biofilm surface

γ_{sl} : **interfacial tension** between solid film surface and sessile droplet

γ_{lv} : **surface tension** of sessile droplet

Θ : **contact angle** between biofilm surface and sessile droplet

Figure 4.1 Schematic illustration of the contact angle (Θ) and interfacial tension of three surfaces at three phase boundary (γ); γ_{sv} ; between surface (s) and vapor (v), γ_{lv} ; droplet (l) and vapor (v), γ_{sl} ; surface (s) and droplet (l), respectively.

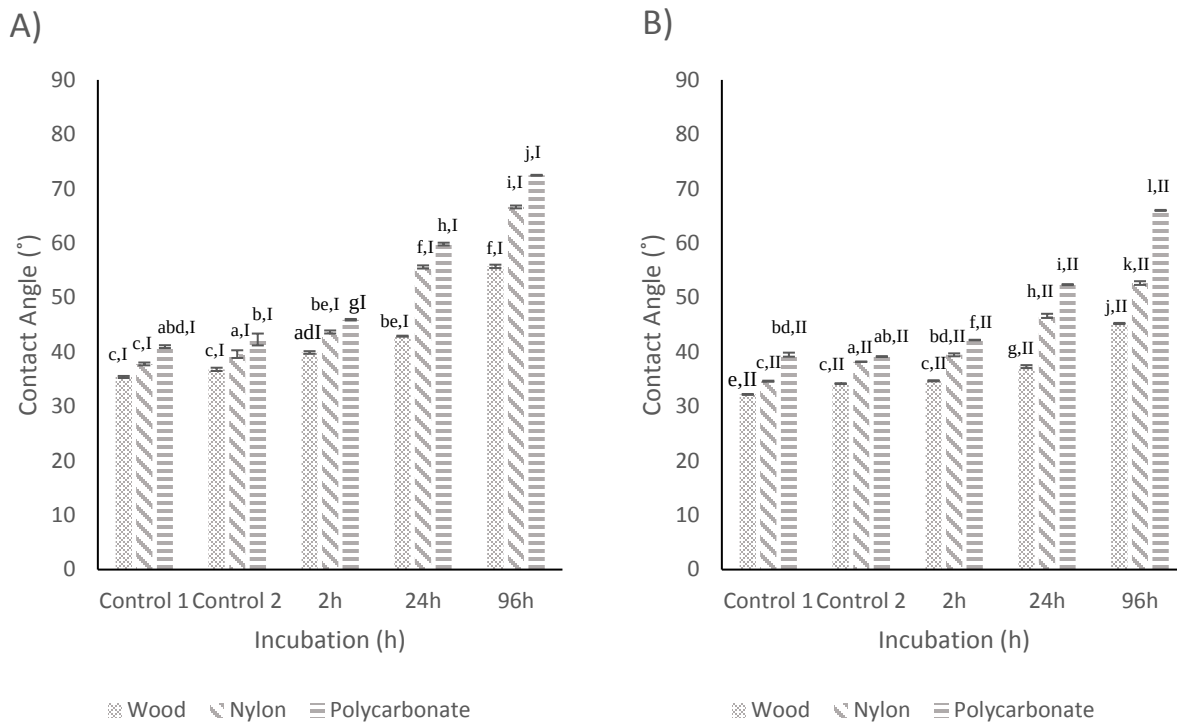


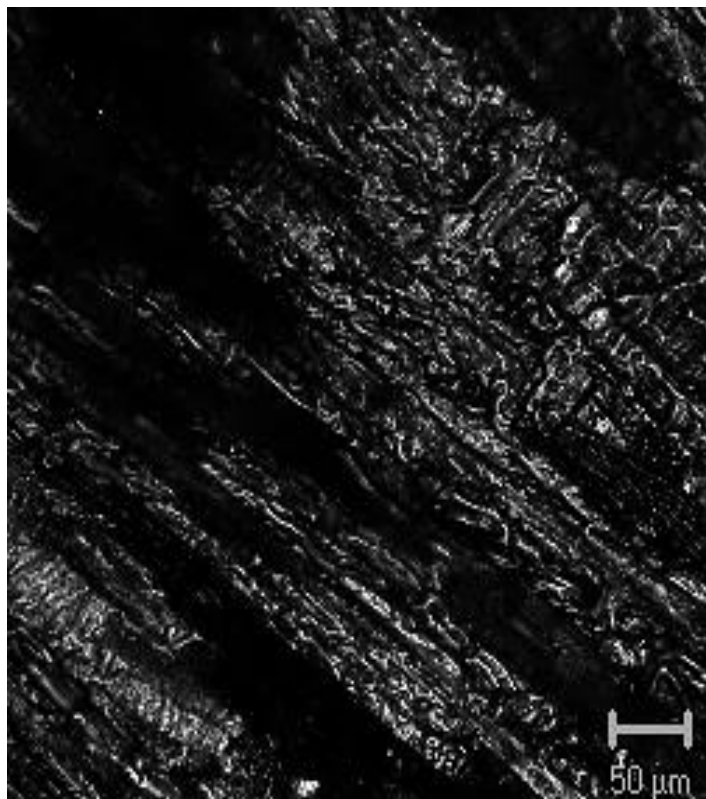
Figure 4.2 Average contact angle measurements of biofilms grown on wood, nylon, and polycarbonate coupons using two different solvents (A) water and (B) formamide. Control 1 represents contact angle values measured on dry coupons with no inoculation of cells. Control 2 represents contact angle values measured after materials were soaked in TSB for 96-h with no inoculation of cells. Small letter represents statistical significance within each solvent group. Roman numeral shows significant difference between solvents formamide and water.

Table 4.2 Interfacial tension of biofilms grown on wood, nylon, and polycarbonate coupons using two different solvents: water and formamide. control 1 represents contact angle values measured on dry coupons with no inoculation of cells. Control 2 represents contact angle values measured after materials were soaked in TSB for 96-h with no inoculation of cells. Small letters and Roman numerals indicate Tukey test for each column and row, respectively.

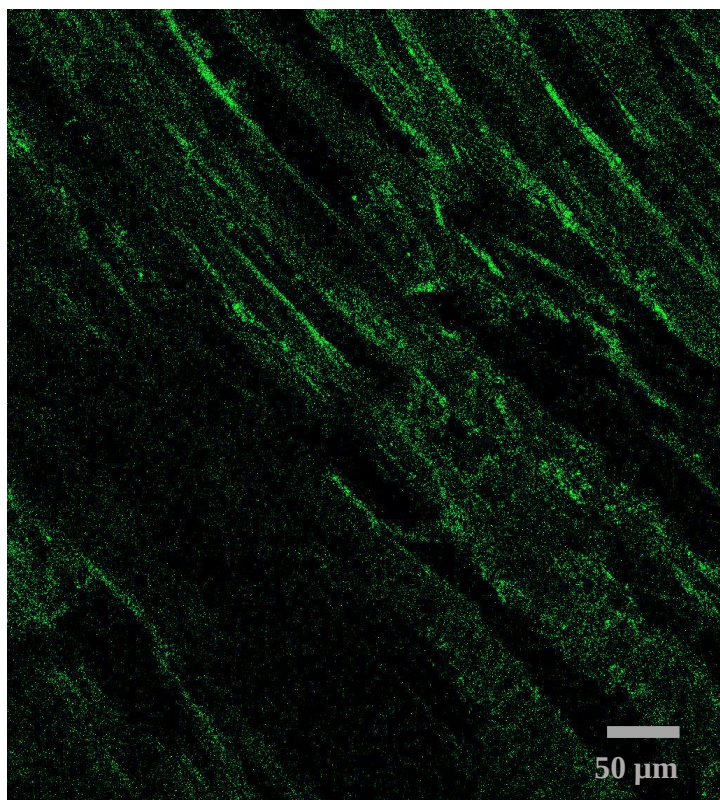
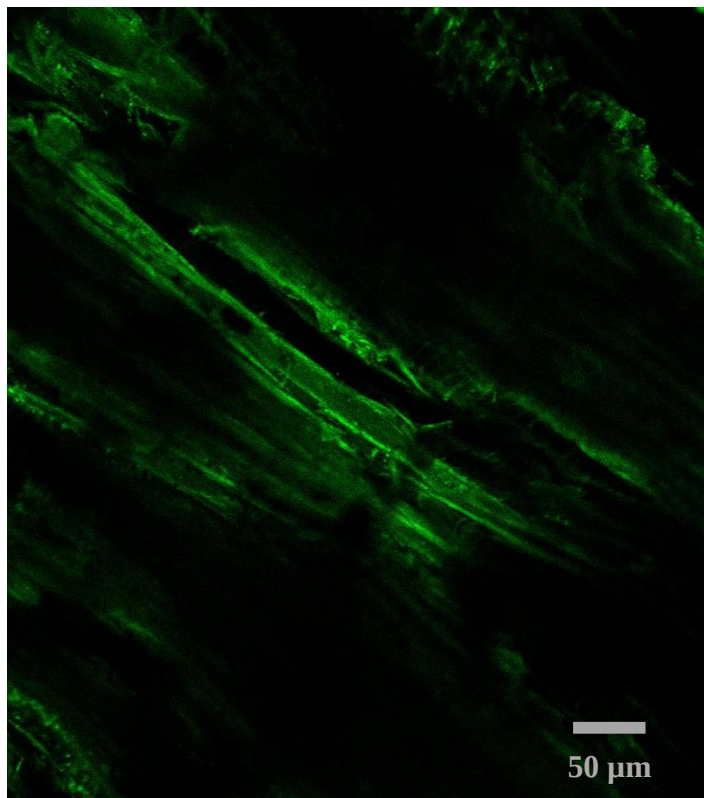
Solvent		Wood	Nylon	Polycarbonate
Water	Control 1	67.5 ± 3.3 ^{a,I}	57.2 ± 1.6 ^{a,I}	51.9 ± 2.2 ^{a,I}
	Control 2	59.3 ± 1.2 ^{a,I}	55.4 ± 1.4 ^{a,I}	47.2 ± 1.5 ^{a,II}
	2h Biofilm Incubation	56.9 ± 2.3 ^{a,I}	44.2 ± 2.2 ^{b,II}	36.2 ± 2.7 ^{b,III}
	24h Biofilm Incubation	40.9 ± 0.5 ^{b,I}	35.0 ± 0.3 ^{bc,II}	29.1 ± 0.3 ^{c,III}
	96h Biofilm Incubation	30.2 ± 1.1 ^{c,I}	25.7 ± 0.6 ^{c,II}	19.6 ± 0.7 ^{d,II}
Formamide	Control 1	67.1 ± 0.8 ^{a,I}	60.1 ± 1.1 ^{a,I}	54.8 ± 0.9 ^{a,II}
	Control 2	62.6 ± 0.1 ^{a,I}	57.05 ± 0.2 ^{a,II}	51.4 ± 1.5 ^{a,II}
	2h Biofilm Incubation	59.5 ± 0.8 ^{a,I}	46.1 ± 0.9 ^{b,II}	36.2 ± 0.1 ^{b,III}
	24h Biofilm Incubation	50.4 ± 1.8 ^{b,I}	44.1 ± 2.4 ^{b,II}	37.4 ± 1.1 ^{b,II}
	96h Biofilm Incubation	41.6 ± 0.8 ^{c,I}	35.8 ± 1.9 ^{c,II}	28.6 ± 2.5 ^{c,II}

