

Understanding *Salmonella* biofilm formation and sanitizer efficacy in the produce industry

by

Colton Ivers

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Approved by:

Major Professor
Valentina Trinetta, Ph.D.

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Abstract

In recent years, *Salmonella* has caused multiple foodborne outbreaks linked to fresh produce. The ability of *Salmonella* to form biofilms is predicted to be a strategy to increase persistence and survival. When harvesting equipment is not effectively cleaned and sanitized, microorganisms can persist and potentially cross-contaminate produce. Preventing contamination during harvest is therefore important to ensure food safety. The objectives of this study were to: 1) study attachment properties of *Salmonella* biofilms on nylon, HDPE, and wood; and 2) evaluate the efficacy of five commercially available sanitizers to control *Salmonella* biofilms on experimentally inoculated coupons representative of harvesting bins and picking bags.

For objective 1, multi-strain *Salmonella* biofilms were grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor at $22 \pm 2^\circ\text{C}$ on nylon, HDPE, and wood coupons for up to 96-hour. Cell attachment and biofilm hydrophobicity were evaluated by a rinse assay and contact angle measurements, respectively. Laser Scanning Confocal Microscopy (LSCM) was utilized to visualize biofilm formation and architecture. Nylon and wood surfaces showed higher hydrophilic properties as compared to HDPE and resulted in greater *Salmonella* attachment ($P < 0.05$).

For objective 2, multi-strain *Salmonella* biofilms were grown on coupons following the protocol developed in objective 1. Biofilms were exposed to 500 ppm free chlorine, 500 ppm peroxyacetic acid, 75psi steam, 5% silver dihydrogen citrate (SDC) for 1 or 2 minutes and 100 ppm chlorine dioxide gas for 24-hour. After treatment, coupons were neutralized, and surviving cells were enumerated. Nylon, wood, and HDPE control coupons resulted in initial *Salmonella* biofilm populations of 9.59 ± 0.29 , 9.34 ± 0.40 , and 8.77 ± 0.34 log CFU/coupon, respectively.

Overall sanitizer, surface type, and application time significantly ($P<0.05$) impacted the remaining *Salmonella* population. All treatments resulted in a significant reduction of *Salmonella* cells when compared to the control ($P<0.05$). Chlorine dioxide gas was the only sanitizer to completely inactivate *Salmonella* biofilm on all surfaces tested.

Overall, the results obtained in this research highlight the importance of linking the physiochemical and attachment characteristics of biofilms with the antimicrobial properties of sanitizers in order to control *Salmonella* biofilm formation on harvesting equipment commonly used in the produce industry.

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Dedication

I dedicate this thesis to my parents and siblings who have supported and inspired me my entire life; to my friends who became family during my time here at K-State. Thank you.

Chapter 1 - Literature Review

1.1 Foodborne diseases and *Salmonella*

Six hundred million people fall ill due to contaminated food annually (Havelaar et al., 2015). Children under the age of 5 years old account for 40% of foodborne disease-related deaths worldwide (Havelaar et al., 2015); in the United States, 48 million cases are associated with people becoming sick from foodborne outbreak episodes annually (CDC, 2011).

Among foodborne pathogens, *Salmonella* is the largest economic burden and leading cause of hospitalizations and deaths linked to 19,500 hospitalizations and 378 deaths annually in the United States (Hoffmann et al., 2021; Scallan et al., 2011). *Salmonella* infections continue to stay unchanged in recent years (CDC, 2011). Foodborne outbreaks caused by *Salmonella* are most associated with poultry products followed by fruit products (CDC, 2019). Fresh produce has been linked to multiple *Salmonella* outbreaks (Bennett et al., 2018; CDC, 2023). In 2020 peaches contaminated with *Salmonella* enteritidis resulted in 101 confirmed cases and 28 hospitalizations across 17 states (CDC, 2021). In 2008, jalapeno peppers, serrano peppers and tomatoes were contaminated with *Salmonella* Saintpaul and caused 1,442 confirmed infection cases across 48 states, the District of Columbia, and Canada (CDC, 2008). Although *Salmonella* infections can occur through various routes, 86% of the cases are linked to contaminated food (Majowicz et al., 2010). These infections commonly occur through the fecal oral route and are usually attributed to poor sanitation (Marriott & Gravani, 2006; Ohl & Miller, 2001).

Salmonellosis is a food infection resulting from the ingestion of the pathogen. The endotoxins produced by *Salmonella* irritates the intestinal wall and can cause nausea, vomiting, and diarrhea (Marriott & Gravani, 2006). Symptoms associated with salmonellosis generally exhibit within 8 to 72 hours and may last 1 or 2 days (Etienne, 2006).

The pathogenicity of *Salmonella* relies on its ability to invade human epithelial cells, survive, and replicate intracellularly (Eng et al., 2015). Pathogenicity island-1 (SPI-1) and pathogenicity island-2 (SPI-2) encode type III secretion systems that are critical for the invasion of host cells, intracellular survival, and replication (Wang et al., 2020). The type III secretion system encoded by SPI-1 injects effector proteins into the host cell's cytoplasm which promotes *Salmonella* invasion (Wang et al., 2020; Eng et al., 2015). Then a distinct type III secretion system, encoded by SPI-2, secretes effectors into the host cytosol that alter the structure of the host vacuole preventing the fusion of lysosomes thus, enabling *Salmonella* to survive and replicate intracellularly (Eng et al., 2015; Waterman & Holden, 2003). This process induces a typical acute inflammatory response resulting in diarrhea, fever, and abdominal pain (Eng et al., 2015). Most cases of salmonellosis caused by non-typhoidal *Salmonella* remain restricted to the intestine; however, in severe cases, usually infants and immunocompromised patients, the infection can become systemic and infect other organs (Eng et al., 2015).

The classification of *Salmonella* includes two main species: *Salmonella bongori* and *Salmonella enterica* (Löfström et al., 2016). *Salmonella enterica* comprises 6 subspecies: *S. enterica* ssp. *enterica* (I), *S. enterica* ssp. *salamae* (II), *S. enterica* ssp. *arizonae* (IIIa), *S. enterica* ssp. *diarizonae* (IIIb), *S. enterica* ssp. *houtenae* (IV), and *S. enterica* ssp. *indica* (VI). *S. enterica* ssp. *enterica* (I) is responsible for 99% of human and animal infections whereas other subspecies are found in cold blooded animals and rarely result in salmonellosis in humans (Löfström et al., 2016; Lamas et al., 2018). Additionally, *S. enterica* ssp. *I enterica* is divided into typhoidal *Salmonella* and nontyphoidal *Salmonella*. Nontyphoidal *Salmonella* serovars generally cause gastroenteritis whereas typhoidal *Salmonella* serovars (Typhi and Paratyphi) cause typhoid fever.

The remainder of this chapter will focus on *Salmonella enterica* ssp. I *enterica* nontyphoidal strains as they account for most foodborne infections (Scallan et al., 2011).

1.2 *Salmonella* in the produce industry

Fresh produce can act as vector of transmission for *Salmonella* (Lamas et al., 2018) and represents a food safety concern as these commodities are often consumed raw (Fatica & Schneider, 2011). From 1998 to 2013, in the United States, there were 972 foodborne outbreaks associated with raw produce and 21% were attributed to *Salmonella*. (Bennett et al., 2018). The percentage of all foodborne outbreaks contributed to raw produce increased from 8% during 1998 to 2001 to 16% during 2010 to 2013 (Bennett et al., 2018). Many factors may play a role in the proportional increase in raw produce outbreaks such as increased consumption, distribution, surveillance, consumer behavior, and larger scale production (Olaimat & Holley, 2012).

Although there are various interventions that can reduce populations of pathogenic microorganisms on raw produce, preventing contamination is critical to further reduce risk (Etienne 2006; Beuchat & Ryu, 1997). Produce can be contaminated at numerous points in the food supply chain such as pre-harvest, post-harvest, and processing (Beuchat & Ryu, 1997). Therefore, it is important to highlight these potential sources to control and prevent contamination. Spreading livestock/poultry manure on soil is a common practice in agriculture to recycle this waste product and improve soil fertility; however, enteric pathogenic microorganisms are commonly present in manure and may contaminate soil, irrigation water, and subsequently, produce (Islam et al., 2004). Furthermore, *Salmonella* has been shown to survive for over 300 days in manure-amended soil (You et al., 2006) and persist on lettuce and parsley plants for up to 63 and 161 days, respectively when grown in the presence of inoculated compost (Islam et al., 2004). Irrigation water also serves as a potential source of contamination and has

been linked to multiple outbreaks in produce (Fatica & Schneider, 2011; Patel et al., 2013; Liu et al., 2018). In 2002 tomatoes contaminated with *Salmonella* Newport caused 510 illnesses in 26 states and later in 2005, the same strain caused 72 illnesses in 16 states. Traceback efforts isolated the outbreak strain to pond water used for irrigation (Greene et al., 2007). A study conducted by Patel et al. (2013) demonstrated that *Salmonella* could persist on spinach leaves up to 7 days after being contaminated via irrigation water. Pre-harvest contamination can potentially favor the entrance and introduce pathogens into processing facilities (Wang & Ryser, 2014). Produce processing facilities use various systems such as rollers, conveyors, and totes to wash and pack produce. This equipment can serve as contamination points, if not adequately cleaned and sanitized. Newman et al. (2017) evaluated the microbial load of produce equipment in packing facilities by utilizing indicator organisms. Interestingly, the study included harvest bins used to transport produce from the field to packing house. Coliforms were present on 22% of harvest bins. The highest prevalence of coliforms was found on conveyor belts: 56% tested positive. *Salmonella* was found on 2.1% of packing house equipment sampled (Duffy et al., 2005). Wang & Ryser, (2014) demonstrated that plastic rollers contaminated with *Salmonella* cross-contaminated 96% of tomatoes whereas, brush rollers cross contaminated only 8% of tomatoes. Another study conducted by Allen et al. (2005) inoculated stainless steel, PVC, sponge rollers, conveyor belts, and unfinished oak wooden surfaces with a five-serovar *Salmonella* cocktail and conducted a challenge study for 28 days under two different temperature and relative humidity conditions to represent Florida's fall/winter and late spring tomato production seasons. The study concluded *Salmonella* was able to persist on surfaces such as stainless steel, PVC, and unfinished oak for an extended period. Interestingly, the study also suggested the properties that make a surface easy to clean may also make the surface more likely to transfer

pathogens. Furthermore, Moore et al. (2007) investigated the transfer efficacy of *Salmonella* across stainless steel, Formica (laminated), polypropylene, and wooden surfaces. Stainless steel and Formica surfaces transferred a significantly greater amount of *Salmonella*, as compared to polypropylene and wood.

1.3 *Salmonella* biofilms

Salmonella has the ability to form and develop biofilms on food contact surfaces common in processing environments (Joseph et al., 2001; Patel et al., 2013; Byun et al., 2021). A biofilm is defined as a group of organized cells attached to a surface and bound together by the Extracellular Polymeric Substances (EPS) (Donlan, 2002). This complex microenvironment expresses unique properties and characteristics that cannot be understood through the study of planktonic cells (Flemming et al., 2016). Cells within a biofilm may experience reduced growth rates, up and down regulation of specific genes, gene exchange, quorum sensing, and increased tolerance and or resistance to environmental stressors such as antibiotics and sanitizers (Donlan, 2002; Flemming et al., 2016). The life cycle of biofilms is generally viewed as cyclical and consists of five stages: initial attachment, irreversible attachment, development, maturation, and dispersion (Stoodley et al., 2002). Initial bacterial attachment can be highly influenced by the physical and chemical properties of the environment and bacterial cell (Palmer et al., 2007, Wang et al., 2011). Environmental factors that influence bacterial attachment may include shear force, surface and cell hydrophobicity, surface conditioning and roughness, pH, and nutrient levels (Donlan, 2002; Palmer et al., 2007). In this initial stage bacterial cells can easily detach or be removed from the surface. Irreversible attachment occurs when cells begin to form a matrix primarily composed of EPS (Palmer et al., 2007; Rumbaugh & Sauer, 2020, Flemming et al., 2016). Moreover, the EPS matrix provides necessary structure to stabilize the biofilm and

interact with the surface to form a robust connection (Flemming et al., 2016). At this point cells are firmly attached to the surface and cannot be easily removed. Interestingly, Stoodley et al. (2002) observed that single cells and microcolonies exhibit flagellar motility and continuous detachment in mature biofilms and the term irreversible attachment refers only to the robust surface attachment. After irreversible attachment, the biofilm continues to develop the complex structure through the creation of channels and pores (Stoodley et al., 2002; Rumbaugh & Sauer, 2020). The development of biofilm structure, specifically the EPS matrix, allows the biofilm community to enhance cell to cell communication and to increase tolerance/resistance to adverse environmental conditions such as the exposure of sanitizers and antimicrobials (Flemming et al., 2016). The EPS matrix serves as a barrier to inactivate and or slow the diffusion of antimicrobials and is suggested to enhance antimicrobial resistance in biofilm cells (Flemming et al., 2016). Cellular gene regulation and expression are different in biofilms compared to planktonic cells (Stoodley et al., 2002): a study conducted with *P. aeruginosa* observed substantial differences of expression in up to 50% of detectable proteins between planktonic and biofilm samples (Sauer et al., 2022). Speranza et al., (2011) evaluated the effect of temperature, pH, and nutrient concentration on *Salmonella* Typhimurium biofilm formation on stainless steel. The objective was to understand the impact of some environmental factors *Salmonella* faces in food processing facilities. Temperature ranged from 20 to 40°C, pH from 4.5 to 7.5, and buffered peptone from 0.5 to 2.5 g/L. The aptitude of biofilm formation (ABF) was defined as the time necessary to start adhering to the stainless steel. *Salmonella* attached to stainless steel surfaces most rapidly when temperature was close to 30°C, pH close to 6.0, and reduced media (1.0 to 1.5g/l). However, maximum biofilm formation occurred under slightly different conditions (25°C; 6.6pH; BP 1.0g/l) and (30°C; pH 5.9; BP 0.5g/l). Nevertheless, *Salmonella* biofilm

formation was favored when temperature was near 30°C, close to neutral pH, and media devoid of nutrients. Additionally, *Salmonella* strain affects the impact of pH, NaCl concentrations, and temperature on biofilm formation (Lianou & Koutsoumanis, 2012). Biofilm formation differences among strains was more apparent as environmental conditions became less favorable (Lianou & Koutsoumanis, 2012). Moreover, Vestby et al. (2009) reported that biofilm formation on polystyrene microtiter plates at 20°C was significantly impacted by *Salmonella* serovar. *Salmonella* Agona and Montevideo resulted in significantly more biofilm formation than *Salmonella* Senftenberg and Typhimurium (Vestby et al., 2009). Furthermore, Patel et al. (2013) observed biofilm formation differences between *Salmonella* serovars isolated from produce when grown on polycarbonate surfaces; *Salmonella* Thompson resulted in significantly greater biofilm formation than *Salmonella* Newport and Negev. Patel et al. (2013) also reported *Salmonella* isolated from produce sources resulted in greater biofilm formation than those from poultry sources. Overall, Obe et al. (2022) concluded biofilm formation can vary between different *Salmonella* serovars.

1.4 Biofilm models

Consistent *in vitro* methods to develop biofilms are necessary to evaluate and understand biofilm properties and characteristics. Currently, there is a wide range of biofilm models each with specific attributes that can influence biofilm development. Therefore, it is imperative to understand the advantages and limitations of the various system. The distinction between batch, continuous, and semi-continuous models is important to clarify; batch models provide a fixed amount of nutrients in a confined vessel, while continuous and semi-continuous models utilize fluid flow to add nutrients periodically or consistently to the environment.

The suspended substratum models can be utilized in batch and or continuous phases. Under static conditions, this model relies on planktonic cells to form a biofilm at solid-liquid interface (McBain, 2009). Culture and the desired substratum are added to a sterile vessel and incubated for the desired time. The construction of this model is simple, cost effective, and can be used to evaluate biofilm properties on a wide range of surfaces. Wang et al. (2020) and Yang et al. (2017) utilized a similar model, under static conditions, to grow *Salmonella* biofilms on stainless steel coupons to evaluate the impact of sanitizer treatments on *Salmonella* biofilms. However, it must be noted that this model is limited in terms of its ability to simulate the natural environment, since it does not simulate the movement of bulk liquid and shear force conditions bacteria may experience in processing environments.

The Center for Disease Control and Prevention (CDC) biofilm reactor is a type of suspended substratum model and was developed by CDC researchers (Johnson et al., 2021) (Figure 1.1). This specific model is comprised of a vessel, eight removable rods, stir baffle, import valve, and waste valve. Additionally, each rod can hold three 1.3-cm-diameter coupons; furthermore, rods can be removed independently of each other without disrupting biofilm formation on the remaining rods. Additionally, this design enables researchers to evaluate different materials at once and control shear force and fluid dynamics (Gomes et al., 2018). Materials such as stainless steel, nylon, Teflon, glass, and plastics manufactured by Biosurface Technologies (Bozeman, MT) are commercially available to use in this system. The CDC biofilm reactor is commonly used to develop robust biofilms and evaluate sanitizers and disinfectants (Gomes, et al., 2018). The CDC biofilm reactor is the only biofilm model recommended by the EPA to test disinfectants with claims against bacterial biofilms (MB19-06, MB-20-04) (EPA, 2023). Specifically, in the medical industry the CDC bioreactor mimics the

shear force conditions of catheters and implantable cardiac devices (Gomes, et al., 2018).

Mendez et al. (2020) confirmed that the CDC bioreactor produces robust, repeatable results under similar conditions of natural environments and industrial systems. However, this system is labor and capital intensive and can only evaluate one inoculum at a time.

Microtiter plates are a time efficient method for high-throughput analysis (Figure 1.2). This system is the most used model to study biofilm formation (Azeredo et al. 2017). The model allows for a large quantity of biofilms to be grown rapidly under different conditions such as strain, temperature, pH, and nutrient (Coffey et., al 2014; Djordjevic et al., 2002; Gomes et al., 2018). Growth media and bacteria are added to the wells and incubated. Bacteria then adhere to the surface in the wells and form a biofilm. The microtiter plate is rinsed to remove unattached bacteria. Then crystal dye is added to visualize the biofilm and perform optical density measurements. Cells can also be directly quantified through detachment and plating (Azeredo et al., 2017). Nevertheless, microtiter plates are limited to batch mode experiments, surface substrates, and early-stage biofilm formation (Azeredo et al., 2017).

Flow cell models and the Modified Robbins model utilize the flow of liquid culture in a tube or cell to generate biofilms on coupons or pegs (Figure 1.3 and Figure 1.4) (McBain, 2009). These models can be used to visualize biofilm formation in real time (McBain, 2009). Coupons are placed in the flow cell and media is pumped through the flow cell continuously (McBain, 2009). Flow cell devices utilize a glass coverslip to close the system and allow a microscope to be mounted and image the biofilm over an extended period without disturbing the biofilm (McBain, 2009). The Modified Robbins device is a pipe designed with removable pegs that does not disturb the flow characteristics and can operate from laminar to turbulent flow (McBain, 2009). This model has been used to simulate the environment of industrial pipe systems and it

can sustain biofilm growth for several weeks (McBain, 2009; Azeredo et al., 2017). However, previous knowledge of flow dynamics and stabilization time is needed to compare biofilm formation between coupons (Azeredo et al., 2017).

1.5 Cleaning and sanitation

As the food industry continues to expand into a globalized food system, food safety practices such as cleaning and sanitation are critically important to ensure consumer safety. Cleaning is the “physical removal of soil from surfaces which can include the use of water and detergent” (Produce Safety Alliance, 2022). Cleaning is an important pre-step for sanitation, as dirty surfaces cannot be effectively sanitized. The cleaning process is important to help minimize biofilm formation. The optimization of cleaning results in enhanced sanitizer efficacy and biofilm control (Grinstead, 2009). Interestingly, soils differ in composition and respond to various cleaning compounds differently (Grinstead, 2009). Biofilms are mostly made up of carbohydrates, proteins, lipids, and nucleic acids. Grinstead (2009) suggested that biofilms should be treated as additional soil that needs to be cleaned.

Nevertheless, the biofilm formation capabilities of *Salmonella* create unique sanitation challenges. Wang et al., (2017), isolated 89 strains of *Salmonella enterica* from beef trim and evaluated biofilm formation, antimicrobial resistance, and biofilm cell survival/recovery post sanitizer exposure. Biofilm formation is highly strain dependent and in this study 85% of the strains were classified as strong or superior biofilm formers (Wang et al., 2017). Differences within *Salmonella* serovar were also noted as a majority of *Salmonella* Dublin strains were classified as weak to moderate biofilm formers whereas all *Salmonella* Newport, Montevideo, and Typhimurium strains were classified as strong or superior biofilm formers (Wang et al., 2017). Strong and superior biofilm formers experienced significantly higher survival and

recovery post sanitation than moderate and weak biofilm formers when exposed to chlorine, ClO₂, or quaternary ammonium compound (QAC) based sanitizer (Wang et al., 2017). Chylkova et al. (2017) reported that *Salmonella* biofilms were 2 to 16 times more tolerant to PAA than their planktonic forms. Therefore, the ability of *Salmonella* to form strong biofilms could impact sanitizer efficacy.

1.5.1 Chlorine dioxide

Chlorine dioxide (ClO₂) is a strong oxidizer with broad-spectrum antimicrobial activity (Marriott & Gravani, 2006; Gomez & Vicente, 2012). The primary mode of action relies on oxidizing compounds to disrupt the cellular membrane and cellular respiration. ClO₂ is sensitive to light, humidity, temperature, and poses potential safety and toxicity risks for users (Han et al., 2001; Marriott & Gravani, 2006; Gomez & Vicente, 2012). ClO₂ can be applied in either gas or aqueous forms and it has been successfully used to control pathogens in various food-processing environments. Aqueous solutions intended for sanitizing food processing equipment and utensils must not exceed 200 ppm chlorine dioxide (40 CFR 180.940). The gaseous application of chlorine dioxide presents various advantages compared to aqueous application. ClO₂ gas can penetrate the biofilm matrix and inactivate biofilms on food contact surfaces and leaves less residual ClO₂ (Nam et al., 2014). This was further demonstrated by Kim & Park (2022) who observed complete inactivation of *Salmonella* biofilms when ClO₂ gas, at a concentration of 50ppmv, was applied for 20 minutes. Similarly, *Salmonella* biofilms grown on stainless steel and plastic treated with 100ppm ClO₂ for one-minute were reduced below the detectable limit and resulted in a minimum reduction of 5.20 log CFU/cm² and 5.49 log CFU/cm², respectively (Byun et al., 2021). The effectiveness of ClO₂ gas may be due to its ability to penetrate the biofilm matrix (Nam et al., 2014; Prodduk et al., 2014). Additionally, ClO₂ has been proven

effective on reducing *Salmonella* on the surface of produce (Mahmoud & Linton, 2008; Trinetta et al., 2010). Tomatoes treated with ClO₂ gas for 180-second resulted in nearly a 5 log CFU/cm² reduction of *Salmonella* (Trinetta et al., 2010). Interestingly, no negative effects on color were observed on tomatoes treated with ClO₂ gas whereas bleaching has been observed on other produce such as strawberry and lettuce leaves (Trinetta et al., 2010).

1.5.2 Silver dihydrogen citrate (SDC)

Silver dihydrogen citrate (SDC) is a broad spectrum, non-toxic, and ready to use antimicrobial manufactured by Pure Bioscience (El Cajon, CA). SDC must be applied for a minimum of 1-minute for sanitizing food contact surfaces following product label instructions (Pure Bioscience, Inc., 2016). SDC utilizes silver ions as the main antimicrobial compound, that work towards the disruption of the cellular membrane and DNA (Pure Bioscience, Inc., 2016; Mijndonckx et al, 2013). Silver ions bind with sulfhydryl groups which block respiration and electron transfer, thus effectively restricting the proton motive force and causing the cellular membrane to break down (Gerba, 2013). The phenomenon allows silver ions to enter the cell and interfere with the DNA replication by forming complexes with nucleic acids (Gerba, 2013). Silver ions also promote the formation of Reactive Oxygen Species (ROS) damaging proteins, DNA, RNA, and lipids. The production of ROS in the cell is highly toxic (Mijndonckx et al, 2013). Additionally, bacterial cells may recognize the citrate compound as a food source and pump the compound into the cell (Pure Bioscience Inc., 2016). However, residual water from cleaning has the potential to lower the effectiveness of SDC as a sanitizer (Pure Bioscience Inc., 2016). Chloride is commonly found in water at levels of 50-100ppm can interact with the silver ions present in SDC to form less soluble silver salts which are much less effective at controlling pathogens (Mijndonckx et al, 2013; Pure Bioscience Inc., 2016) According to the SDC

technical data sheet, 1ppm of chloride can bind up to 3ppm silver (Pure Bioscience, Inc., 2016). Limited research has evaluated the efficacy of SDC on *Salmonella*. Nevertheless, SDC achieved around a 5 log CFU/cm² reduction of *Listeria monocytogenes* when applied to stainless steel and cast aluminum surfaces for 2-minute (Masuku et al., 2014). Mendes-Oliveira et al. (2022) evaluated the effectiveness of a novel silver dihydrogen citrate, glycerin, and lactic acid (SGL) based sanitizer to control *Escherichia coli* on leafy greens during washing. SGL resulted in a 3.15 log reduction of *E. coli* post wash (Mendes-Oliveira et al., 2022). Manuel et al. (2017) studied the effectiveness of SDC on human norovirus on stainless steel. Moreover, after a 30-minute application SDC achieved 2.0 and 3.0 log₁₀ RNA copy number reductions for strains GI.6 and GII.4. When SDC was applied to the same strains in the presence of soil it did not reduce RNA copy number regardless of application time. The study concluded that SDC exhibits efficacy towards human norovirus in the absence of soil. Nevertheless, previous research suggests that SDC may be an effective, non-toxic, antimicrobial in the absence of soil.

1.5.3 Peroxyacetic acid (PAA)

Peroxyacetic acid (PAA) is a strong oxidizer with a broad antimicrobial spectrum (Gomez & Vicente, 2012). It is hypothesized that PAA denatures membrane proteins and increases permeability (Finnegan et al., 2010). The increase in permeability allows PAA molecules to freely enter the cell and oxidize proteins, enzymes, and DNA resulting in cell death (Finnegan et al., 2010). Commercially available PAA products contain an equilibrium of acetic acid, hydrogen peroxide and peracetic acid (EPA, 2012). PAA is environmentally friendly as it does not form harmful by-products and rapidly dissociates into acetic acid and hydrogen peroxide (EPA, 2012). PAA is also minimally affected by pH and organic soils compared to chlorine (Marriott & Gravani, 2006). Peroxyacetic acid can be applied to food contact surfaces as

a sanitizing solution at a concentration of up to 500ppm according to 40CFR 190.1196.

Nevertheless, manufacturer's label instruction for use must be followed.

Nahar et al. (2022) reported PAA was effective against *Salmonella* biofilms grown on plastic and observed a 3.7 log CFU/cm² reduction. Additionally, Belessi et al. (2011) reported 2% PAA was more effective in controlling *Listeria monocytogenes* biofilms on stainless steel coupons than 0.46% sodium hypochlorite. The same study also observed biofilms grown at 20° C were more resistant to PAA than those grown at 5°C. Singh, et al, (2018) also compared the efficacy of sodium hypochlorite and PAA solutions in postharvest wash water and reported that PAA at 100mg/L was equally or more effective at controlling *Salmonella* Typhimurium on produce than sodium hypochlorite.

1.5.4 Chlorine (sodium hypochlorite)

Chlorine is an inorganic chemical compound, strong oxidizer, relatively inexpensive which has made it a popular antimicrobial in the food industry specifically, the produce industry (Marriott & Gravani, 2006; Rosenblum et al., 2012; Gomez & Vicente, 2012). The antimicrobial activity of chlorine is largely dependent on the amount of hypochlorous acid and pH plays an important role in the amount of hypochlorous acid in the solution: low pH favors hypochlorous acid formation (Gomez & Vicente, 2012; Marriott & Gravani, 2006). Nevertheless, at a pH below 4.0 toxic and corrosive gases can form (Marriott & Gravani, 2006). Although chlorine is more stable at a higher pH, it is more effective as pH decreases due to the increase of hypochlorous acid (Marriott & Gravani, 2006). Therefore, the pH of chlorine sanitizers must be monitored to maintain optimal efficacy and worker safety (Marriott & Gravani, 2006). It is generally recognized that hypochlorous acid disrupts glucose by the oxidation of sulfhydryl groups important to carbohydrate metabolism (Marriott & Gravani, 2006; Gomez & Vicente,

2012). The main limitations of chlorine sanitizers are pH and the presence of organic matter, which significantly decrease sanitizing potential (Gomez & Vicente, 2012). Sodium hypochlorite sanitizing solutions may not exceed 200 ppm available chlorine when applied to food-processing equipment and utensils according to 21 CFR 178.1010.