A)

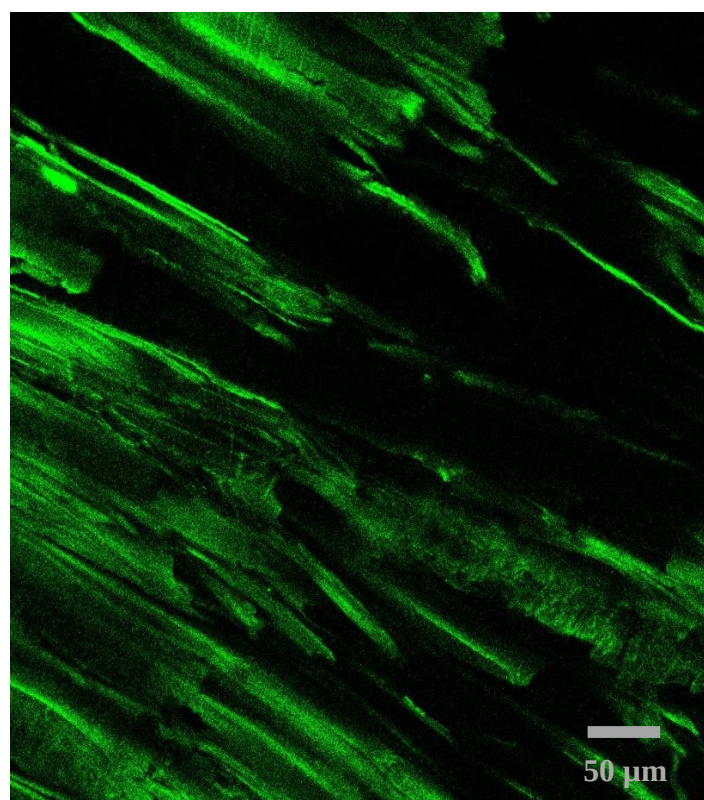
0-h



2-h



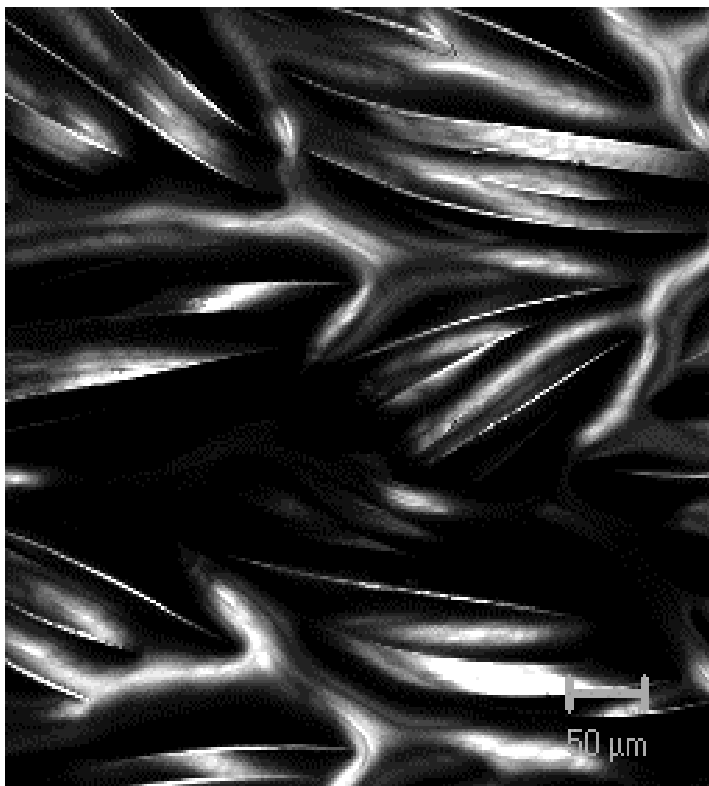
24-h



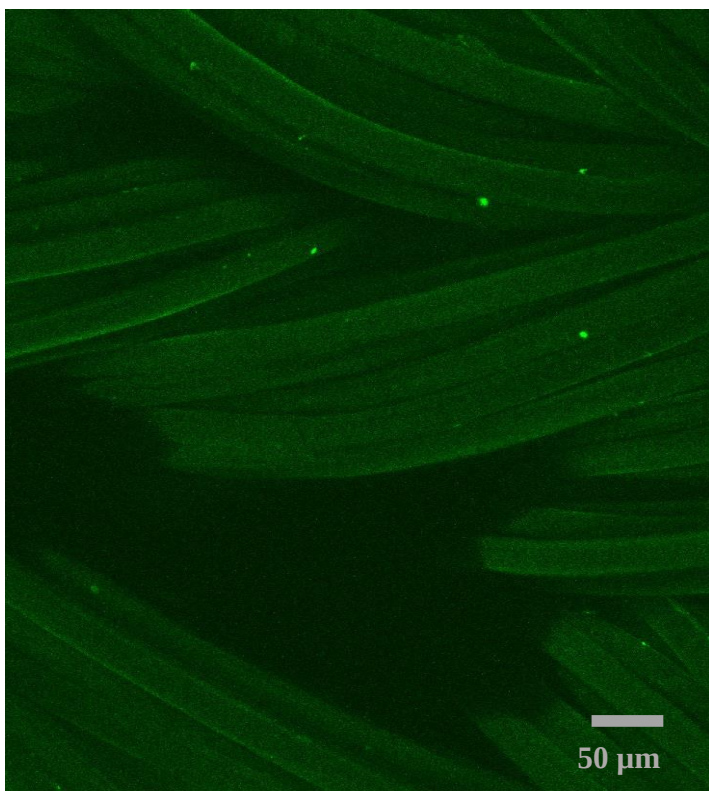
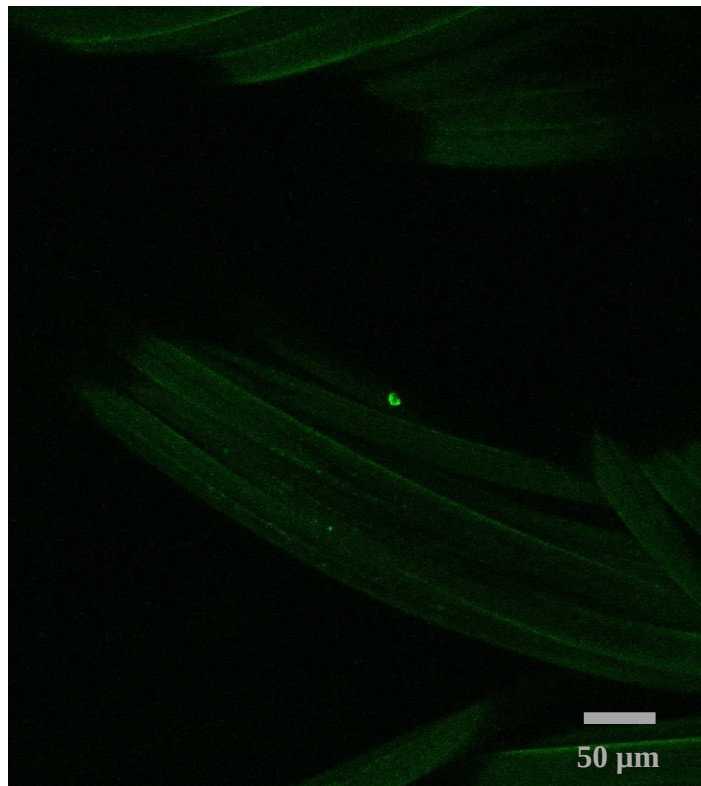
96-h