Chlorine has been shown to be an effective sanitizer to reduce the microbial load in the food industry. Byun et al., 2021 showed that 100ppm chlorine solution with a contact time of one-minute reduced *S. Enteritidis* by more than 5 log CFU/mL under clean conditions. However, under dirty conditions 100ppm chlorine with a contact time of one-minute only reduced *S. Enteritidis* by 1.53 log CFU/mL (Byun et al., 2021). When *S. Enteritidis* biofilms on stainless steel and plastic were treated with 100ppm for one-minute a reduction of 3.77 CFU/cm² and 3.50 CFU/cm² was observed, respectively (Byun et al., 2021).

1.5.5 Steam

Heat is the oldest agent for inactivating microorganisms (CDC, 2019). Steam causes structural and physiological change to the bacterial membrane, RNA, DNA, and proteins resulting in cell death (Russell, 2003). Nevertheless, steam is generally regarded as an expensive and ineffective measure to control pathogens in the food industry as it is highly affected by organic material and may generate condensation (Marriott & Gravani, 2006). Steam has been shown to reduce *Salmonella* by nearly 6.0 log on shelled almonds (Lee et al., 2006). Additionally, superheated steam at 200°C for 30-second reduced *Salmonella* by 5.5 log on pistachios (Ban & Kang, 2016). *Salmonella* biofilms grown on stainless steel and PVC and treated with steam for 20-second resulted in a reduction of 2.52 and 2.12 log, respectively (Ban et al., 2012). Based on the literature no studies evaluated the efficacy of steam on *Salmonella* biofilms grown in a CDC biofilm reactor. However, the effectiveness of steam vapor was

assessed on *E. coli* biofilms grown in a CDC biofilm reactor (Song et al., 2012). Results confirmed that steam may be an effective treatment to reduce biofilms on food contact surfaces, since *E. coli* biofilms on polycarbonate coupons were completely inactivated after 10 second application (Song et al., 2012).

1.6 Conclusion

Based on the Literature review the presence and persistence of *Salmonella* in processing environments can be attributed to many factors, such as its biofilm forming ability and tolerance/resistance to sanitizers. Few studies investigated the effect of commonly used sanitizers on multi-strain *Salmonella* biofilms grown under turbulent conditions and on surfaces other than stainless steel and plastic. Nevertheless, due to the range of models used to develop biofilms it is difficult to accurately compare results from different studies.

Figures and Tables



Figure 1.1 CDC Biofilm Reactor from Biosurface Technologies Corporation (Bozeman, MT).



Figure 1.2 Microtiter plate from Thermo Fisher Scientific (Waltham, MA).



Figure 1.3 Flow cell device from Biosurface Technologies Corporation (Bozeman, MT).

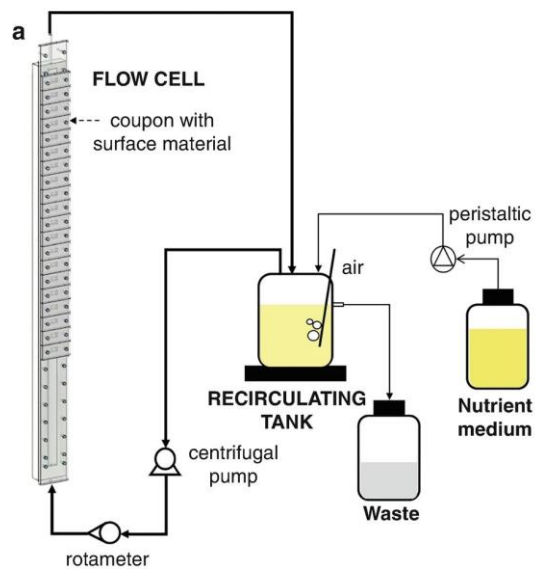


Figure 1.4 Schematic of Modified Robbins device adapted from Gomes et al. (2022)
 (photograph of Modified Robbins device removed)
 (<http://creativecommons.org/licenses/by/4.0/>).

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Chapter 2 - Research Importance

Research regarding sanitizer efficacy on *Salmonella* is plentiful (Byun et al., 2022; Byun et al., 2021; Aryal & Muriana, 2019). Nevertheless, these studies did not grow biofilm in a Centers for Disease Control and Prevention (CDC) Biofilm Reactor. This system is a high shear reactor that produces consistent biofilms. The reactor is a continuous system that expose cells to shear stress and renewable nutrients in order to mimic industrial system. Another advantage of this system is the possibility to test multiple growing surfaces at once and enumerate samples at different time points; and provide repeatable results that can be used by industry.

Therefore, the objectives of this research were to:

- Set up a repeatable protocol for *Salmonella* biofilms growth in a CDC biofilm reactor;
- Evaluate attachment, hydrophobicity and biofilm architecture of *Salmonella* biofilms grown on nylon, wood, and high-density polyethylene coupons;
- Evaluate the efficacy of five commercially available sanitizers, chlorine, chlorine dioxide gas, peroxyacetic acid (PAA), silver dihydrogen citrate (SDC), and steam, on *Salmonella* biofilms grown on food contact surfaces representative of the produce industry: nylon, wood, and high-density polyethylene.

Chapter 3 - Evaluation of *Salmonella* Biofilm Attachment and Hydrophobicity on Common Food Contact Surfaces in the Tree-Fruit Industry

Abstract

Salmonella can attach, form biofilms, and persist on food contact surfaces which creates a food safety challenge for the tree-fruit industry as these surfaces may serve as points of contamination for produce. Once a biofilm is formed traditional cleaning and sanitation protocols may be inadequate to effectively remove the biofilm. This study evaluated attachment characteristics, hydrophobic properties, and structure of *Salmonella* biofilms grown on food contact surfaces specific to the tree-fruit industry (e.g., nylon, wood, and high-density polyethylene [HDPE]). Multi-strain *Salmonella* biofilms were grown on each material in a Centers for Disease Control and Prevention (CDC) biofilm reactor at $22 \pm 2^\circ\text{C}$ for 2, 24, and 96 hours to evaluate *Salmonella* attachment and structure characteristics. After each incubation period coupons were removed and gently rinsed to remove loosely adhered cells and remaining cells were enumerated. Biofilm structure was analyzed with Laser Scanning Confocal Microscopy (LSCM). The hydrophobicity of biofilms and materials were obtained by contact angle measurements which used water and formamide as reference solvents; *Salmonella enterica* serovar Typhimurium LT2 was used as a BSL 1 surrogate. Material type and biofilm age significantly influenced attachment and biofilm hydrophobicity ($P < 0.05$). The greatest attachment, across all biofilm ages, was observed on nylon followed by wood and HDPE. Uninoculated nylon coupons exhibited the most hydrophilic material followed by wood and HDPE; $27.55 \pm 1.13^\circ$, $34.53 \pm 0.45^\circ$, and $55.29 \pm 0.57^\circ$, respectively ($P < 0.05$). The highest

contact angle measurements were observed after 96-hour of biofilm formation on each material. Understanding the attachment and hydrophobicity characteristics of *Salmonella* may help tree-fruit producers make informed decisions when selecting food contact surfaces to minimize biofilm formation and subsequent potential for cross-contamination.

3.1 Introduction

Salmonella foodborne infections are a major public health concern and account for an annual economic burden of \$4.1 billion in the United States (Hoffman et al., 2021). *Salmonella* causes more hospitalizations and deaths than any other foodborne pathogen. Fruit and vegetable products may serve as vectors of transmission, and they are often linked to over 25% of *Salmonella* outbreaks (IFSAC, 2022). *Salmonella* can attach to various surfaces in the food industry and form biofilms (Joseph et al., 2001). A biofilm is an intricate structure of organized cells attached to a surface and bound together by an Extracellular Polymeric Substances (EPS) (Donlan, 2002). The EPS matrix is responsible for adhering the microbial community together and firmly to the surface (Flemming et al., 2016). The EPS matrix is known to increase horizontal gene transfer and decrease the diffusion of antimicrobials (Flemming et al., 2016). Therefore, *Salmonella* biofilm formation is a major food safety issue as biofilms may increase persistence in processing environments (Vestby et al., 2009), contaminate products, and resist routine cleaning protocols (Joseph et al., 2001).

The ability for bacteria to adhere to a surface is a complex process and involves van der Waals, electrostatic, and hydrophobic interactions (Berne et al., 2018). Attachment can only occur if the bacteria overcome these physiochemical forces (Berne et al., 2018). Van der Waals interactions act on bacteria from the longest range and promote attachment (Berne et al., 2018). As bacteria move closer to the surface the magnitude of van der Waals increases, thus promoting attachment (Berne et al., 2018). Electrostatic interactions are dependent upon ionic strength and the pH of the liquid environment (Berne et al., 2018). Furthermore, hydrophobic interactions are impacted by the chemistries of both the bacteria and surface and can be repulsive or attractive (Berne et al., 2018). Surface hydrophobicity is thought to impact bacterial attachment and

biofilm formation (Berne et al., 2018). However, the degree to which surface hydrophobicity may affect bacterial attachment is controversial in the literature: previous researchers were not able to correlate surface hydrophobicity and *Salmonella* attachment (Casarin et al., 2016); whereas others found some correlation on select surfaces (Chia et al., 2009). Nevertheless, many agree that cellular attachment is highly complex and influenced by several factors: serovar, material, nutrient, temperature, and pH (Schlisselberg & Yaron, 2013; Patel et al., 2013; Obe et al., 2022; Roy et al., 2021; Speranza et al., 2011; Stepanovic et al., 2003).

Harvesting equipment such as nylon picking bags and wooden and high-density polyethylene (HDPE) harvest bins are commonly used in the tree-fruit industry. The prevalence of *Salmonella* (Duffy et al., 2005) and indicator organisms (Newman et al., 2017) on produce processing equipment coupled with pathogen's potential to form strong biofilms (Amrutha et al., 2017; Wang et al., 2022) imply that these surfaces may serve as reservoirs and cross contaminate produce (Joseph et al., 2001). To my knowledge, no previous research evaluates *Salmonella* biofilm formation and the hydrophobic/hydrophilic properties of its biofilms grown on nylon, wood, and HDPE coupons. Therefore, this study aims to evaluate *Salmonella* biofilm formation and hydrophobicity of biofilms grown on nylon, wood, and HDPE at three different times (2, 24, and 96-hour) of growth.

3.2 Material and Methods

3.2.1 Bacterial strains

Three *Salmonella* serovars were used in this research. *Salmonella* Enteritidis ATCC BAA-1045 isolated from raw almonds, *S. Agona* LJH 517 from alfalfa sprouts, and *S. Newport* ATCC 6962 from a clinical isolate. Each strain was preserved in CryoCare Bacteria Preservers system (Scientific, Stamford, TX), and stored at -80°C until experiment. A bacterial cocktail was

obtained by using a modified protocol developed in our lab (Mendez et al., 2020). Bacteria solutions were incubated for 24-hour at 37°C with 70 rpm shaking. The *Salmonella* cocktail consisted of 1 ml of each overnight culture. Initial cocktail population was determined as ~ 9 log CFU/ml.

3.2.2 Biofilm growth

Biofilms were grown in a Center for Disease Control and Prevention (CDC) biofilm reactor (Biosurface Technologies, Bozeman, Montana) for up to 96-hour using a modified protocol developed in our lab (Mendez et al., 2020). For the batch phase, Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) at a concentration of 3g/l was added to the reactor then the reactor was inoculated with 1 ml of *Salmonella* cocktail. After 24-hour, TSB at a concentration of 1.5g/l was pumped at a rate of 7 ml/min for 72-hour. The reactor was maintained at 22 ± 2 °C and the stir plate was set to 120rpm.

3.2.3 Coupon material

The following materials were selected to represent picking bags and harvest bins in the tree fruit industry: nylon, wood, and high-density polyethylene (HDPE). All coupons had 1.27 cm diameter on both sides for biofilm growth. Nylon material was cut from used apple harvest picking bags. Sheets of basswood were purchased from a local store and cut into 1.27 cm diameter circles with laser cutter (Glowforge, WA). HDPE coupons were purchased from Biosurface Technologies Corporation (Bozeman, MT). All coupons were scrubbed with soapy water, sonicated for five-minute, and rinsed with tap water. This process was repeated a total of three times. Clean coupons were placed under UV light for 30-minute before transferred in the biofilm reactor. The fully assembled reactor was then autoclaved at 121°C for 15-minute.

3.2.4 Biofilm attachment

The method described by Manville et al. (2023) and Cerca et al. (2005) was followed to evaluate attachment of *Salmonella*. Briefly, a *Salmonella* cocktail of $\sim 9 \log \text{CFU/ml}$ was added to a fully assembled bioreactor. At the appropriate time (2, 24 and 96 h), one coupon for each material was removed for analysis. Coupons were gently placed in 50 ml of sterilized deionized water for 10-second. Next, coupons were transferred in a new beaker for 10-second, followed by a third rinse to remove loosely attached cells. Afterwards, coupons were allowed to dry for 30-min prior to enumeration. Coupons were then placed in 10 ml of Phosphate-Buffered Saline solutions (PBS, VWR, Solon, OH). Biofilms were detached from coupons following ASTM method E2871-19 (ASTM, 2019). Positive control coupons were not rinsed, and coupons placed in TSB for 96-hour served as negative controls. Serial dilutions were performed in 0.1% peptone water (Bacto, Sparks, MD) and spread plated in duplicate on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD). Colonies were counted and reported as $\log \text{CFU/cm}^2$ after 18-24 hours of incubation at 37°C .

3.2.5 Hydrophobicity assay

Hydrophobicity experiments were conducted in the Food Analytical Chemistry Laboratory at Kansas State University. *Salmonella enterica* serovar Typhimurium LT2 was provided by Dr. Bergholz, Michigan State University, Department of Food Science and Human Nutrition and was used as a BSL 1 surrogate for *S. Enteritidis* ATCC BAA-1045, *S. Agona* LJH 517, and *S. Newport* ATCC 6962 as this experiment took place in a BSL 1 laboratory. The hydrophobicity of *Salmonella* biofilms grown on nylon, wood, and HDPE were evaluated at three different times (2, 24, and 96-hour). Additionally, two controls were evaluated for each material: clean not-inoculated coupons and clean not-inoculated coupons soaked in TSB for 96-

hour and dried for 1-hour. All coupons were allowed to dry for 1-hour prior to assay. The contact angle values were obtained by the sessile-drop contact angle technique and automatically captured by optical drop tensiometer (Attention Theta Flex C311, Nanoscience Instruments, Phoenix, Arizona) over a 30-second period (Manville et al., 2023). Deionized water and formamide (10 μ m) served as reference liquids (Manville et al., 2023). Measurements were taken in quadruplicate and at two different locations on each coupon.

3.2.6 Laser Scanning Confocal Microscopy (LSCM)

Biofilms grown on nylon, wood, and HDPE were imaged under Laser Scanning Confocal Microscopy (LSCM) (LSM-5 Pascal associated with a Zeiss Axioplan 2) at the Microscopy Facility of the Biology Department at Kansas State University. Samples were imaged at 0, 2, 24, and 96-hour. The samples were stained with SYTO 9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR) to differentiate live and dead cells as described by Manville et al. (2023). Images and 3D projections were developed using ImageJ (NIH, USA).

3.2.7 Statistical analysis

Coupons were randomly assigned treatments across experiments. Attachment assay and hydrophobicity assay were performed in triplicate and quadruplicate, respectively. Statistical differences were determined at $P < 0.05$. All results were analyzed in SAS Studio (Cary, NC) using the GLM procedure with Tukey's multiple comparison.

3.3 Results

3.3.1 Biofilm attachment

Salmonella biofilm attachment was influenced by incubation time, material type, and their interaction ($P < 0.05$). *Salmonella* attachment was greatest on nylon followed by wood then

HDPE ($P<0.05$) and increased over time (2-, 24-, and 96-hour) ($P<0.05$). Control coupons with 96-hour biofilms and no rinse step resulted in *Salmonella* populations of 10.00 ± 0.07 , 9.72 ± 0.14 , and 8.86 ± 0.19 CFU/cm² on nylon, wood, and HDPE, respectively. No microbes were detected on non-inoculated coupons soaked in TSB for 96-hour. *Salmonella* populations in respect to incubation time and coupon type is presented in Figure 3.1. The 2-hour incubation period resulted in different *Salmonella* populations on nylon, wood, and HDPE; 7.69 ± 0.25 , 6.66 ± 0.43 , and 4.73 ± 0.23 log CFU/cm², respectively ($P<0.05$). When incubation time increased from 2-hour to 24-hour, *Salmonella* attachment increased on each coupon; nylon, wood, and HDPE resulted in populations of 9.43 ± 0.02 , 8.41 ± 0.59 , and 7.06 ± 0.28 log CFU/cm², respectively ($P<0.05$). Nevertheless, the populations recovered from 24-hour HDPE were comparable to those of 2-hour nylon and wood ($P>0.05$); similar populations were also observed between 24-hour wood and 2-hour nylon ($P>0.05$). *Salmonella* populations on wood and HDPE rose further when incubated for 96-hour; 9.68 ± 0.08 and 8.59 ± 0.10 log CFU/cm², respectively ($P<0.05$); however, nylon only increased slightly to 10.03 ± 0.06 log CFU/cm² ($P>0.05$). Attachment after 96-hour on nylon and wood was similar to 24-hour nylon; furthermore, 96-hour HDPE and 24-wood exhibited a similar degree of attachment.

3.3.2 Hydrophobic properties of surfaces and biofilms

Contact angle measurements are presented in Table 3.1. Deionized water and formamide were selected as reference solvents for contact angle measurements to depict the physiochemical characteristics of the biofilm. Solvent polarity is known to influence contact angle; therefore, formamide and deionized water, the latter being less polar, were selected as reference solvents. Previous research observed significant difference in contact angle between formamide and deionized water on similar materials (Manville et al., 2023; Meijer et al., 2000). Similarly, this

study observed solvent type significantly influenced contact angle values ($P<0.05$); formamide resulted in lower contact angle values than water. This section will describe the contact angles obtained from water; formamide followed a similar trend with lower values. Material type and condition also impacted contact angle values ($P<0.05$). All materials evaluated presented contact angles of $<90^\circ$ and are therefore classified as hydrophilic or wetting surfaces (Verplanck et al., 2007). The contact angle measurements obtained from clean, uninoculated, coupons indicate HDPE presented the most hydrophobic surface ($55.29 \pm 0.57^\circ$) followed by wood ($34.56 \pm 0.44^\circ$) and then nylon ($27.55 \pm 1.21^\circ$). When soaked in sterile TSB for 96-hour contact angle increased on nylon and HDPE; $30.13 \pm 0.78^\circ$ and $61.93 \pm 0.43^\circ$, respectively ($P<0.05$); the contact angle observed on wood increased slightly; $34.82 \pm 0.21^\circ$ ($P>0.05$). Nevertheless, clean and TSB control coupons resulted in the lowest contact angles. The formation of 2-hour old biofilms on nylon and HDPE significantly increased contact angle; $33.99 \pm 0.15^\circ$ and $64.62 \pm 0.64^\circ$, respectively ($P<0.05$); however, the contact angle observed on wood remained similar to the controls; $36.20 \pm 0.29^\circ$ ($P>0.05$). Contact angle values further increased with 24-hour old biofilms, indicative of increased in hydrophobic surface properties. The trend of increasing hydrophobicity continued as 96-hour biofilms formed on nylon, wood, and HDPE resulted in the highest contact angle values; 40.83 ± 0.98 , 47.82 ± 0.73 , and 71.10 ± 0.98 , respectively ($P<0.05$). Overall, *Salmonella* biofilm formation altered the physiochemical properties and increased the hydrophobicity of the surfaces evaluated.

3.3.3 Laser Scanning Confocal Microscopy (LSCM)

The intensity of fluorescently stained *Salmonella* cells was measured by Laser Scanning Confocal Microscopy (LSCM) and subsequently rendered into 3D models to visualize biofilm development (Figure 3.2). The 3D models complement the quantitative data in Figure 3.1 and

provide insight on the attachment and aggregation characteristics of *Salmonella* biofilms over a 96-hour period. Biofilms grown on nylon coupons displayed a dense accumulation of cells after 2-hour of formation (Figure 3.2 A). Further aggregation of cells was observed on more mature, 24- and 96-hour, biofilms (Figure 3.2 A). Wood has been shown to autofluorescence during LSCM (Munir et al., 2021). Similarly, I observed slight autofluorescence of the wood at 0-hour in Figure 3.2 B. Nevertheless, biofilms grown on wood initially appeared to form smaller colonies dispersed across the coupon and then exhibited increased surface coverage as biofilm maturity increased. Initial biofilm formation on HDPE resulted in the least fluorescence (Figure 3.2 C). After 24-hour aggregates of cells were dispersed widely across the materials and surface coverage was the greatest after 96-hour of biofilm formation. Overall, these models further show the physical and physiochemical properties of nylon and wood are more favorable to *Salmonella* attachment than HDPE.

3.4 Discussion

It is important to understand *Salmonella* attachment and characterize biofilm formation to help control pathogen contamination on food contact surfaces commonly used in the tree-fruit industry, e.g., nylon, wood, and HDPE. This study is the first to evaluate *Salmonella* biofilm formation on these types of materials. *Salmonella* was able to adhere to different materials commonly used in the tree-fruit industry. Similar to previous research, attachment was significantly impacted by surface type (Wan Norhana et al., 2009; Chia et al., 2009; Sinde & Carballo, 2002) and incubation time (Manville et al., 2023; Wang et al., 2011). Patel et al. (2013) observed *Salmonella* isolates from poultry sources attached greater on polycarbonate surfaces compared to stainless steel. Similarly, Schlisselberg & Yaron (2013) also found that the initial

surface coverage of *Salmonella* was dependent on stainless steel finish and increased over a 5-day incubation period. In the current study, *Salmonella* attachment also increased over time.

Some researchers have suggested that *Salmonella* attachment is favored on hydrophobic surfaces (Sinde & Carballo, 2002); Chia et al. (2009) positively correlated *Salmonella* attachment and material hydrophobicity between select materials but was not able to correlate attachment and hydrophobicity of all evaluated materials. Whereas Casarin et al. (2016) found no correlation between *Salmonella* attachment and hydrophobicity of AISI 304 and 316 stainless steel. Manville et al. (2023) observed *Listeria* biofilms grown for 96-hour exhibited enhanced hydrophobic properties than young 2-hour biofilms. Similarly, this study observed that hydrophobicity values increased as the biofilm matured.

Thames et al. (2023) reported that increased fluidic shear stress negatively impacted the biofilm forming ability of *Salmonella* on ultra-high molecular weight polyethylene. In the current study, a Center for Disease Control and Prevention (CDC) biofilm reactor was used to form *Salmonella* biofilm under high-shear conditions. James et al. (2019) also used a CDC biofilm reactor to evaluate bacterial adhesion and observed that surface roughness enhanced attachment and biofilm formation. Interestingly, when total surface area was adjusted to account for the increased surface area of rough surfaces bacterial populations were similar to those observed on the smooth surfaces. These high-shear conditions are known to increase the movement of cells towards the surface of the coupons (Bakker et al., 2003); however, bacteria must form stronger bonds to overcome these forces and remain attached to the coupon (Saur et al., 2017). Nevertheless, the increased surface area and niche formation may have favored attachment for bacteria cells on wood and nylon under the high-shear environment. Conversely, a lower attachment was observed for HDPE coupons potentially due to the nature of the material

(no niche areas) and a lower surface area (James et al., 2019). Typically, cell hydrophobicity increases overtime for biofilm formation as compared to planktonic cells (Mirani et al. 2018). My results indicate the coupons with biofilms displayed greater contact angle values as compared to their controls. Overtime contact angles continued to increase and after 96-hour, the highest contact angles were recorded.

In order to illustrate distinct differences in *Salmonella* attachment on surfaces over time LSCM images were taken. Schlisselberg & Yaron (2013) utilized LSCM to quantify *Salmonella* biofilm depth and surface coverage on various stainless-steel surfaces. They observed that biofilms formed for 1-day on electropolished stainless steel exhibited significantly less surface coverage and similar depth as compared to non-treated stainless-steel surfaces. Interestingly, after 5-days, biofilm surface coverage and depth increased dramatically on both surface types. However, non-treated stainless steel presented significantly greater surface coverage and biofilm depth. These observations further demonstrate the impact of surface type and time on biofilm formation characteristics. In the current study, I observed that 2-hour biofilms on wood and HDPE formed dense, widely dispersed colonies and increased surface coverage with extended incubation times. Whereas, 2-hour biofilms grown on nylon displayed complete surface coverage and increased overall biofilm mass with extended incubation times. HDPE resulted in the least colonization of *Salmonella* across all time periods. This is not surprising as it was the only hard non-porous food contact surface evaluated and is typically regarded as an acceptable surface in terms of hygienic design.

3.5 Conclusion

These results should be considered by the tree-fruit growers when selecting materials used for harvesting bins and bags. Overall, *Salmonella* attachment and biofilm formation was

significantly impacted by surface type and incubation time; biofilm formation influenced hydrophobicity properties of the coupons; and biofilm formation were influenced by surface type and incubation time. This research suggests that alternative materials should be considered in place of wood and nylon, since they are difficult to clean if biofilms establish. Therefore, material selection should be made with care to help provide information to produce growers and prevent *Salmonella* attachment and subsequent biofilm formation.

Figures and Tables

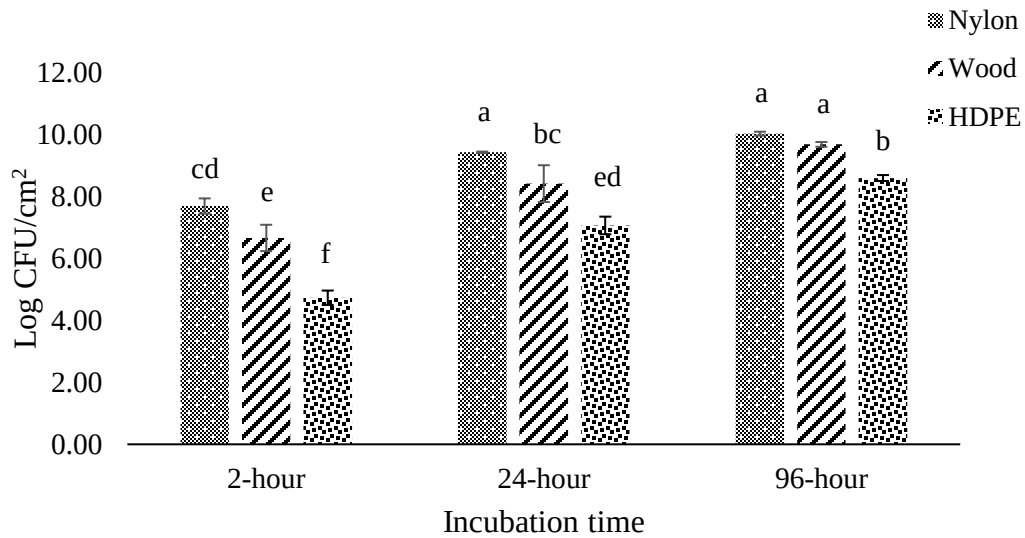
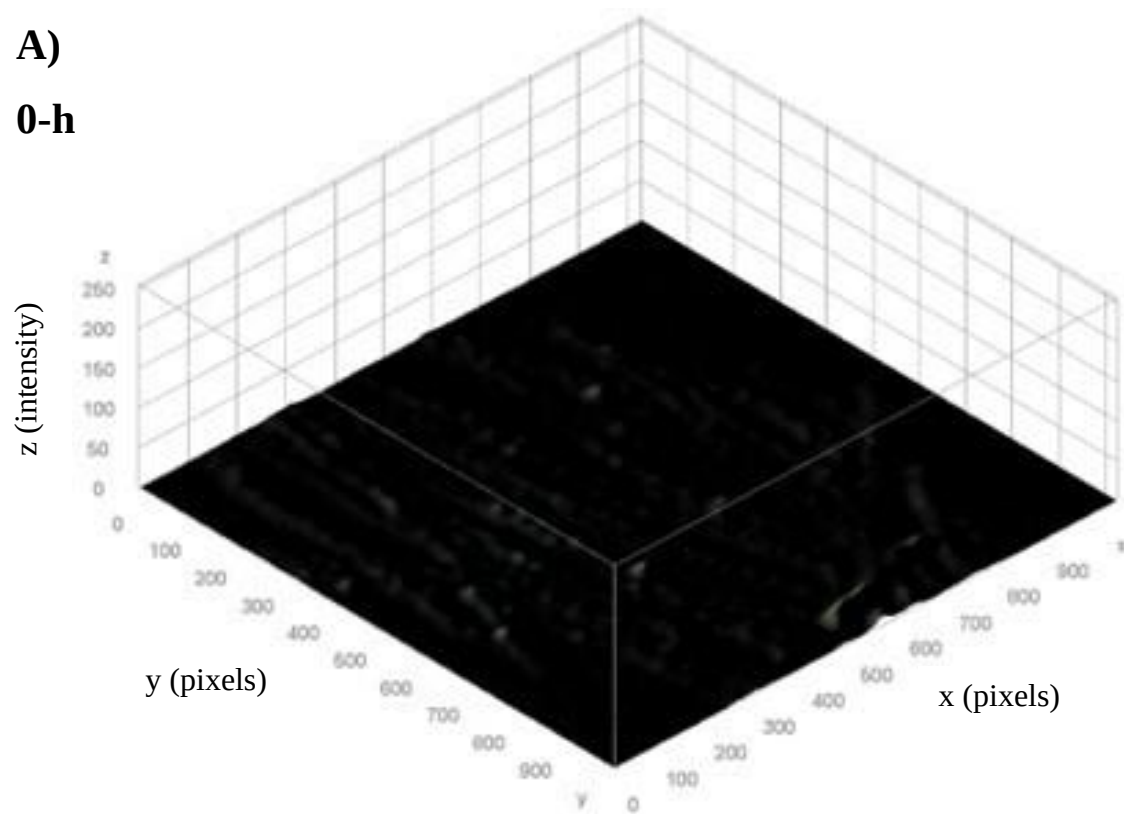
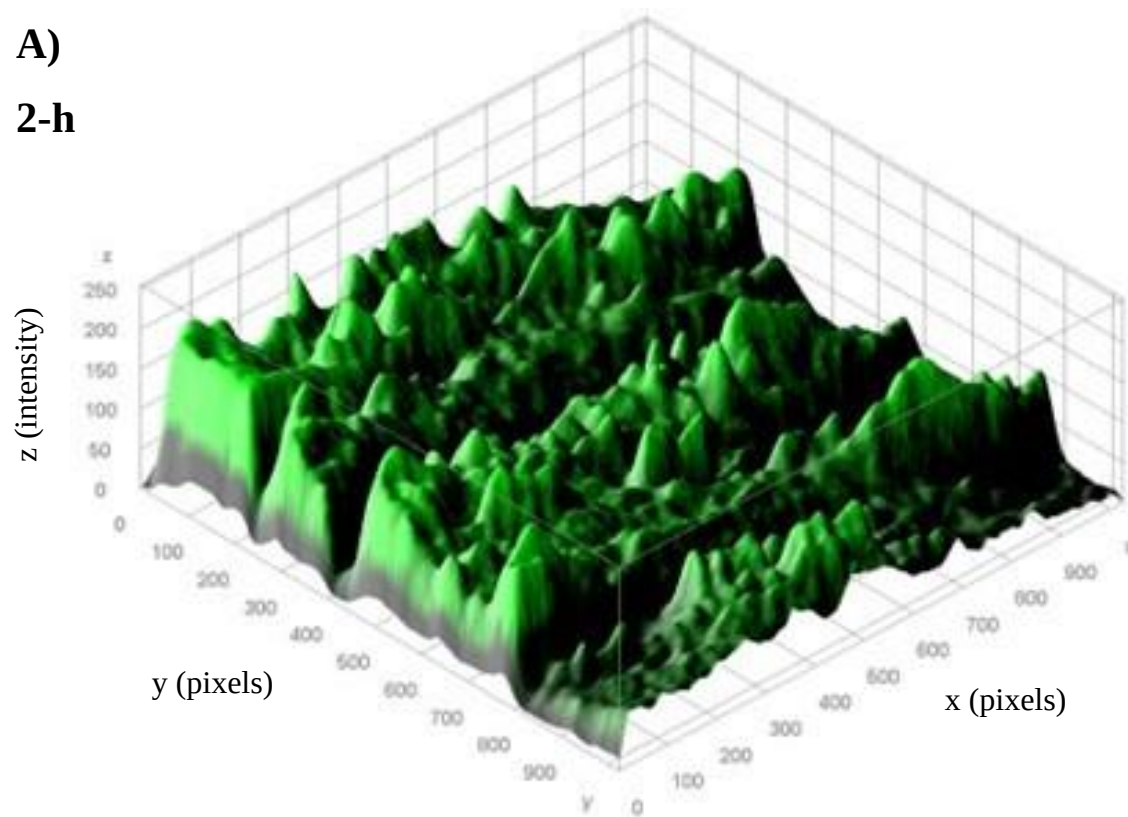