B)

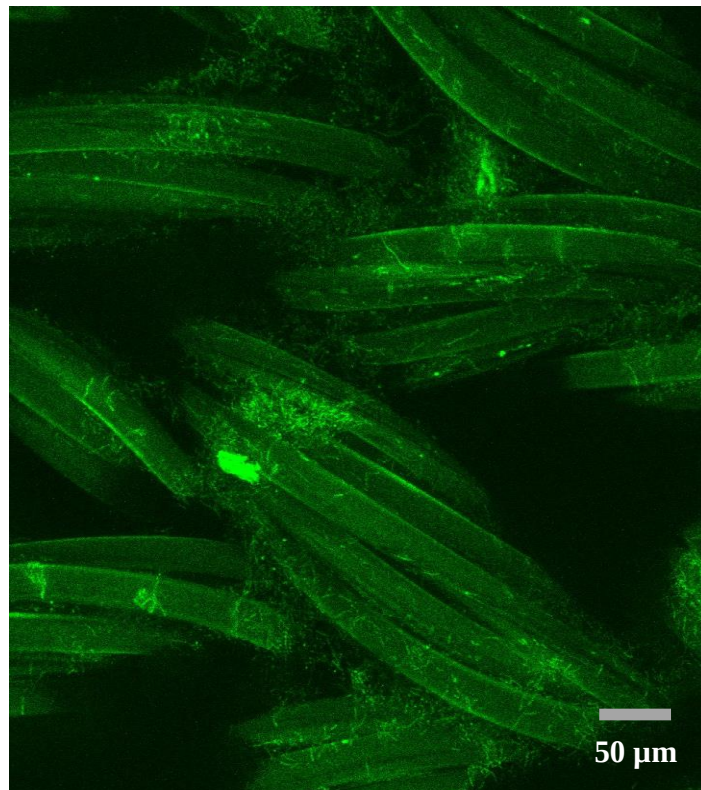
0-h



2-h



24-h



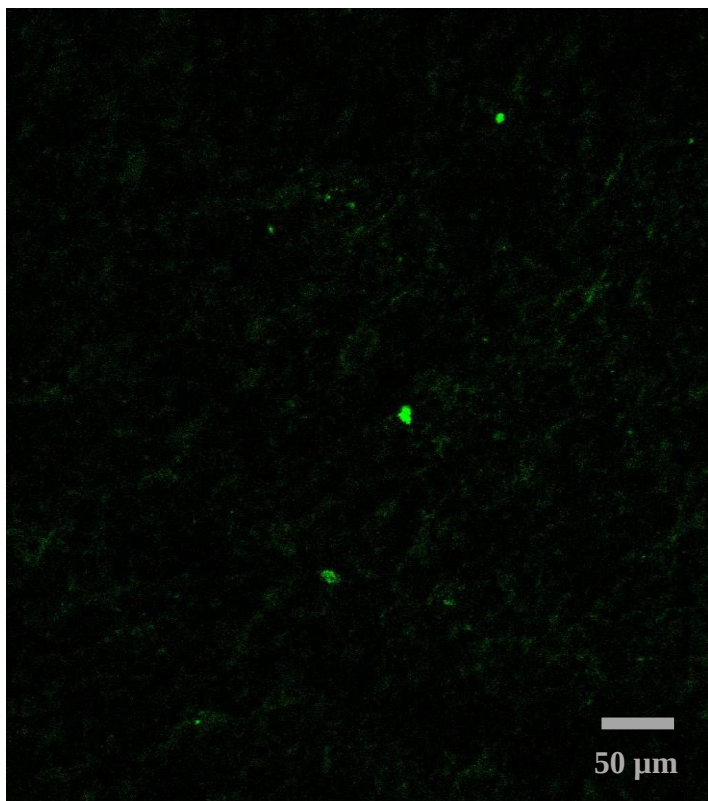
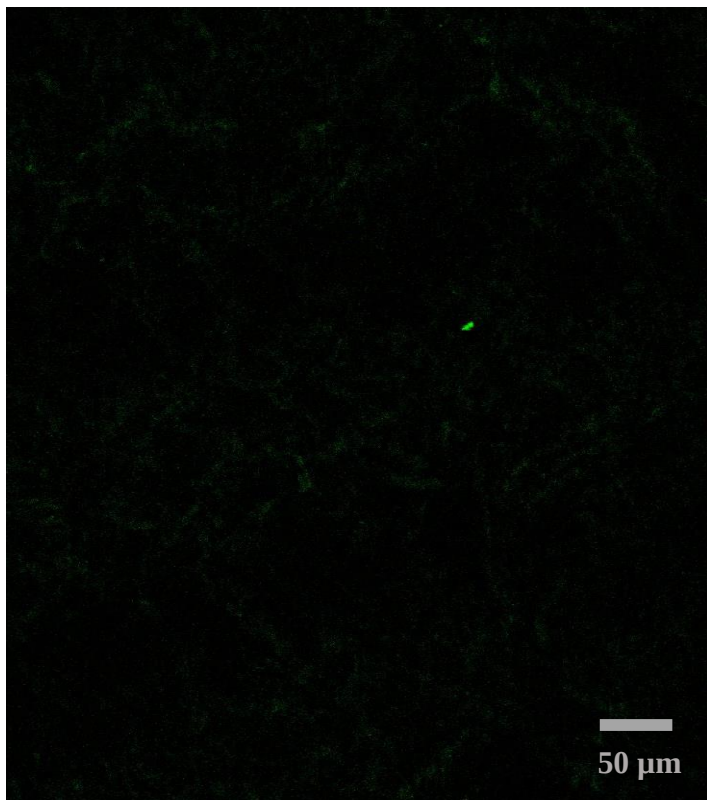
96-h

C)

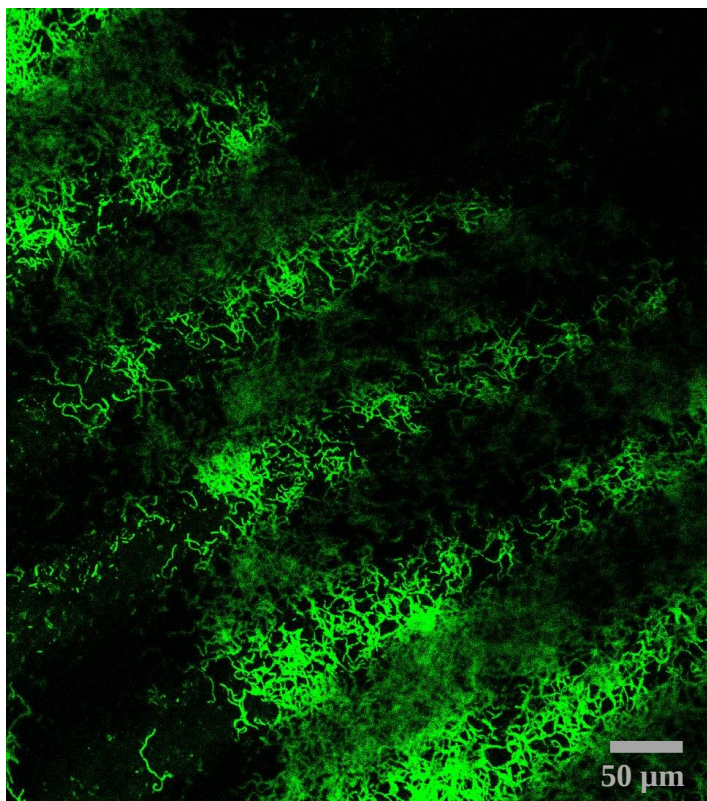
0-h



2-h



24-h



96-h

Figure 4.3 Images show *L. monocytogenes* biofilm development on (A) wood, (B) nylon, and (C) polycarbonate after growth in a CDC Biofilm Reactor at 2, 24, and 96-h under a LSCM. Live bacterial populations are shown in green after live/dead staining with Syto 9. At 0-h, in black and white, material surfaces (wood, nylon, and polycarbonate) can be observed with no biofilm development. All 2D images were constructed using ImageJ.