Figure 3.1 *Salmonella* (Log CFU/cm²) attachment to nylon, wood, and HDPE after 2, 24, and 96-hour incubation and rinse step. The following counts were recovered from mature 96-hour biofilm with no rinse step; 10.00 ± 0.07 , 9.72 ± 0.14 , and 8.86 ± 0.19 CFU/cm² on nylon, wood, and HDPE, respectively. Small letters indicate statistical difference between treatments.

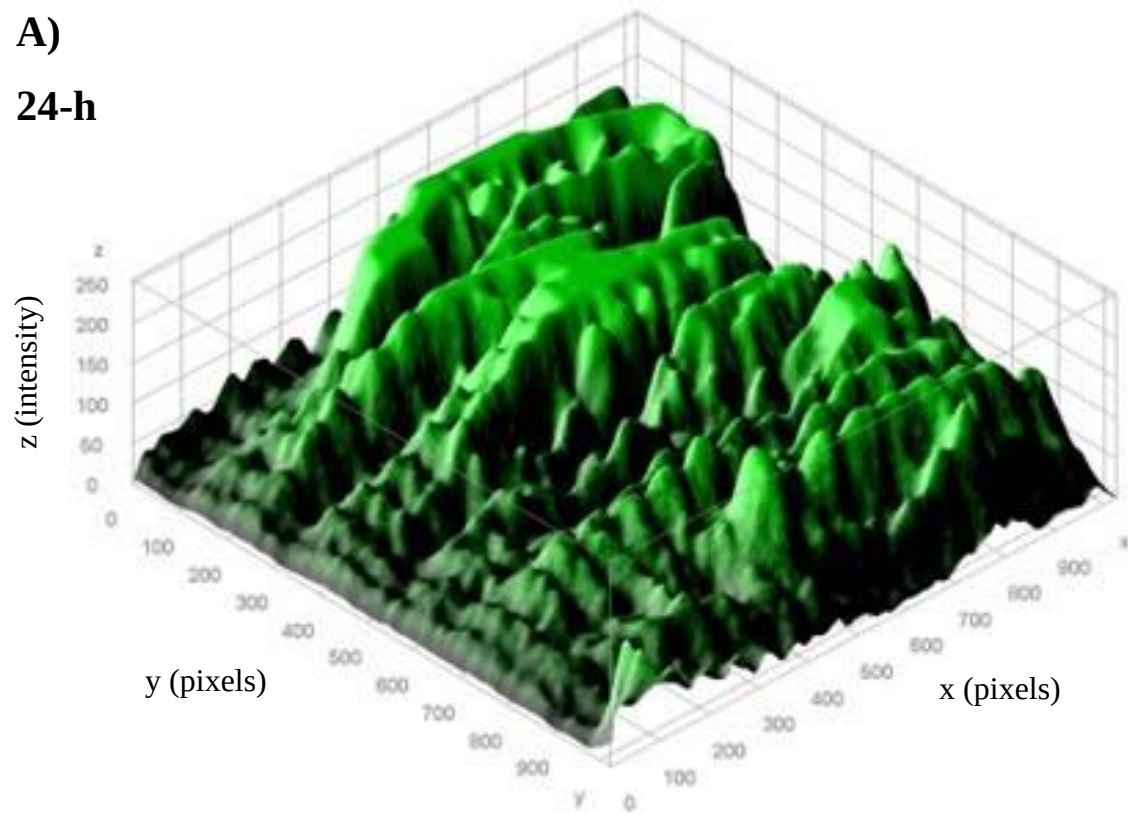
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0-h