References

- Abdallah, M., Benoliel, C., Jama, C., Drider, D., Dhulster, P., & Chihib, N.-E. (2014). Thermodynamic Prediction of Growth Temperature Dependence in the Adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to Stainless Steel and Polycarbonate. *Journal of Food Protection*, 77(7), 1116–1126.
<https://doi.org/10.4315/0362-028X.JFP-13-365>
- Abebe, G. M. (2020). The Role of Bacterial Biofilm in Antibiotic Resistance and Food Contamination. *International Journal of Microbiology*, 2020, 1–10.
<https://doi.org/10.1155/2020/1705814>
- Abu-Lail, N. I., & Camesano, T. A. (2006). The effect of solvent polarity on the molecular surface properties and adhesion of *Escherichia coli*. *Colloids and Surfaces B: Biointerfaces*, 51(1), 62–70.
<https://doi.org/10.1016/j.colsurfb.2006.05.009>
- Ashokkumar, S., Adler-Nissen, J., & Moller, P. (2012). Factors affecting the wettability of different surface materials with vegetable oil at high temperatures and its relation to cleanability. *Applied Surface Science*, 263(December), 86–94.
<https://doi.org/10.1016/j.apsusc.2012.09.002>
- ASTM. (2019). E2871-19 Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method. ASTM International. <https://doi.org/10.1520/E2871>
- Bonsaglia, E. C. R., Silva, N. C. C., Fernandes Júnior, A., Araújo Júnior, J. P., Tsunemi, M. H., & Rall, V. L. M. (2014). Production of biofilm by *Listeria monocytogenes*

- in different materials and temperatures. *Food Control*, 35(1), 386–391.
<https://doi.org/10.1016/j.foodcont.2013.07.023>
- Briandet, R., Herry, J. M., & Bellon-Fontaine, M. N. (2001). Determination of the van der Waals, electron donor and electron acceptor surface tension components of static Gram-positive microbial biofilms. *Colloids and Surfaces B: Biointerfaces*, 21(4), 299–310. [https://doi.org/10.1016/S0927-7765\(00\)00213-7](https://doi.org/10.1016/S0927-7765(00)00213-7)
- Center for Disease Control and Prevention (CDC). (2022). Listeria (listeriosis) people at risk.
<https://www.cdc.gov/listeria/risk.html#:~:text=CDC%20estimates%20that%20Listeria%20is,year%2C%20and%20about%20260%20die.>
- Cerca, N., Pier, G. B., Vilanova, M., Oliveira, R., & Azeredo, J. (2005). Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. *Research in Microbiology*, 156(4), 506–514. <https://doi.org/10.1016/j.resmic.2005.01.007>
- Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.-N., & Hebraud, M. (2002). *Listeria monocytogenes* LO28: Surface Physicochemical Properties and Ability To Form Biofilms at Different Temperatures and Growth Phases. *Applied and Environmental Microbiology*, 68(2), 728–737.
<https://doi.org/10.1128/AEM.68.2.728-737.2002>
- Chmielewski, R. A. N., & Frank, J. F. (2003). Biofilm Formation and Control in Food Processing Facilities. *Comprehensive Reviews in Food Science and Food Safety*, 2(1), 22–32. <https://doi.org/10.1111/j.1541-4337.2003.tb00012.x>
- Connors, T. (2015). Introduction to Wood Structure and Characteristics. *Agriculture and*

Natural Resources Publications., 12, 104.

http://uknowledge.uky.edu/anr_reports/104

da Silva, E. P., & De Martinis, E. C. P. (2013). Current knowledge and perspectives on biofilm formation: The case of *Listeria monocytogenes*. *Applied Microbiology and Biotechnology*, 97, 957–968. <https://doi.org/10.1007/s00253-012-4611-1>

De-la-Pinta, I., Cobos, M., Ibarretxe, J., Montoya, E., Eraso, E., Guraya, T., & Quindós, G. (2019). Effect of biomaterials hydrophobicity and roughness on biofilm development. *Journal of Materials Science: Materials in Medicine*, 30(7). <https://doi.org/10.1007/s10856-019-6281-3>

Doijad, S. P., Barbuddhe, S. B., Garg, S., Poharkar, K. V., Kaloirey, D. R., Kurkure, N. V., Rawool, D. B., & Chakraborty, T. (2015). Biofilm-Forming Abilities of *Listeria monocytogenes* Serotypes Isolated from Different Sources. *PLOS ONE*, 10(9), e0137046. <https://doi.org/10.1371/journal.pone.0137046>

Epstein, A. K., Pokroy, B., Seminara, A., & Aizenberg, J. (2011). Bacterial biofilm shows persistent resistance to liquid wetting and gas penetration. *Proceedings of the National Academy of Sciences of the United States of America*, 108(3), 995–1000. <https://doi.org/10.1073/pnas.1011033108>

Erkan, M., & Dogan, A. (2019). Harvesting of Horticultural Commodities. In *Postharvest Technology of Perishable Horticultural Commodities* (pp. 129–159). Elsevier. <https://doi.org/10.1016/B978-0-12-813276-0.00005-5>

Faria, S. I., Teizeira-Santos, R., Romeu, M. J., Morais, J., Vasconcelos, V., & Mergulhao, F. J. (2020). The Relative Importance of Shear Forces and Surface

- Hydrophobicity on Biofilm Formation by Coccoid Cyanobacteria. *Polymers*, 12(3). <https://doi.org/10.3390/polym12030653>
- Ferreira, V., Wiedmann, M., Teixeira, P., & Stasiewicz, M. J. (2014). *Listeria monocytogenes* Persistence in Food-Associated Environments: Epidemiology, Strain Characteristics, and Implications for Public Health. *Journal of Food Protection*, 77(1), 150–170. <https://doi.org/10.4315/0362-028X.JFP-13-150>
- Hebbar, R. S., Isloor, A. M., & Ismail, A. F. (2017). Contact Angle Measurements. In *Membrane Characterization* (pp. 219–255). Elsevier. <https://doi.org/10.1016/B978-0-444-63776-5.00012-7>
- Hemmatian, T., Lee, H., & Kim, J. (2021). Bacteria Adhesion of Textiles Influenced by Wettability and Pore Characteristics of Fibrous Substrates. *Polymers*, 13(2), 223. <https://doi.org/10.3390/polym13020223>
- Khelissa, S. O., Jama, C., Abdallah, M., Boukherroub, R., Faille, C., & Chihib, N.-E. (2017). Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells. *AMB Express*, 7(1), 191. <https://doi.org/10.1186/s13568-017-0492-0>
- Kushwaha, K., & Muriana, P. M. (2009). Adherence Characteristics of *Listeria* Strains Isolated from Three Ready-to-Eat Meat Processing Plants. *Journal of Food Protection*, 72(10), 2125–2131. <https://doi.org/10.4315/0362-028X-72.10.2125>
- Lee, B.-H., Hébraud, M., & Bernardi, T. (2017). Increased Adhesion of *Listeria monocytogenes* Strains to Abiotic Surfaces under Cold Stress. *Frontiers in Microbiology*, 8, 2221. <https://doi.org/10.3389/fmicb.2017.02221>

- Lunden, J. M., Miettinen, M. K., Autio, T. J., & Korkeala, H. J. (2000). Persistent *Listeria monocytogenes* Strains Show Enhanced Adherence to Food Contact Surface after Short Contact Times. *Journal of Food Protection*, 63(9), 1204–1207.
- Mendez, E., Walker, D. K., Vipham, J., & Trinetta, V. (2020). The use of a CDC biofilm reactor to grow multi-strain *Listeria monocytogenes* biofilm. *Food Microbiology*, 92, 103592. <https://doi.org/10.1016/j.fm.2020.103592>
- Peltonen, J., Järn, M., Areva, S., Linden, M., & Rosenholm, J. B. (2004). Topographical parameters for specifying a three-dimensional surface. *Langmuir*, 20(22), 9428–9431. <https://doi.org/10.1021/la0400252>
- Ruiz-Llacsahuanga, B., Hamilton, A., Zaches, R., Hanrahan, I., & Critzer, F. (2021). Prevalence of *Listeria* Species on Food Contact Surfaces in Washington State Apple Packinghouses. *Applied and Environmental Microbiology*, 87(9), e02932-20. <https://doi.org/10.1128/AEM.02932-20>
- Satpute, S. K., Bhawsar, B. D., Dhakephalkar, P. K., & Chopade, B. A. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. *Indian Journal of Marine Sciences*, 37(3), 243–250.
- Stein, R. A., & Chirilă, M. (2017). Routes of Transmission in the Food Chain. In *Foodborne Diseases* (pp. 65–103). Elsevier. <https://doi.org/10.1016/B978-0-12-385007-2.00003-6>
- Silva, S., Teixeira, P., Oliveira, R., & Azeredo, J. (2008). Adhesion to and Viability of *Listeria monocytogenes* on Food Contact Surfaces. *Journal of Food Protection*, 71(7), 1379–1385.
- Takahashi, H., Suda, T., Tanaka, Y., & Kimura, B. (2010). Cellular hydrophobicity of

- Listeria monocytogenes* involves initial attachment and biofilm formation on the surface of polyvinyl chloride. *Letters in Applied Microbiology*, 50(6), 618–625.
<https://doi.org/10.1111/j.1472-765X.2010.02842.x>
- Teixeira, P., Lima, J., Azeredo, J., & Oliveira, R. (2008). Adhesion of *Listeria monocytogenes* to materials commonly found in domestic kitchens. *International Journal of Food Science & Technology*, 43(7), 1239–1244.
<https://doi.org/10.1111/j.1365-2621.2007.01598.x>
- Tolker-Nielsen, T., & Molin, S. (2000). Spatial Organization of Microbial Biofilm Communities. *Microbial Ecology*, 40(2), 75–84.
<https://doi.org/10.1007/s002480000057>
- Wang, H., Feng, H., Liang, W., Luo, Y., & Malyarchuk, V. (2009). Effect of Surface Roughness on Retention and Removal of *Escherichia coli* O157:H7 on Surfaces of Selected Fruits. *Journal of Food Science*, 74(1), E8–E15.
<https://doi.org/10.1111/j.1750-3841.2008.00998.x>
- Wassmann, T., Kreis, S., Behr, M., & Buegers, R. (2017). The influence of surface texture and wettability on initial bacterial adhesion on titanium and zirconium oxide dental implants. *International Journal of Implant Dentistry*, 3(1).
<https://doi.org/10.1186/s40729-017-0093-3>
- Xu, L. C., & Siedlecki, C. A. (2014). Staphylococcus epidermidis adhesion on hydrophobic and hydrophilic textured biomaterial surfaces. *Biomedical Materials (Bristol)*, 9(3). <https://doi.org/10.1088/1748-6041/9/3/035003>
- Zammuto, V., Rizzo, M. G., Spanò, A., Spagnuolo, D., Di Martino, A., Morabito, M., Manghisi, A., Genovese, G., Guglielmino, S., Calabrese, G., Capparucci, F.,

- Gervasi, C., Nicolò, M. S., & Gugliandolo, C. (2022). Effects of crude polysaccharides from marine macroalgae on the adhesion and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Algal Research*, 63(February). <https://doi.org/10.1016/j.algal.2022.102646>
- Zhao, Q., Liu, Y., & Wang, C. (2005). Development and evaluation of electroless Ag-PTFE composite coatings with anti-microbial and anti-corrosion properties. *Applied Surface Science*, 252(5), 1620–1627. <https://doi.org/10.1016/j.apsusc.2005.02.098>

Chapter 5 - The Combined Effect of Sanitizers and UV-C Light on

***Listeria monocytogenes* Biofilms Growth and Survivability of**

Surfaces

Abstract

Listeria monocytogenes is an aggressive biofilm former that can establish and persists in food-processing environments. Commonly associated with ready-to-eat (RTE) foods, dairy and cold stored meat products in recent years, this organism has also been increasingly linked to fresh produce outbreaks. The purpose of this study was to investigate the application of lactic acid, thymol, silver citric acid, and UV-C light alone or in combination on biofilms on different food-contact surfaces for 2 or 5-min. Multi-strain *L. monocytogenes* biofilms were grown in a CDC reactor for 96-h on wood, nylon, and polycarbonate coupons at $20 \pm 2^\circ\text{C}$. After each treatment, coupons were neutralized, and remaining cells enumerated. Results showed that the most effective treatment was the simultaneous use of UV-C light and silver citric acid (4 log reduction) and the least effective UV-C light alone ($P < 0.05$). Nevertheless, the combined applications of UV and sanitizers did not have a greater effect in microbial log reduction as compared to the application of sanitizer alone. Material was found to play a significant role in the efficacy of the sanitizers ($P < 0.05$). Conversely no significant difference was observed between application times. The present study demonstrates the ability of *L. monocytogenes* to grow and form biofilms on different surfaces and contributes to the understanding of the response of this food safety threat to antimicrobial intervention strategies.

5.1 Introduction

Listeria monocytogenes is a foodborne pathogen commonly associated with Ready-To-Eat (RTE) foods, dairy, and cold stored meat products (Zhu et al. 2017). This ubiquitous bacterial pathogen is the cause of listeriosis, an invasive infection resulting in high rates of mortality and morbidity (Garner et al. 2016). *L. monocytogenes* is a saprophyte able to invade the cytosol of eukaryotic cells (Freitag et al. 2009). This bacterium can adapt and grow at refrigeration temperatures, low pH, and high salinity (Townsend et al., 2021). *L. monocytogenes* biofilm forming capabilities enable this pathogen to survive sanitation treatments indicating concern for the safety of food products (Srey et al., 2013). Researchers have been focusing efforts on understanding this microorganism's aptitude to survive, persist and infect in food-processing environments and equipment. However, the rising numbers of *L. monocytogenes* outbreaks linked to fresh produce have increased the need to understand how to effectively control this pathogen.

The prevalence, persistence, and diversity through which *L. monocytogenes* infects fresh produce is a growing concern for many, especially considering the zero-tolerance regulation put in place by the US Food and Drug Administration (FDA) for *L. monocytogenes* in RTE products (Archer, 2018). Equipment used during the harvesting and handling of produce are known reservoirs of microbial biofilms (Kallipolitis et al., 2020; Schoder et al., 2011; FDA, 2022). These irregular food-contact surfaces provide niche environment for biofilms to persist even after sanitizer application.

The use of chemical sanitizers is common practice within the produce industry when controlling *Listeria* spp. Most studies analyzed the effect of commercially available sanitizers such as peroxyacetic acid (PAA), ozone, halogen-based compounds, hydrogen peroxide, acid

anionic compounds and quaternary ammonium compounds on food processing surfaces (Aryal et al., 2019; Hua et al., 2019; Fagerlund et al., 2019). The implementation of technologies such as UV-C light have provided a method for disinfecting surfaces that may eliminate the need for mechanical scrubbing (Adhikari et al. 2015). Today, food safety is moving toward synergistic processes of simultaneous or sequential germicidal applications or hurdles to obtain greater pathogen reduction (Berdejo et al., 2019). Studies analyzing the combined effects of UV-C light and chemical treatment have found that combined applications have higher antimicrobial effects as compared to single application. A study conducted by Ding et al. (2018) found the simultaneous application of UV-A light and benzoic acid when treating leafy greens was most effective. The combined use of chemical and physical means of sanitation has been presented as a positive strategy to overcome contamination in food processing environments. Therefore, the objective of this study was to investigate the survival of *Listeria monocytogenes* biofilms on produce harvesting materials after treatment with chemical sanitizers alone and/or concurrent with UV-C light. The chemical sanitizers (lactic acid, silver dihydrogen citrate, and thymol) were selected to represent non-toxic or generally recognized as safe (GRAS) chemicals readily available to produce farmers.

5.2 Material and Methods

5.2.1 Bacterial strains

Three strains of *L. monocytogenes* were used in this study to form biofilms: L2624 (serotype 1/2b) and L2626 (serotype 1/2a), isolated from the 2001 U.S. cantaloupe outbreak, and J2230 (serotype 4b) from a clinical sample (Mendez et al., 2020). All strains were kept on CyroCare Bacteria Preserver beads (Key Scientific, Stamford, TX) stored at -80°C until experiments. A *L. monocytogenes* cocktail as previously described by Mendez et al. (2020) was

created by combining individual strains in equal ratio to reach an initial population of $\sim 1 \times 10^8$ CFU/ml.

5.2.2 Substrate material

The materials selected for this study included wood, nylon, and polycarbonate all representative of substrates used during the harvesting and handling of fresh produce. Coupons made of polycarbonate (1.27cm diameter) were purchased from BioSurface Technologies Corporation (Bozeman, MT). Wood coupons were made of plywood of unfinished basswood purchased from a local store and cut into coupons of 1.27 cm in diameter. Nylon fabric was obtained from a local store and was cut into 2cm x 2cm squares to fit within the rods of the reactor.

5.2.3 Biofilm formation

Based on a protocol developed in our laboratory (Mendez et al., 2020) with slight modifications, biofilms were grown in a CDC Biofilm reactor (BioSurface Technologies, Bozeman, MT) for up to 96-h. To begin the 24-h batch phase, 1ml of *L. monocytogenes* cocktail was used to inoculate the reactor. This was followed by an additional 72-h of continuous nutrient flow at a rate of 8ml/min using tryptic soy broth (TSB, BD Difco, Sparks, MD). This reactor was maintained at $20 \pm 2^\circ\text{C}$ for 96-h of incubation.

5.2.4 Chemical sanitizer and UV-C light

The following chemical sanitizers were used to evaluate efficacy against *L. monocytogenes* biofilm: 4% lactic acid solution (Purac, Corbion, Blair, NE) confirmed using a lactic acid test kit (ChemWorld, Kennesaw, GA); 5% silver dihydrogen citrate (SDC)-based sanitizer as per label (PURE Bioscience, El Cajon, CA); and 0.23% thymol solution as per label (Bioesque solutions, Lighthouse Point, FL). The lamp used as the emission source of the UV-C

light was set at 254nm (Lumalier, Memphis, TN). Power output was monitored using a radiometer (Sper Scientific Ltd, Scottsdale, AZ) during all the experiments.