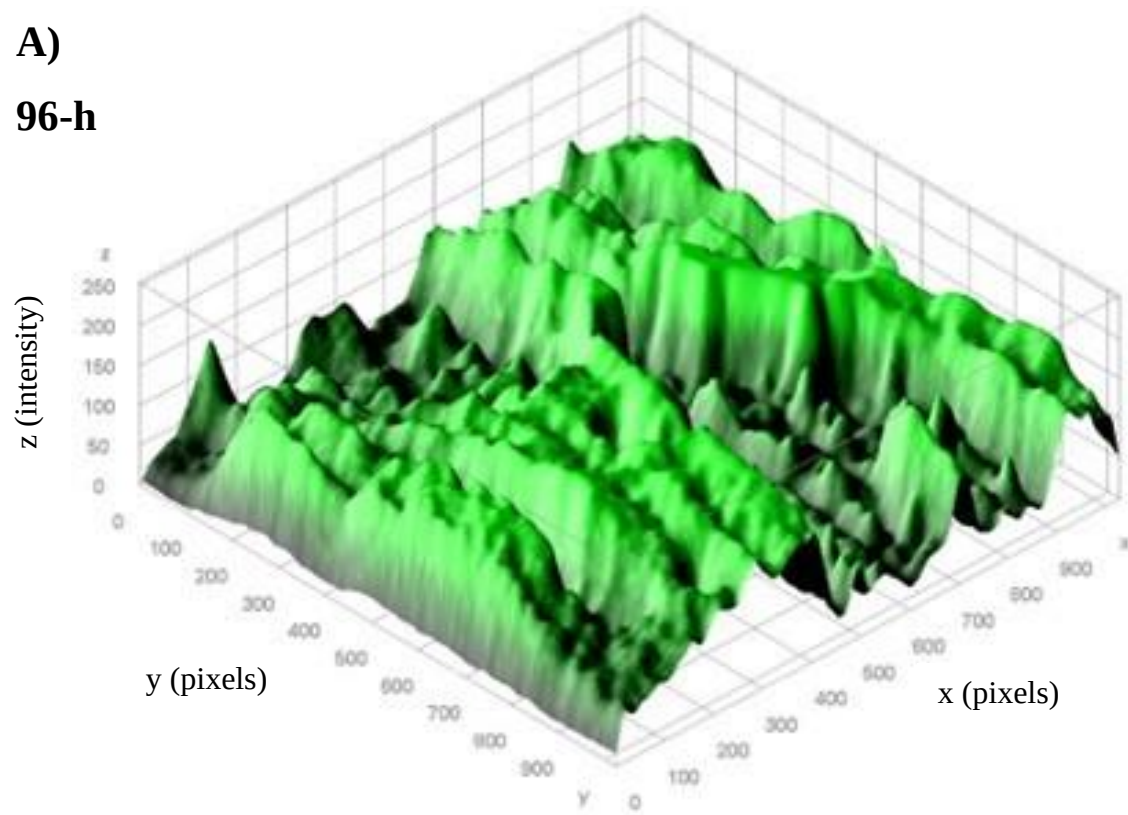
A)
2-h



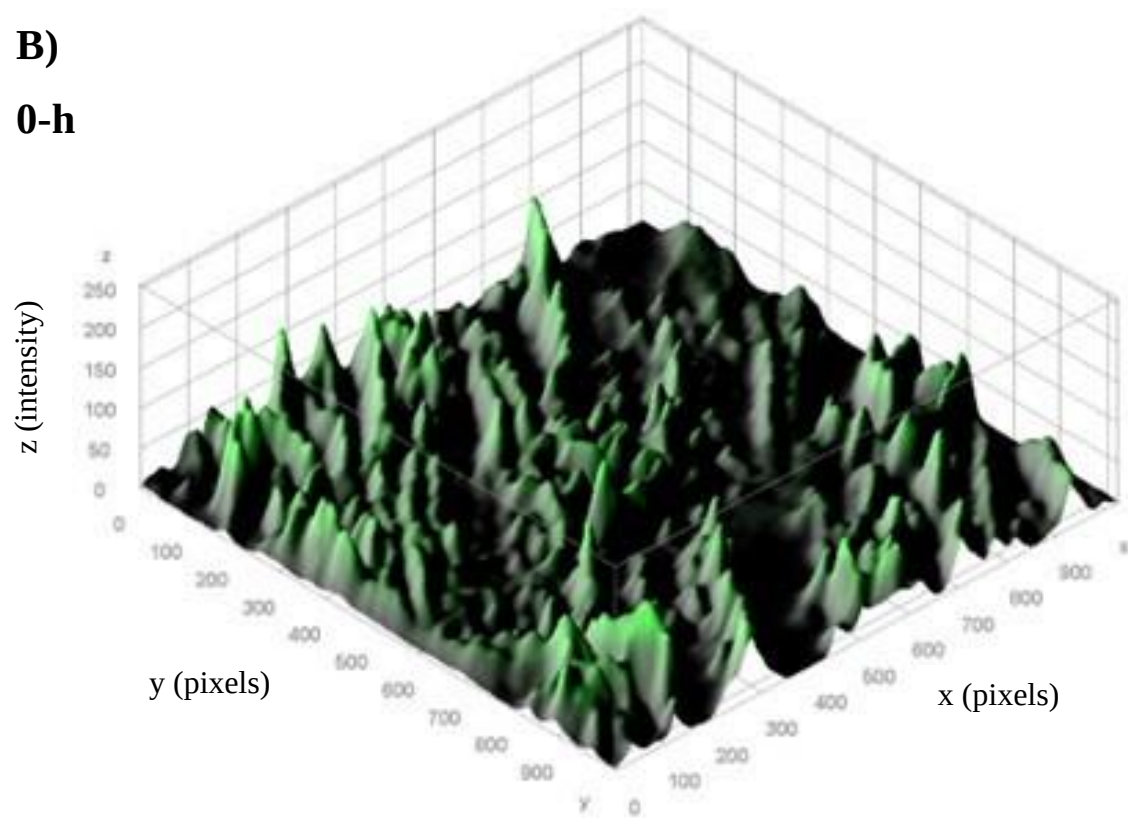
A)
24-h



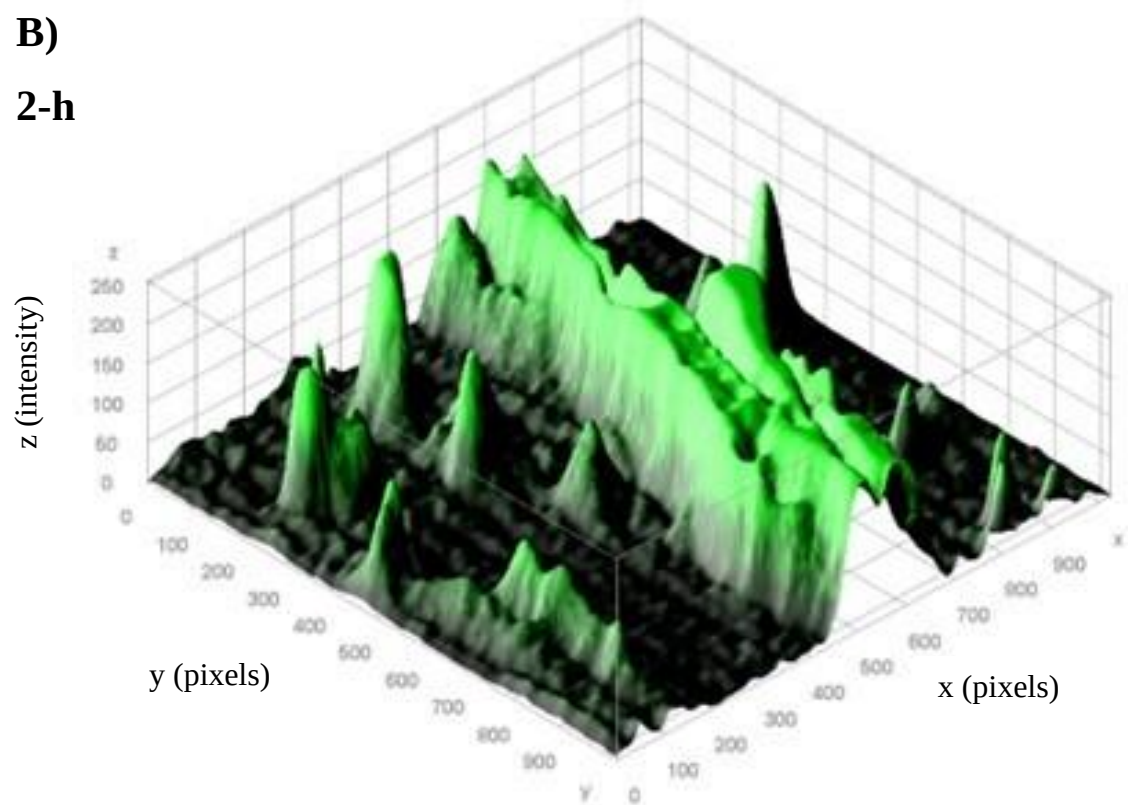
A)
96-h



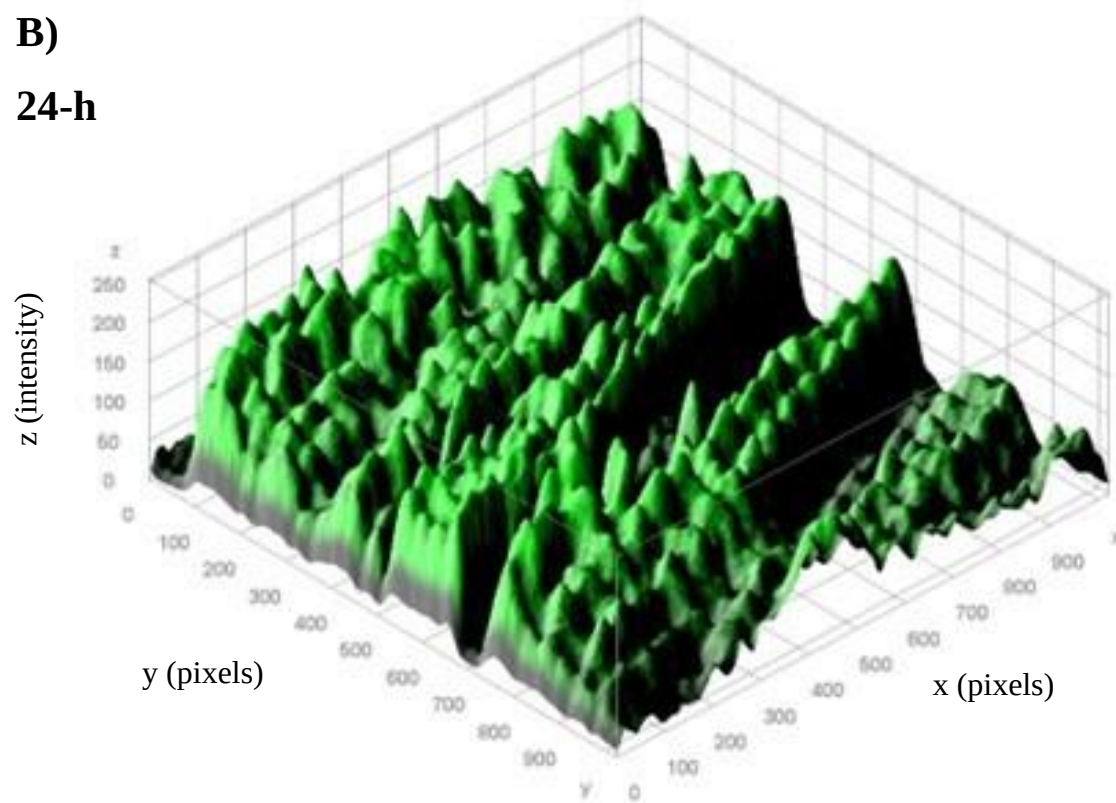
B)
0-h



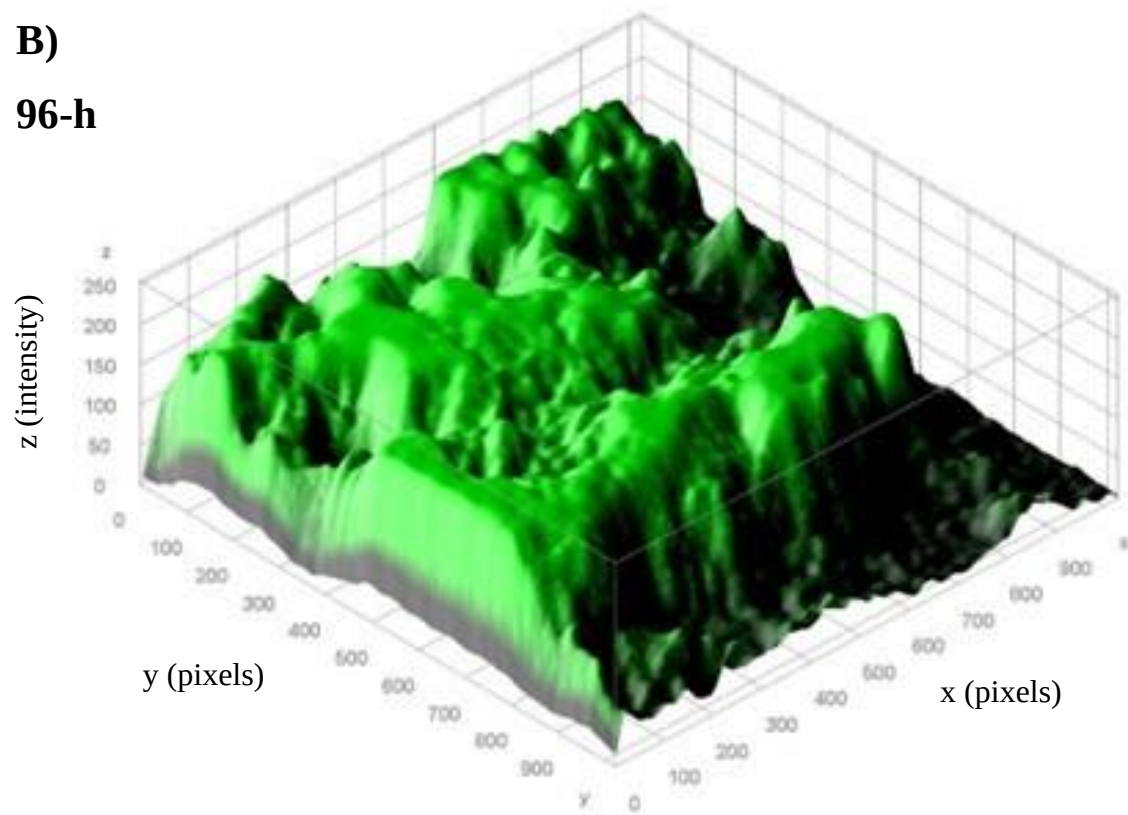
B)
2-h



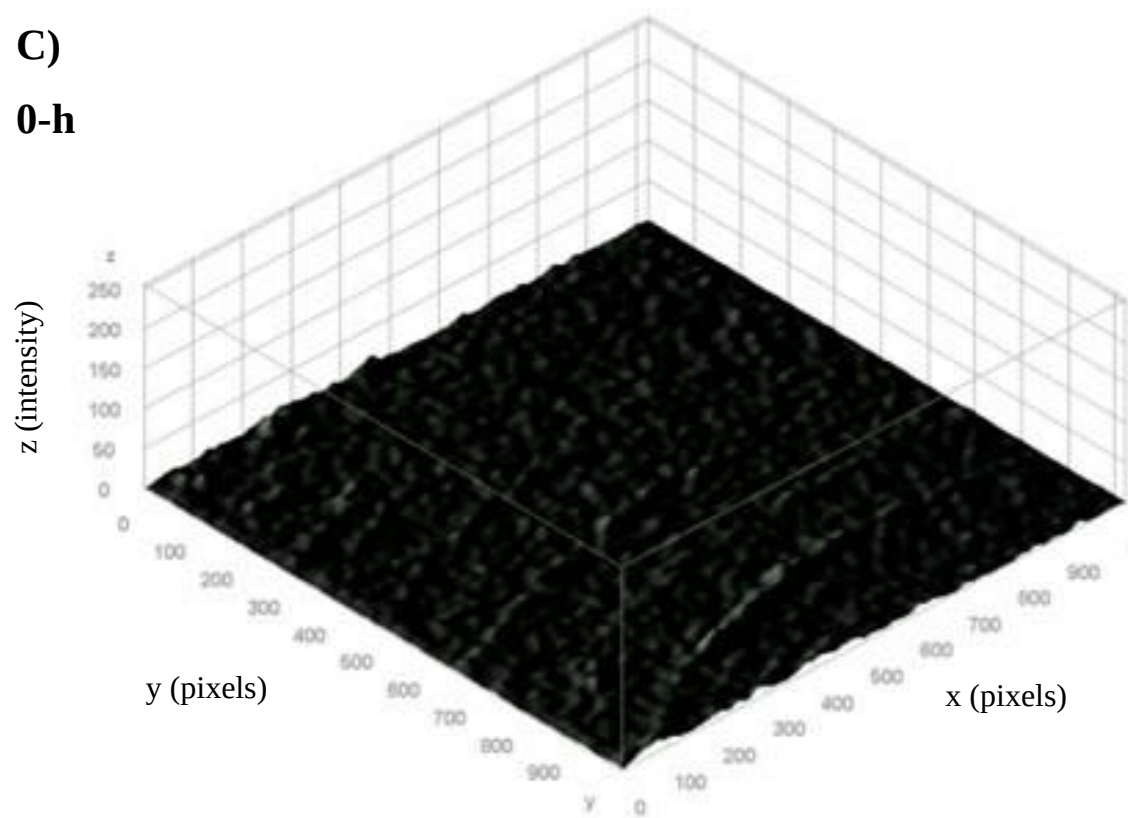
B)
24-h



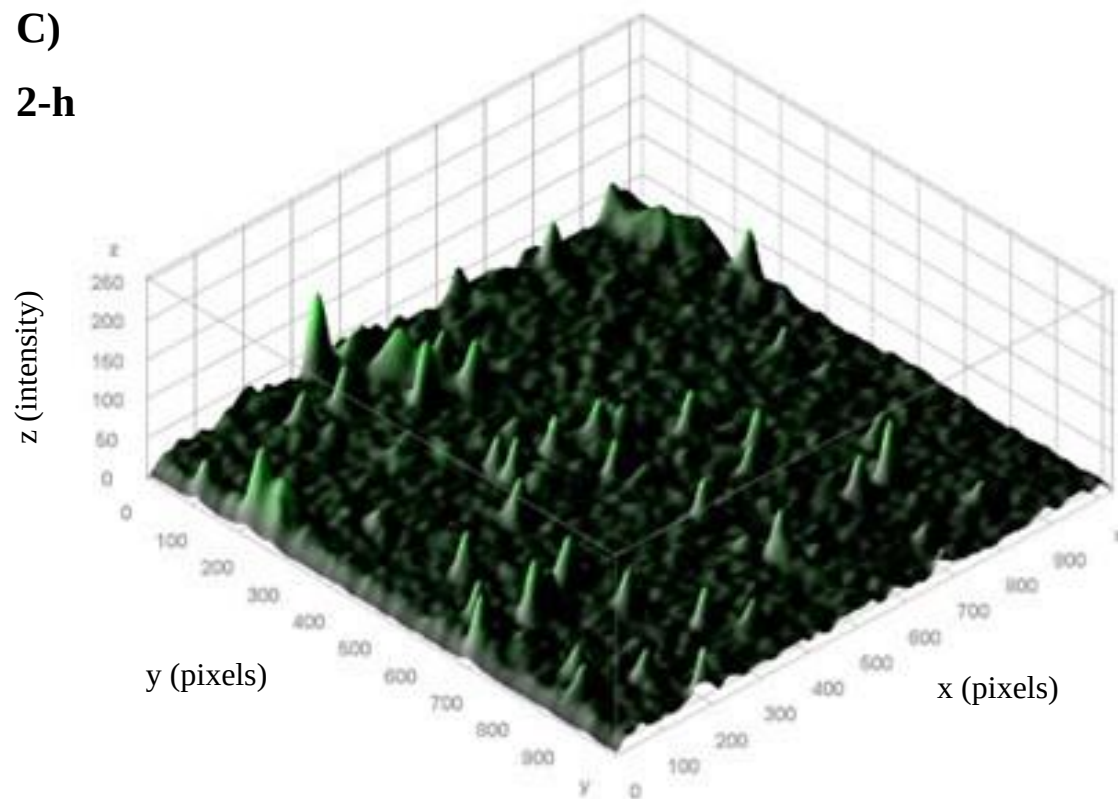
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96-h