5.2.5 Application of treatments

To simulate industrial conditions, a UV-C lab bench apparatus was built to demonstrate the effectiveness of dual antimicrobial processes (Figure 5.1). The apparatus was designed to deliver uniform and quantified irradiation and an even distribution of sanitizer to ensure coverage of all coupons. Sanitizers were applied with a steady flow rate of 266.67 ml/min delivered through a diaphragm pump feeding into a food grade nozzle. The UV-C lamp was 16cm away from the coupon with an average output intensity of 850 $\mu\text{W}/\text{cm}^2$ emitting light at 254nm. At the two application times 2 and 5 minutes the UV dose was found to be 102,000 $\mu\text{WSec}/\text{cm}^2$ and 255,000 $\mu\text{WSec}/\text{cm}^2$ respectively based on the average intensity multiplied by the exposure time (seconds) (Bolton et al., 2003). After 4 days of biofilm development, the rods containing coupons (wood, nylon, polycarbonate) were removed from the reactor and placed in the sanitizing apparatus. The preferred sanitizer treatment was applied ($20\pm 2^\circ\text{C}$). Half-way through the application time, the rod was rotated to ensure treatment exposure to both sides of the coupon.

5.2.6 Recovery and enumeration of microbial cells

After treatment exposure, coupons treated with a chemical sanitizer were individually placed in 10ml of Dey and Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA), while controls were placed in phosphate-buffered saline (PBS, VWR, Solon, OH). To recover cells, coupons underwent 30s of sonication (40kHz and 100% amplitude resulting 110W of power) followed by 30s of vortexing; this procedure was repeated 3 times to ensure full recovery of remaining cells (ASTM, 2019). Serial dilutions were then performed in 0.1% peptone water

(Bacto, Sparks, MD) and spread plated on Tryptic Soy Broth (TSA, Difco, Sparks, MD) in duplicate. After 24h of incubation at $37\pm 2^{\circ}\text{C}$ colonies were counted, and results reported as log CFU/cm².

5.2.7 Experimental design and statistical analysis

Seven different treatment combinations including the application of chemical sanitizers and UV-C light alone and simultaneously were analyzed in this study. Each treatment was randomized across the 8 rods residing within the CDC reactor and all experiments were completed 4 times. The statistical significance was defined by a $P < 0.05$. A mixed model was used for the analysis of log reduction among treatments, materials, and application time. All data was analyzed using SAS 9.4TS Level 1M5 (SAS Institute, Cary, North Carolina).

5.3 Results

Control coupons of wood, nylon, and polycarbonate were enumerated after 96-h resulting in 8.87 ± 0.13 log CFU/cm², 9.60 ± 0.32 log CFU/cm², and 7.68 ± 0.06 log CFU/cm² respectively. Overall, the most effective treatment was the simultaneous application of UV-C light and SDC ($P < 0.05$). This combination resulted in average reductions of 1.2 log CFU/cm² on wood, 3.4 log CFU/cm² on nylon, and 4 log CFU/cm² on polycarbonate. UV-C light exposure was found to be the least effective strategy for all materials regardless of application time ($P < 0.05$). As shown in Table 5.1, the individual applications of material, sanitizer, and time were all significant factors against *L. monocytogenes* biofilms ($P < 0.05$). However, materials and sanitizers, were the only interaction found to be significant ($P < 0.05$). This highlights that the effectiveness of the sanitizer on mature biofilms is influenced by the substrate material.

The effects of single and simultaneous sanitizer applications of lactic acid, SDC, thymol, and UV-C light on experimentally inoculated wood, nylon and polycarbonate coupons can be

observed in Figure 5.2. Biofilms developed on wood were found to be the most resistant to treatments when compared to nylon and polycarbonate, resulting in ≤ 1 log CFU/cm² reduction for all selected sanitizers at each application time. Single sanitizers applied were highly effective on biofilms grown on polycarbonate with reductions nearing 3.6 log CFU/cm². Thymol (0.23%) and SDC (5%) alone were found to cause cell reductions up to 3 log CFU/cm² depending on the substrate on which the biofilm was developed. For example, nylon exposed to thymol for 5min exhibited a 3.7 log CFU/cm² microbial reduction and polycarbonate exposed for 5 min exhibited a 3.6 log CFU/cm² reduction. SDC caused a 3.6 log CFU/cm² reduction of cells after 5min on polycarbonate and a 2.8 log CFU/cm² reduction on nylon after 5min exposure. Lactic acid (4%) alone resulted in up to a 2.5 log CFU/cm² reduction of *L. monocytogenes* depending on the substrate: after 2min of exposure on nylon there was 2.5 log CFU/cm² reduction and after 2min exposure to polycarbonate there was a 2 log CFU/cm² reduction. No significant difference was found when comparing the effectiveness of single use sanitizers to simultaneous applications ($P < 0.05$).

5.4 Discussion

The fresh produce industry is constantly battling *L. monocytogenes* on surfaces that are difficult to clean and sanitize. With more recent outbreaks being associated with pre- and post-harvest practices, evaluating the effectiveness of interventions that are efficient and practical for small produce growers is becoming more prudent to food safety.

The substrate where a biofilm is formed has been observed to greatly influence the efficacy of sanitizers. In this study, wood was highly resistant to both sole sanitizer applications and simultaneous applications, followed by nylon and polycarbonate respectively. In a study by Bang et al. (2014), similar results could be observed where *E. coli* O157:H7 biofilms were most

persistent on wood surfaces after treatments of NaOCl and ClO₂. Yang et al. (2009), reconfirms this finding when studying *L. monocytogenes* biofilms on smooth and rough high-density polyethylene (HDPE): sanitizers were found to be less effective on rough HDPE. Both studies infer that surface texture provides protection to cells and limits sanitizer penetration. Textural discrepancies could also explain the low success of the application of UV-C light alone. UV-C light shows germicidal properties when UV light is absorbed by DNA, creating cyclobutane pyrimidine dimers that stop cellular transcription and replication. UV-C's effectiveness as a sanitizer is dependent on its ability to reach genetic material (Gayan et al., 2014; Ravant et al., 2001).

In our study, there was high variability among bacterial reduction based on surface material and sanitizer. Lactic acid is an organic compound that permeates the cell membrane leading to extreme pH distortion causing loss of cell contents, lysis, and eventual cell death (Zhang et al., 2021). The effectiveness of lactic acid to control *L. monocytogenes* biofilms was previously studied by Ban et al., (2012) on 6-day old biofilms developed on polyvinyl chloride and stainless steel. Using 0.5-2.0% lactic acid, researchers were able to achieve a 0.19-0.94 log reduction after 5-30s immersion in sanitizer. In our study, a greater reduction could be observed due to the longer exposure time (2 and 5min). The strong antibacterial properties of thymol are due to the high concentration of phenolic compounds that can disrupt the cytoplasmic membrane interrupting the proton motive force, the flow of electrons, and active transport as well as causing the congealing of cell contents (Burt s. 2004). Desai et al. (2012), found 4-day old biofilms (7 log CFU/coupon) to be completely eradicated on stainless steel coupons using 0.5% thymol after 24h of exposure. Our study using 0.23% thymol was able to achieve up to a 3-log reduction after only 5 min of exposure, depending on material. Few studies can be found regarding the efficacy

of SDC against mature *L. monocytogenes* biofilms. Masuku et al. (2014) applied SDC for 2min against 4-h old *L. monocytogenes* cells on stainless steel and aluminum and achieved a 5-log reduction. Instead within our study a >3 log reduction of *L. monocytogenes* biofilms after 5min exposure was observed on polycarbonate. Many of the discrepancies between our results and the study conducted by Masuku et al. (2014) are probably due to biofilm growing conditions, the biofilm age, sanitizer concentrations, substrate material, and sanitizer contact time.

As previously stated, the simultaneous applications of sanitizers have been predicted to improve efficacy against target microorganisms. Studies analyzing concurrent sanitizer use have found that two stressors on an organism result in higher bacterial lethality. Ding et al. (2018) reported that treatment with benzoic acid (BA) and UV-A light on *E. coli* resulted in a ~6 log CFU/cm² reduction of cells after 30min. Silva-Espinoza et al. (2020) found that the simultaneous use of clove essential oil and UV-C light was highly effective against *Salmonella* biofilms. Nevertheless, when Tajik et al., (2015) treated *L. monocytogenes* biofilm using UV-C light and *zataria multiflora* boiss (Shirazi thyme) essential oil simultaneously for 15 and 45 min no significant difference was observed as compared to single use treatments. This aligns with the results of our study where no significant difference between simultaneous and single applications were observed. These results could be linked to the fact that irregular liquid particles from the chemical sanitizers could scatter light shielding cells from UV wavelengths (McKinney et al., 2009); or sanitizers could interfere with the free radicals that are being produced with ultraviolet light (Tajik et al., 2015) and/or the biofilm EPS acts as a shield from UV light and limits the diffusion of disinfectants (Yin et al., 2019; Jahid and Ha, 2012). Nevertheless, in our study we reported that UV light and SDC when applied simultaneously were the most effective at reducing

L. monocytogenes biofilms: SDC is a poor reflector of UV light which could help explain the increased effectiveness when compared to the other applications (Pure Bioscience, Inc, 2011).