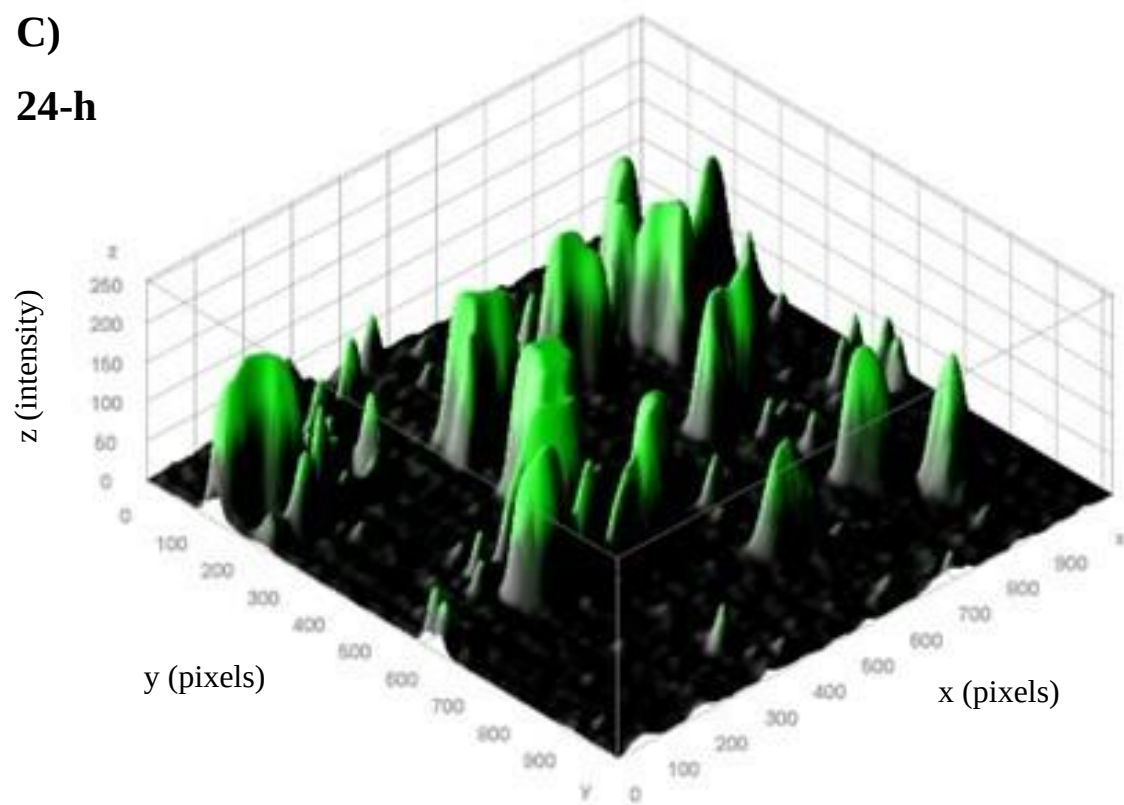
C)
0-h



C)
2-h



C)
24-h



C)
96-h

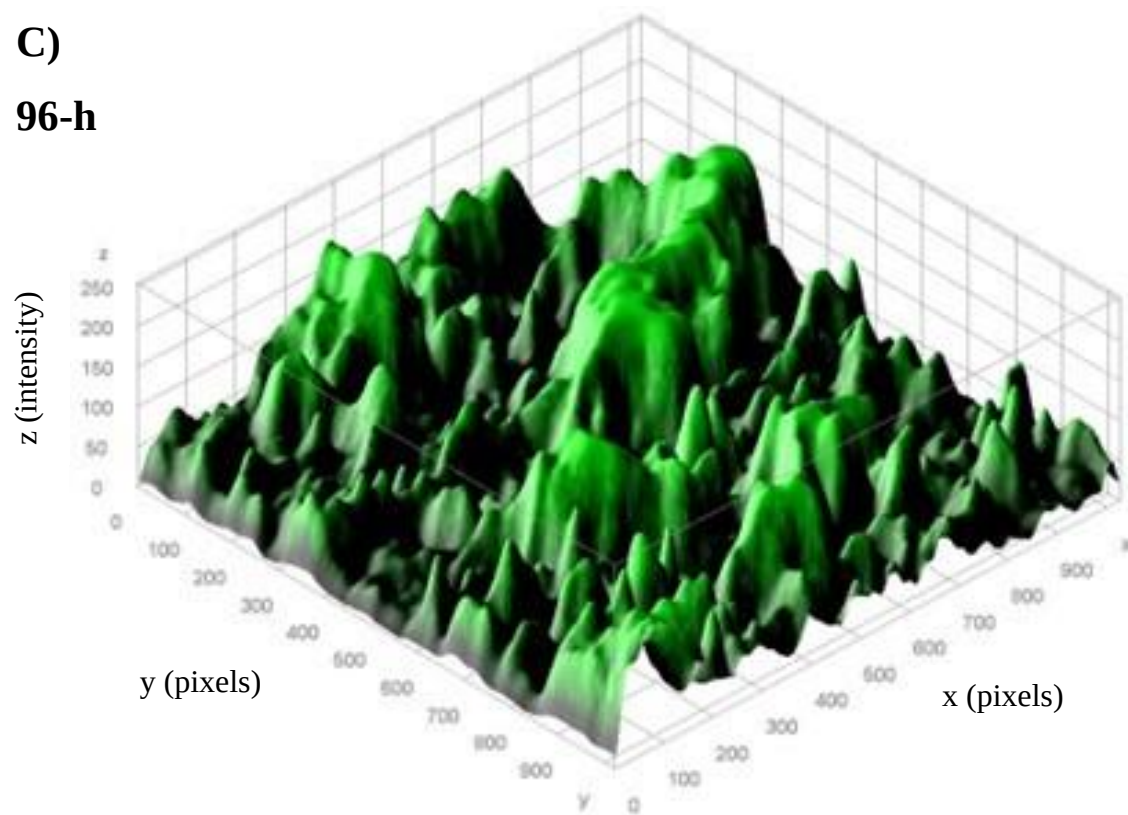


Figure 3.2 3D surface projections of *Salmonella* biofilms on (A) nylon, (B) wood, and (C) HDPE after 0, 2, 24, and 96-h of growth in CDC biofilm reactor. Samples imaged with Laser Scanning Confocal Microscopy (LSCM). Green represents living *Salmonella* cells. Z-axis represents fluorescent intensity; X and Y-axis represent pixels. All images were processed with ImageJ.

Table 3.1 Contact angle measurements of biofilms grown on wood, nylon, and HDPE coupons using two different solvents (A) water and (B) formamide. Control 1 represents contact angle values measured on dry coupons without inoculum. Control 2 represents contact angle values measured after materials were soaked in TSB for 96-h without inoculum. Small letters and Roman numerals indicate values with statistical difference ($P < 0.05$) for each column and row, respectively.

Solvent	Conditions	Contact angle [°]		
		Nylon [°]	Wood [°]	HDPE [°]
Water	Control 1	27.55 ± 1.13 ^{ab,I}	34.53 ± 0.45 ^{b,II}	55.29 ± 0.57 ^{a,III}
	Control 2	30.13 ± 0.78 ^{c,I}	34.83 ± 0.21 ^{b,II}	61.93 ± 0.43 ^{e,III}
	2 h	33.99 ± 0.16 ^{de,I}	36.20 ± 0.29 ^{b,II}	64.62 ± 0.64 ^{f,III}
	24 h	35.85 ± 0.21 ^{e,I}	42.25 ± 0.39 ^{e,II}	66.96 ± 0.66 ^{g,III}
	96 h	40.83 ± 0.98 ^{f,I}	47.82 ± 0.74 ^{f,II}	71.10 ± 0.98 ^{h,III}
Formamide	Control 1	25.89 ± 1.39 ^{a,I}	32.50 ± 0.38 ^{a,II}	52.42 ± 0.59 ^{b,III}
	Control 2	28.16 ± 0.26 ^{b,I}	32.42 ± 0.38 ^{a,II}	57.33 ± 0.55 ^{c,III}
	2 h	30.35 ± 0.29 ^{c,I}	34.93 ± 0.13 ^{b,II}	59.56 ± 0.37 ^{d,III}
	24 h	32.73 ± 0.41 ^{d,I}	39.44 ± 0.4 ^{c,II}	59.74 ± 1.18 ^{d,III}
	96 h	38.56 ± 0.57 ^{g,I}	44.00 ± 1.24 ^{e,II}	67.95 ± 0.71 ^{g,III}

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Chapter 4 - Efficacy of Commercially Available Sanitizers to Control *Salmonella* Biofilms on Harvesting Bins and Picking Bags

Abstract

Salmonella ability to form biofilms is predicted to be a strategy to increase persistence and survival. When harvesting tools and bins are not effectively cleaned and sanitized, microorganisms can persist and potentially cross-contaminate produce. This study evaluated the efficacy of five commercially available sanitizers to reduce *Salmonella* biofilms on experimentally inoculated materials representative of harvesting bins and picking bags in the fresh produce industry. Multi-strain *Salmonella* biofilms were grown in a Centers for Disease Control and Prevention (CDC) reactor at $22 \pm 2^\circ\text{C}$ for 96-hour. Samples were exposed to 500 ppm free chlorine, 500 ppm peroxyacetic acid (PAA), 75psi steam, and 5% silver dihydrogen citrate (SDC) for 1 or 2-minute and 100 ppm chlorine dioxide gas for 24-hour. Nylon, wood, and high-density polyethylene (HDPE) control coupons resulted in initial *Salmonella* biofilm populations of 9.59 ± 0.29 , 9.34 ± 0.40 , and 8.77 ± 0.34 log CFU/coupon, respectively. Sanitizer, surface type, and application time significantly affected the viability of *Salmonella* ($P < 0.05$). All treatments resulted in a significant reduction of *Salmonella* when compared to the control ($P < 0.05$). Surface type also impacted sanitizer efficacy ($P < 0.05$). Understanding the efficacy of sanitizers to control *Salmonella* may improve the safety of fresh produce by increasing available treatment strategies to sanitize picking bags and harvesting bins.

4.1 Introduction

Salmonella contamination continues to be a challenge for the fresh produce industry (Faticia & Schneider, 2011; Carstens et al., 2019). This foodborne pathogen is the largest economic burden and leading cause of hospitalizations and deaths annually in the United States of America (Hoffmann et al., 2021; Scallan et al., 2011). It is estimated from 1998 to 2020, over 26% of foodborne *Salmonella* illnesses were associated with fruits and vegetables (IFSAC, 2022): in 2008, jalapeno peppers, serrano peppers and tomatoes were contaminated with *Salmonella* Saintpaul and caused 1,442 confirmed infection cases across 48 states, the District of Columbia, and Canada (CDC, 2008); in 2020 peaches contaminated with *Salmonella* enteritidis resulted in 101 confirmed cases and 28 hospitalizations across 17 states (CDC, 2021).

Produce can be contaminated through multiple sources during harvesting and processing. When harvesting equipment is not effectively cleaned and sanitized, it may serve as a reservoir for *Salmonella* biofilm formation and potentially cross-contaminate fresh produce. Preventing contamination during harvest is vital for produce safety. *Salmonella* has been shown to persist on packing line materials for over 28 days under environmental conditions consistent with Florida's fall/winter tomato production season (Allen et al., 2005). The same research also reported that surfaces such as stainless steel, PVC, and unfinished oak wood could support the survival of *Salmonella* over an extended period of time. It is during these situations of harsh environmental conditions that microorganisms tend to aggregate and form biofilms to increase survival, contribute to antimicrobial tolerance and persist in processing environment (Trampari et al., 2021; Obe et al., 2021). Biofilms are complex microenvironments and express unique challenges as compared to planktonic cells (Park et al., 2012; Schalfer & Myer, 2017). A biofilm is defined as a complex structure of organized cells attached to a surface and bound together by

Extracellular Polymeric Substances (EPS) (Donlan, 2002). The development of a biofilm, specifically the EPS matrix, allows the microbial community to enhance cell to cell communication and serves as a defense mechanism when exposed to sanitizers and antimicrobials (Flemming et al., 2016; Schalfer & Myer, 2017). The EPS matrix provides a barrier to inactivate and/or slow the diffusion of antimicrobials compounds (Flemming et al., 2016). Biofilms formed under high shear force are usually more resistant to breakage compared to those formed under low shear force (Donlan & Costerton, 2002) and very few studies have developed *Salmonella* biofilm under turbulent conditions and evaluated sanitizer efficacy. Therefore, the objectives of this study were to evaluate the efficacy of chlorine, peroxyacetic acid (PAA), steam, chlorine dioxide (ClO₂) gas, and silver dihydrogen citrate (SDC) on mature 96-hour *Salmonella* biofilms grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor on common food contact surfaces used for harvesting and picking in the fresh produce industry and to provide scientific-based data to producers regarding sanitizer selection.

4.2 Material and Methods

4.2.1 Bacterial strains

Three *Salmonella* serovars were used in this study: *S. Enteritidis* ATCC BAA-1045 isolated from raw almonds, *S. Agona* LJH 517 from alfalfa sprouts, and *S. Newport* ATCC 6962 from a clinical isolate. Each strain was preserved in CryoCare Bacteria Preservers system (Scientific, Stamford, TX) and stored at -80°C until experiment. A bacterial cocktail was obtained by using a modified protocol developed in our lab (Mendez et al., 2020). Briefly, frozen beads were streaked for isolation on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD) and incubated at 37°C for 18-hour. An isolated colony for each serovar was used to inoculate 10ml of Tryptic Soy Broth (TSB, BD Difco, Sparks, MD). Bacteria solutions were incubated for 24-hour

at 37°C with 70 rpm shaking. The *Salmonella* cocktail consisted of 1 ml of each overnight culture. Initial cocktail population was determined as ~ 9 log CFU/ml.

4.2.2 Biofilm growth

Biofilms were grown in a CDC Biofilm Reactor (Biosurface Technologies, Bozeman, Montana) for 96-hour. TSB (350ml), at a concentration of 3g/l was added to a fully assembled and sterile biofilm reactor. For the batch phase, the reactor was then inoculated with 1 ml of *Salmonella* cocktail. After 24-hour, TSB at a concentration of 1.5g/l was pumped at a rate of 7 ml/min for 72-hour. The reactor was maintained at $22 \pm 2^{\circ}\text{C}$ and the stir plate was set to 120rpm for 96-hour to obtain a mature biofilm.

4.2.3 Surface materials

The following materials were selected to represent picking bags and bins commonly used in the produce industry: nylon, wood, and high-density polyethylene (HDPE). All coupons had 1.27 cm diameter surface on both sides for biofilm growth. Nylon material was cut from used apple harvest picking bags. Sheets of basswood were purchased from a local store and cut into 1.27 cm diameter circles with laser cutter (Glowforge, WA). HDPE coupons were purchased from Biosurface Technologies Corporation (Bozeman, MT). All coupons were scrubbed with soapy water, sonicated for five-minute, and rinsed with tap water. This process was repeated a total of three times. Then clean coupons were placed under UV light for 30-minute before being placed in biofilm reactor. The fully assembled reactor was then autoclaved at 121°C for 15-minute.

4.2.4 Sanitizer treatments

The following sanitizers were evaluated as interventions to reduce and control *Salmonella* biofilm: 500 ppm free liquid chlorine (Diversity, Fort Mill, SC) confirmed by

chlorine test kit (ChemWorld, Kennesaw, GA); 500 ppm peroxyacetic acid (PAA) (Pace International, Wapato, WA) measured with peroxyacetic acid test kit (Masters, Wood Dale, IL); 75 psi steam generated by a Hill Injection™ (Dupray, Italy) according to manufacturer specifications; 100ppm chlorine dioxide gas (ICA Trinova, Newnan, GA) measured with portable gas detector (Analytical Technology Inc., Collegeville, PA); and 5% silver dihydrogen citrate (SDC) (Pure Bioscience Inc., El Cajon, CA) according to label.