5.5 Conclusion

In the present study we evaluated the effectiveness of lactic acid, SDC, thymol, and UV-C light alone or simultaneously against 96-h biofilms grown on wood, nylon, and polycarbonate. We found that the biofilm growth substrate is an important factor to consider for the efficacy of sanitizers and that the simultaneous applications of UV-C and chemical sanitizers does not guarantee greater potency, as compared to single sanitizer applications. This research explores unconventional treatment options such as lactic acid, thymol, and silver dihydrogen citrate used alone or simultaneously with UV-C light that could be used to control *L. monocytogenes* biofilms. The most effective mitigation strategy used against *L. monocytogenes* biofilms that resulted in greater cellular reduction was the simultaneous applications of silver dihydrogen citrate and UV-C light. Produce farmers may be able to apply these mitigation strategies to prevent pathogens from entering their products. This research also shows how important it is to select harvesting and handling materials that can be easily sanitized. Materials with numerous textural discrepancies may lead to biofilms that are more resistant to sanitizers as observed with biofilms grown on wood. Produce growers may use the results of this study to make informed decisions on the type of materials they should use for handling their fresh products.

5.6 Figures and Tables

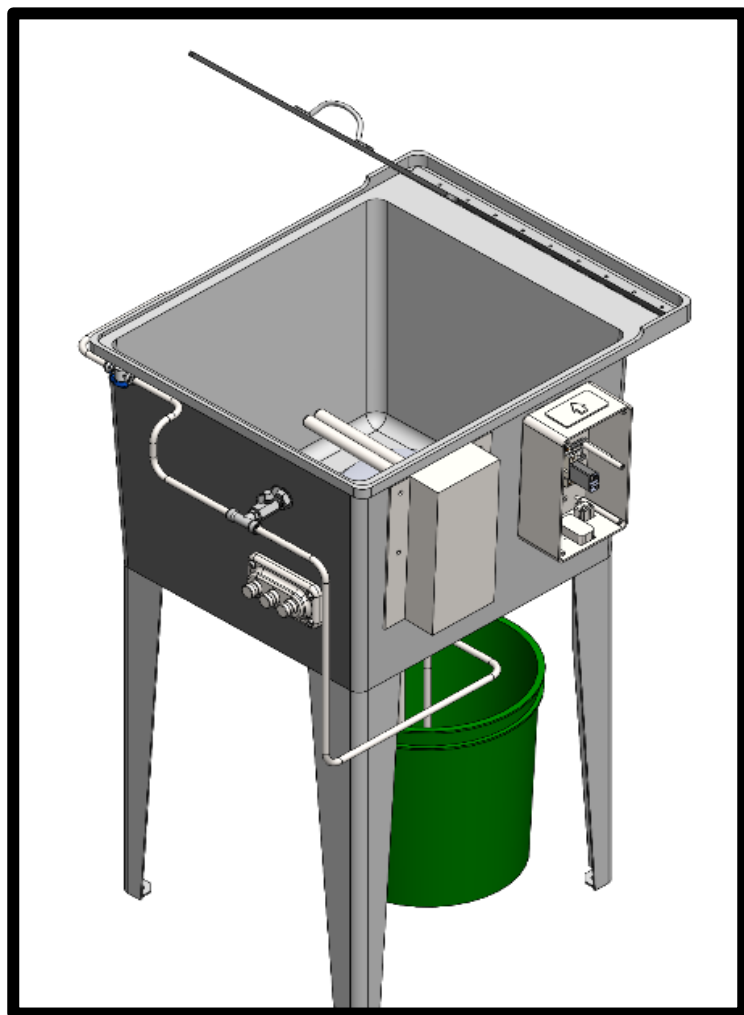


Figure 5.1 Schematic of the scaled lab device equipped with a UV-C lamp and commercial spray system.

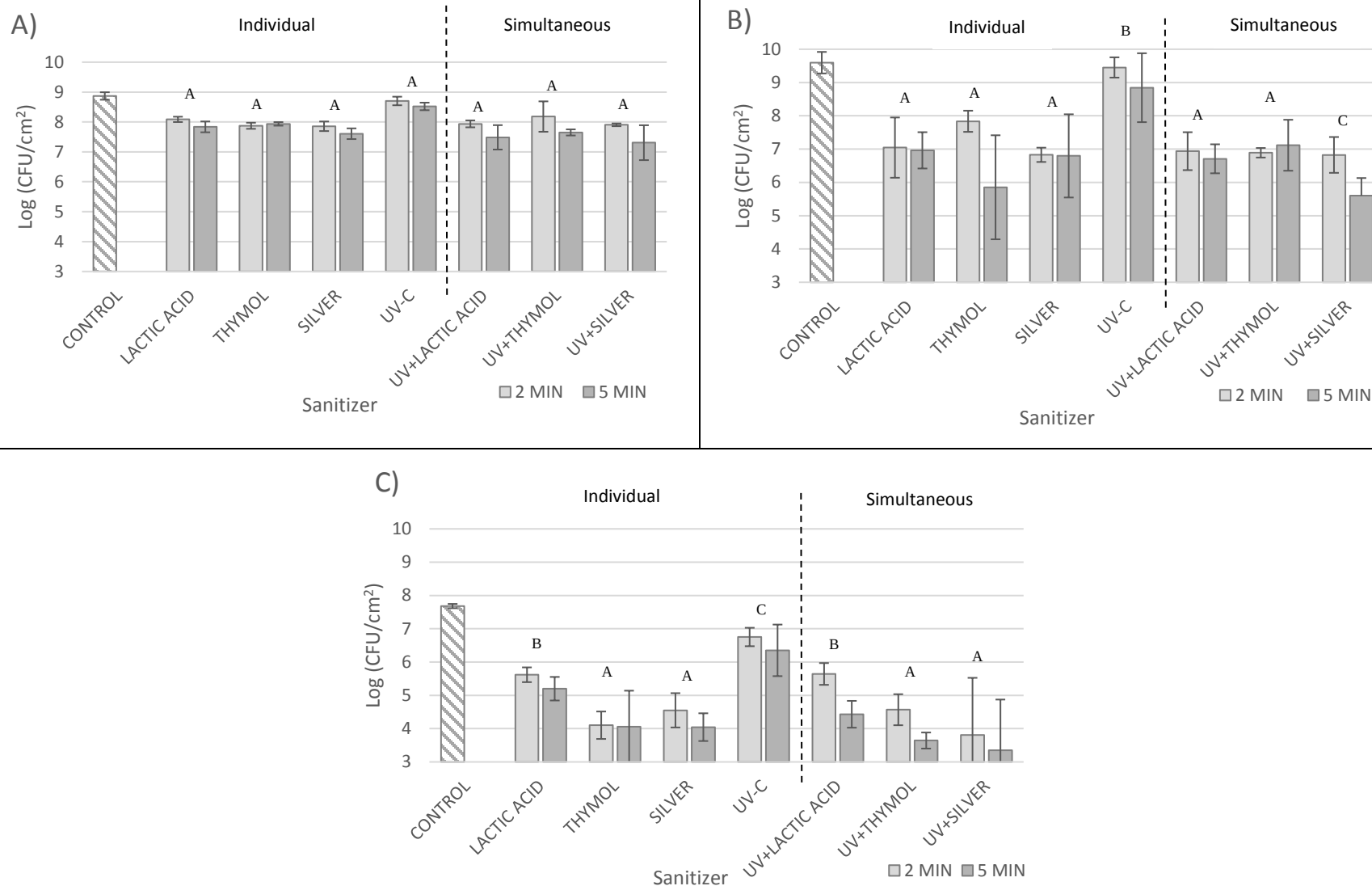


Figure 5.2 Log CFU/cm² of recovered cells on (A) wood, (B) nylon, and (C) polycarbonate after exposure to treatments (lactic acid, silver citrate, UV-C light and the simultaneous use of UV-C light and a chemical sanitizer). Capital letter compares within individual and simultaneous treatments for the same material.

Table 5.1 Statistical output of type 3 tests of fixed effects comparing experimental factors.

Effect	Pr > F
Sanitizer	<0.0001
Time	0.0007
Sanitizer*Time	0.2391
Material	<0.0001
Sanitizer*Material	<0.0001
Time*Material	0.9267
Sanitizer*Time*Material	0.0973

References

- Adhikari, A., Syamaladevi, R. M., Killinger, K., & Sablani, S. S. (2015). Ultraviolet-C light inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on organic fruit surfaces. *International Journal of Food Microbiology*, 210, 136–142. <https://doi.org/10.1016/j.ijfoodmicro.2015.06.018>
- Archer, D. L. (2018). The evolution of FDA's policy on *Listeria monocytogenes* in ready-to-eat foods in the United States. *Current Opinion in Food Science*, 20, 64–68. <https://doi.org/10.1016/j.cofs.2018.03.007>
- Aryal, M., & Muriana, P. M. (2019). Efficacy of Commercial Sanitizers Used in Food Processing Facilities for Inactivation of *Listeria monocytogenes*, *E. Coli* O157:H7, and *Salmonella* Biofilms. *Foods*, 8(12), 639. <https://doi.org/10.3390/foods8120639>
- ASTM. (2019). E2871-19 Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method. ASTM International. <https://doi.org/10.1520/E2871>
- Ban, G.-H., Park, S.-H., Kim, S.-O., Ryu, S., & Kang, D.-H. (2012). Synergistic effect of steam and lactic acid against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. *International Journal of Food Microbiology*, 157(2), 218–223. <https://doi.org/10.1016/j.ijfoodmicro.2012.05.006>
- Bang, J., Hong, A., Kim, H., Beuchat, L. R., Rhee, M. S., Kim, Y., & Ryu, J.-H. (2014). Inactivation of *Escherichia coli* O157:H7 in biofilm on food-contact surfaces by sequential treatments of aqueous chlorine dioxide and drying. *International*

- Journal of Food Microbiology*, 191, 129–134.
<https://doi.org/10.1016/j.ijfoodmicro.2014.09.014>
- Berdejo, D., Pagán, E., García-Gonzalo, D., & Pagán, R. (2019). Exploiting the synergism among physical and chemical processes for improving food safety. *Current Opinion in Food Science*, 30, 14–20.
<https://doi.org/10.1016/j.cofs.2018.08.004>
- Bolton, J. R., & Linden, K. G. (2003). Standardization of Methods for Fluence (UV Dose) Determination in Bench-Scale UV Experiments. *Journal of Environmental Engineering*, 129(3), 209–215. [https://doi.org/10.1061/\(ASCE\)0733-9372\(2003\)129:3\(209\)](https://doi.org/10.1061/(ASCE)0733-9372(2003)129:3(209))
- Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, 94(3), 223–253.
<https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>
- Desai, M. A., Soni, K. A., Nannapaneni, R., Schilling, M. W., & Silva, J. L. (2012). Reduction of *Listeria monocytogenes* Biofilms on Stainless Steel and Polystyrene Surfaces by Essential Oils†. *Journal of Food Protection*, 75(7), 1332–1337.
<https://doi.org/10.4315/0362-028X.JFP-11-517>
- Ding, Q., Alborzi, S., Bastarrachea, L. J., & Tikekar, R. V. (2018). Novel sanitization approach based on synergistic action of UV-A light and benzoic acid: Inactivation mechanism and a potential application in washing fresh produce. *Food Microbiology*, 72, 39–54. <https://doi.org/10.1016/j.fm.2017.11.004>
- Fagerlund, A., Heir, E., Møretrø, T., & Langsrud, S. (2020). *Listeria Monocytogenes* Biofilm Removal Using Different Commercial Cleaning Agents. *Molecules*, 25(4), 792. <https://doi.org/10.3390/molecules25040792>

- Freitag, N. E., Port, G. C., & Miner, M. D. (2009). *Listeria monocytogenes*—From saprophyte to intracellular pathogen. *Nature Reviews Microbiology*, 7(9), 623–628. <https://doi.org/10.1038/nrmicro2171>
- Gayán, E., Condón, S., & Álvarez, I. (2014). Biological Aspects in Food Preservation by Ultraviolet Light: A Review. *Food and Bioprocess Technology*, 7(1), 1–20. <https://doi.org/10.1007/s11947-013-1168-7>
- Garner, D., & Kathariou, S. (2016). Fresh Produce—Associated Listeriosis Outbreaks, Sources of Concern, Teachable Moments, and Insights. *Journal of Food Protection*, 79(2), 337–344. <https://doi.org/10.4315/0362-028X.JFP-15-387>
- Hua, Z., Korany, A. M., El-Shinawy, S. H., & Zhu, M.-J. (2019). Comparative Evaluation of Different Sanitizers Against *Listeria monocytogenes* Biofilms on Major Food-Contact Surfaces. *Frontiers in Microbiology*, 10, 2462. <https://doi.org/10.3389/fmicb.2019.02462>
- Jahid, I. K., & Ha, S.-D. (2012). A review of microbial biofilms of produce: Future challenge to food safety. *Food Science and Biotechnology*, 21(2), 299–316. <https://doi.org/10.1007/s10068-012-0041-1>
- Kallipolitis, B., Gahan, C. G., & Piveteau, P. (2020). Factors contributing to *Listeria monocytogenes* transmission and impact on food safety. *Current Opinion in Food Science*, 36, 9–17. <https://doi.org/10.1016/j.cofs.2020.09.009>
- Masuku, S. M., Babu, D., Martin, E. M., O'Bryan, C. A., Crandall, P. G., & Ricke, S. C. (2014). Lethality of moist heat and silver dihydrogen citrate sanitizer combinations on *Listeria* spp. Adhered to components of a deli meat slicer. *Food Control*, 44, 227–232. <https://doi.org/10.1016/j.foodcont.2014.03.030>

- McKinney, J., Williams, R. C., Boardman, G. D., Eifert, J. D., & Sumner, S. S. (2009). Dose of UV Light Required To Inactivate *Listeria monocytogenes* in Distilled Water, Fresh Brine, and Spent Brine. *Journal of Food Protection*, 72(10), 2144–2150. <https://doi.org/10.4315/0362-028X-72.10.2144>
- Mendez, E., Walker, D. K., Vipham, J., & Trinetta, V. (2020). The use of a CDC biofilm reactor to grow multi-strain *Listeria monocytogenes* biofilm. *Food Microbiology*, 92, 103592. <https://doi.org/10.1016/j.fm.2020.103592>
- Pure Bioscience, Inc. (2011). Petition to the national program to add silver dihydrogen citrate to the national list of allowed and prohibited substances. <https://www.ams.usda.gov/sites/default/files/media/CompletePetitionSilverDihydrogenCitrate.pdf>
- Ravanat, J.-L., Douki, T., & Cadet, J. (2001). UV damage to nucleic acid components. In *Comprehensive Series in Photosciences* (Vol. 3, pp. 207–230). Elsevier. [https://doi.org/10.1016/S1568-461X\(01\)80045-4](https://doi.org/10.1016/S1568-461X(01)80045-4)
- Schoder, D., Melzner, D., Schmalwieser, A., Zangana, A., Winter, P., & Wagner, M. (2011). Important Vectors for *Listeria monocytogenes* Transmission at Farm Dairies Manufacturing Fresh Sheep and Goat Cheese from Raw Milk. *Journal of Food Protection*, 74(6), 919–924. <https://doi.org/10.4315/0362-028X.JFP-10-534>
- Silva-Espinoza, B. A., Palomares-Navarro, J. J., Tapia-Rodriguez, M. R., Cruz-Valenzuela, M. R., González-Aguilar, G. A., Silva-Campa, E., Pedroza-Montero, M., Almeida-Lopes, M., Miranda, R., & Ayala-Zavala, J. F. (2020). Combination of ultraviolet light-C and clove essential oil to inactivate *SALMONELLA*

- Typhimurium* biofilms on stainless steel. *Journal of Food Safety*, 40(3).
<https://doi.org/10.1111/jfs.12788>
- Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control*, 31(2), 572–585.
<https://doi.org/10.1016/j.foodcont.2012.12.001>
- Tajik, H., Naghili, H., Ghasemmahdi, H., Moradi, M., & Badali, A. (2015). Effects of *Zataria multiflora* boiss essential oil, ultraviolet radiation and their combination on *Listeria monocytogenes* biofilm in a simulated industrial model. *International Journal of Food Science & Technology*, 50(9), 2113–2119.
<https://doi.org/10.1111/ijfs.12874>
- Townsend, A., Strawn, L. K., Chapman, B. J., & Dunn, L. L. (2021). A Systematic Review of *Listeria* Species and *Listeria monocytogenes* Prevalence, Persistence, and Diversity throughout the Fresh Produce Supply Chain. *Foods*, 10(6), 1427.
<https://doi.org/10.3390/foods10061427>
- U.S. Food and Drug Administration (FDA) 2022. <https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-listeria-monocytogenes-dole-packaged-salad-december-2021>
- Yang, H., Kendall, P. A., Medeiros, L. C., & Sofos, J. N. (2009). Efficacy of Sanitizing Agents against *Listeria monocytogenes* Biofilms on High-Density Polyethylene Cutting Board Surfaces. *Journal of Food Protection*, 72(5), 990–998.
<https://doi.org/10.4315/0362-028X-72.5.990>

- Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The Microbial “Protective Clothing” in Extreme Environments. *International Journal of Molecular Sciences*, 20(14), 3423. <https://doi.org/10.3390/ijms20143423>
- Zhang, H., HuangFu, H., Wang, X., Zhao, S., Liu, Y., Lv, H., Qin, G., & Tan, Z. (2021). Antibacterial Activity of Lactic Acid Producing *Leuconostoc mesenteroides* QZ1178 Against Pathogenic *Gallibacterium anatis*. *Frontiers in Veterinary Science*, 8, 630294. <https://doi.org/10.3389/fvets.2021.630294>
- Zhu, Q., Gooneratne, R., & Hussain, M. (2017). *Listeria monocytogenes* in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. *Foods*, 6(3), 21. <https://doi.org/10.3390/foods6030021>