Liquid sanitizers (chlorine, PAA, and SDC) were applied using a bench top application system that sprayed sanitizer over coupons in biofilm reactor rod (Figure 4.1). The bench top system applied sanitizers at a constant rate of 400 ml/min for 1- or 2-minute. Coupons were rotated halfway through the application time.

Steam was applied to individual coupons using the Hill Injection™ steamer and lance adaptor nozzle, which generates a precise and intense distribution of steam (Dupray, 2023). Boiler temperature was monitored by digital display on the Hill Injection™ and reached a temperature of 173 °C prior to each application. Additionally, the steam temperature at the nozzle and coupon surface was monitored and recorded by thermocouple (Traceable, Weber, TX). Steam was applied to individual coupons at a distance of 4 cm from nozzle for 1- or 2-minute and rotated halfway through the application time.

For the chlorine dioxide gas treatment, rods were removed from the CDC reactor and placed in 4.7L plastic container (Produce Keeper, OXO, New York, NY). Gas was generated by mixing 0.5g of precursor and 0.5g of activator in a sachet. The sachet was placed in a plastic container with 80 ml of water for humidity (90% RH) and sealed with masking tape. Temperature and humidity were recorded at 2-minute intervals with hygrometer/thermometer

(Traceable®, Thermo Fisher Scientific, Waltham, MA). Chlorine dioxide gas treatment was conducted for 24-hour.

4.2.5 Recovery and enumeration of Salmonella cells in biofilms

After treatment, coupons were transferred into 10 ml of a neutralizing solution. Dey and Engley neutralizing broth (Hardy Diagnostics, Santa Maria, CA) was used for chlorine, PAA, and SDC. Neutralizing buffer (Difco, Sparks, MD) was used for chlorine dioxide gas treatments. A neutralizing assay was performed for each sanitizer to ensure complete neutralization, before starting experiments (Park & Chen, 2011). Control and steam treated coupons were placed in Phosphate-Buffered Saline solutions (PBS, VWR, Solon, OH). Biofilms were removed from coupons following ASTM method E2871-19 (ASTM, 2019). Serial dilutions were performed in 0.1% peptone water (Bacto, Sparks, MD) and spread plated in duplicate on Xylose Lysine Deoxycholate (XLD, Hardy Diagnostics, Santa Maria, CA). Colonies were counted and reported as log CFU/coupon after 18-24 hours of incubation at 37°C.

4.2.6 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to image 96-hour old biofilms on nylon, wood, and HDPE coupons. Prior to SEM, cells were fixed with 500 µl of 10% formalin (Fisher Scientific, Hampton, NH) for 10-minute. Coupons were then rinsed with 500 µl of sterilized deionized water and allowed to dry for 45-minute. A Desk II sputter coater (Denton Vacuum, Moorestown, NJ) was used to freeze dry samples under vacuum and sputter coat with 2 nm of platinum. Samples were imaged with the Hitachi S-3500N (Hitachi, Tokyo, Japan) in the Microscopy Facility of the Biology Department at Kansas State University. All images were processed in ImageJ (NIH, USA).

4.2.7 Experimental design and statistical analysis

Each treatment performed in this study was randomized across 8 rods in the CDC biofilm reactor. Treatments were replicated six times for each material and application time. The statistical significance was defined by $P < 0.05$. A mixed model was used for the analysis of log viable cells among sanitizers, surface type, and application time. All data were analyzed using SAS 9.4TS Level 1M5 (SAS Institute, Cary, North Carolina).

4.3 Results

4.3.1 Sanitizer efficacy

The effects of sanitizer, surface type, and application time were evaluated on mature *Salmonella* biofilms. All main effects (sanitizer, surface type, and application time), 2-way interactions (sanitizer*surface type, sanitizer*application time, and surface type*application time), and the 3-way interactions (sanitizer*surface type*application time) significantly ($P < 0.05$) impacted *Salmonella* viability (Table 4.1). The remaining population after sanitizer application is shown in Table 4.2. The following populations were recovered on nylon, wood, and HDPE control coupons: 9.59 ± 0.29 , 9.34 ± 0.40 , and 8.77 ± 0.34 log CFU/coupon, respectively. Overall, each treatment resulted in significantly lower *Salmonella* populations when compared to the controls ($P < 0.05$).

The efficacy of PAA was significantly impacted by the application time on biofilms grown on nylon and wood surfaces ($P < 0.05$). The 1-minute application resulted in reductions of ~ 3 log CFU/coupon on nylon and wood, whereas after the 2-minute application lower populations were recorded: 3.00 ± 0.97 and 4.90 ± 1.25 log CFU/coupon, respectively ($P < 0.05$). Conversely, application time did not significantly impact PAA efficacy on HDPE ($P > 0.05$). Surface type also impacted PAA efficacy ($P < 0.05$). PAA was more effective on HDPE than

nylon and wood when applied for 1-minute: 2.84 ± 0.65 log CFU/coupon was observed on HDPE, while 6.09 ± 1.29 and 6.41 ± 0.38 log CFU/coupon were recovered on nylon and wood ($P < 0.05$). When applied for 2-minute, similar enumeration counts were observed on nylon and HDPE ($P > 0.05$); whereas a significantly higher population of 4.90 ± 1.25 log CFU/coupon was observed on wood ($P < 0.05$).

The efficacy of chlorine was significantly impacted by application time on nylon and HDPE ($P < 0.05$). The 1- and 2-minute treatments resulted in remaining populations of 7.44 ± 1.15 and 6.54 ± 0.77 log CFU/coupon for nylon and 4.14 ± 0.66 and 3.18 ± 0.74 log CFU/coupon for HDPE ($P < 0.05$). Chlorine was significantly more effective on HDPE than nylon and wood for both times ($P < 0.05$): 3.18 ± 0.74 log CFU/coupon was observed on HDPE after 2 min chlorine treatment, while 6.54 ± 0.77 and 6.91 ± 0.72 log CFU/coupon were recovered on nylon and wood after the same application.

SDC was the least effective sanitizer across all treatments evaluated under the experimental conditions. The populations recovered after SDC was applied for 1-minute were 8.55 ± 0.50 , 7.49 ± 0.41 , and 5.96 ± 0.44 log CFU/coupon on nylon, wood, and HDPE, respectively. A decrease was observed on nylon and HDPE when the sanitizer was applied for 2-minute: 7.87 ± 0.28 and 4.82 ± 0.60 log CFU/coupon ($P < 0.05$); and slight decrease on HDPE: 7.42 ± 0.44 log CFU/coupon.

The efficacy of steam was not significantly impacted by application time or surface ($P > 0.05$). Nylon, wood, and HDPE coupons treated for 2-minute resulted in 2.80 ± 0.50 , 2.52 ± 0.22 , and $< 2.40 \pm 0.00$ log CFU/coupon remaining population. No detectable colonies were observed when steam was applied for 2-minute on HDPE ($P > 0.05$). Similarly, chlorine dioxide gas was not significantly impacted by surface type ($P > 0.05$). Chlorine dioxide gas treatments

reduced cells below the detectable limit ($<2.40 \log \text{ CFU/coupon}$) on all surfaces. Chlorine dioxide gas was the most effective sanitizer followed by steam, PAA, chlorine, and SDC, while PAA was the most effective liquid sanitizer and was the only liquid sanitizer to achieve greater than a 3-log reduction on all surfaces when applied for 2-minute.

4.3.2 Scanning electronic microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to qualitatively visualize surface characteristics (Figure 4.2). Uninoculated nylon and wood surfaces appeared to be porous and rough. Individual strands of nylon could be easily identified. Wood exhibited splinters across the surface. HDPE presented slight ridges and scratches but was not porous (Figure 4.2 A, B, C). After 96-hour, a mature biofilm formed, and the surfaces appeared remarkably different (Figure 4.2 D, E, F). The accumulation of biomass covered the nylon surface and individual threads of nylon could not be easily identified. Similar results were observed on wood and HDPE coupons; biofilm formation covered the entire surface. Interestingly, after 96-hour smooth sections of biomass separated by cracks and crevices were observed on wood and HDPE. These differences in surface characteristics may have influenced biofilm formation and persistence as surface type significantly impacted biofilm survivability during sanitation ($P<0.05$).

4.4 Discussion

Biofilms are more tolerant to sanitizers than planktonic cells which makes controlling them a challenge for the food industry (Park et al., 2012; Wang et al., 2013). It is apparent that *Salmonella* has the ability to attach and form robust biofilms on various surfaces such as stainless steel and plastic (Patel et al., 2013; Wang et al., 2022). Currently, ASTM international does not provide a standard to test disinfectant efficacy specifically for *Salmonella* biofilms. However, ASTM international provides a standard method (ASTM E2871-19) to grow and test

disinfectant efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms grown in a Center for Disease Control and Prevention (CDC) biofilm reactor and is the only method recommended by the EPA to test disinfectant product claims against bacterial biofilms (ASTM, 2019; EPA, 2023). This method was followed in the current study to evaluate the effectiveness of five commercially available sanitizers to control *Salmonella* biofilms on common food contact surfaces used in the fresh produce industry. My results showed the interaction between sanitizer, application time, and surface type is important to consider when implementing cleaning and sanitation protocols on harvesting equipment.

Effective cleaning and sanitation of produce harvest equipment and tools is important as they can harbor pathogens and contaminate produce during harvesting (Carstens et al., 2019). Newman et al. (2017) evaluated the microbial load of produce equipment in packing facilities and included harvest bins used to transport produce from the field to packing house. A total of 86 bins were swabbed for coliform bacteria and resulted in 22% prevalence. Conveyor belts showed the highest prevalence of coliforms with 56% testing positive. Duffy et al. (2005) sampled packing house equipment and reported 2.1% of sites positive for *Salmonella*. These observations suggest that the improvement of cleaning and sanitation protocols are needed in the produce industry, justifying the need for the study.

Biofilm forming capabilities are suggested to influence persistence and resistance to sanitizers (Obe et al., 2021; Wang et al., 2017). Several studies suggested that isolation source can influence biofilm growth and attachment. Patel et al. (2013) evaluated the biofilm formation of *Salmonella* isolates from produce and poultry origins. Similar to my research, Patel et al. (2013) used a CDC biofilm reactor and grew biofilms on stainless steel and polycarbonate surfaces. *Salmonella* isolates of produce origin generated significantly more biofilm load (5.53-

6.84 log CFU/cm²), as compared to biofilm from poultry isolates (3.84-6.38 log CFU/cm²).

However, it was out of the scope of my research to evaluate individual biofilm formation capabilities for the selected strains.

One of the goals of my research was to understand if sanitizer efficacy was influenced by surface type. I observed that surfaces significantly ($P < 0.05$) affect PAA, chlorine and SDC efficacy. Zhang et al. (2019) suggests that chlorine may be more effective at killing thin biofilms whereas PAA may be more effective at killing thick biofilms. My research supports this observation as PAA was equally or more effective in controlling mature *Salmonella* biofilms than chlorine. As observed in the SEM images, the structure of the biofilms formed varied based on surface type, supporting the difference of efficacy among sanitizers. Chlorine has been shown to react rapidly with the biofilm matrix and have reduced penetration; whereas PAA reacts slowly and its penetration rate may be impacted less by the biofilm matrix (Zhang et al., 2019). Chlorine is a strong oxidizer and economical which has made it a popular antimicrobial in the food industry. However, chlorine is highly impacted by pH, organic matter, and may form toxic gases at a low pH (Gomez & Vicente, 2012; Marriott & Gravani, 2006). The efficacy of chlorine on *Salmonella* biofilms formed on stainless steel and plastic was previously investigated (Byun et al., 2021). *Salmonella* biofilms grown on stainless steel and plastic were treated with 100ppm for 1-minute resulted in reductions of 3.77 log CFU/cm² and 3.50 log CFU/cm², respectively (Byun et al., 2021). I observed a slightly greater reduction when chlorine was applied on HDPE for 1-minute, nevertheless a much higher concentration of chlorine (500ppm) was applied.

My research is one of the first evaluating the efficacy of SDC on *Salmonella* biofilms. SDC is a non-oxidative sanitizer that disrupts cell membrane proteins and results in cell death (Pure Bioscience Inc., 2016). Based on my results, SDC was the least effective sanitizer in

reducing *Salmonella* biofilms. Also, Manuel et al. (2017) observed minimal reduction against human norovirus on stainless steel when SDC was applied. In addition to disrupting the cell membrane, SDC may also be accepted into the cell as a ‘food source’ and cause cell death (Pure Bioscience Inc., 2016). The metabolic activity of cells within biofilms differs based on location within the EPS matrix; cells near the top are more metabolically active than those near the bottom (Li, et al., 2020). Therefore, I hypothesized that the effect of this SDC may be limited to metabolically active cells near the top of the biofilm thus, contributing to the overall biofilm resistance.

Extensive research is available for chlorine dioxide (ClO₂) gas applications, as it can readily penetrate and inactivate biofilms on food contact surfaces and raw produce. Trinetta et al., (2012) observed complete inactivation of *L. monocytogenes* biofilm on meat slicers and peelers when 2mg/l ClO₂ was applied for 30-minute. *Salmonella* biofilms were completely inactivated at 50ppm within 20-minute (Kim & Park, 2022). The current study achieved complete inactivation when 100ppm ClO₂ was applied for 24-hour on all surfaces tested, demonstrating its ability to penetrate the biofilm matrix (Nam et al., 2014; Prodduk et al., 2014), independently of the surface type (Kim & Park, 2022).

The efficacy of steam was also evaluated. Steam is a non-chemical intervention generated by heating water to a high temperature under pressure. When applied steam rapidly increases the temperature of the surface (Ban et al., 2014) and causes cell death. Kozempel & Goldberg (2003) suggest steam can more effectively penetrate small pores and lesions on surfaces whereas liquid sanitizers may be limited due to surface tension. Steam has been shown to be an effective intervention to control *Salmonella* biofilms on PVC and stainless-steel surfaces (Ban et al., 2014). Similar results were obtained in my research when steam was applied for 1 min. In

another study conducted by Song et al., (2012) *E. coli* biofilms grown on polycarbonate surface in a CDC biofilm reactor were treated with steam for 10 sec: more than a 6 log reduction was observed. My research agrees that steam is an effective intervention to control biofilms. Nevertheless, since the intervention was applied to a small area for an extended period (2 min), further experiments are necessary to confirm the efficacy of this sanitizer when applied into industry conditions.

4.5 Conclusion

Based on the results of the current study, I conclude the differences in sanitizer efficacy are probably due to the biofilm structure formed on the different surfaces used in this study. The rough/porous nature of nylon and wood, as compared to the smooth surface structure of HDPE might offer hiding niches for cells to aggregate; therefore, making it difficult for sanitizers to reach. These results show the interactions between surface materials, sanitizers, and application times should be accounted for when selecting cleaning and sanitation strategies.

Figures and Tables

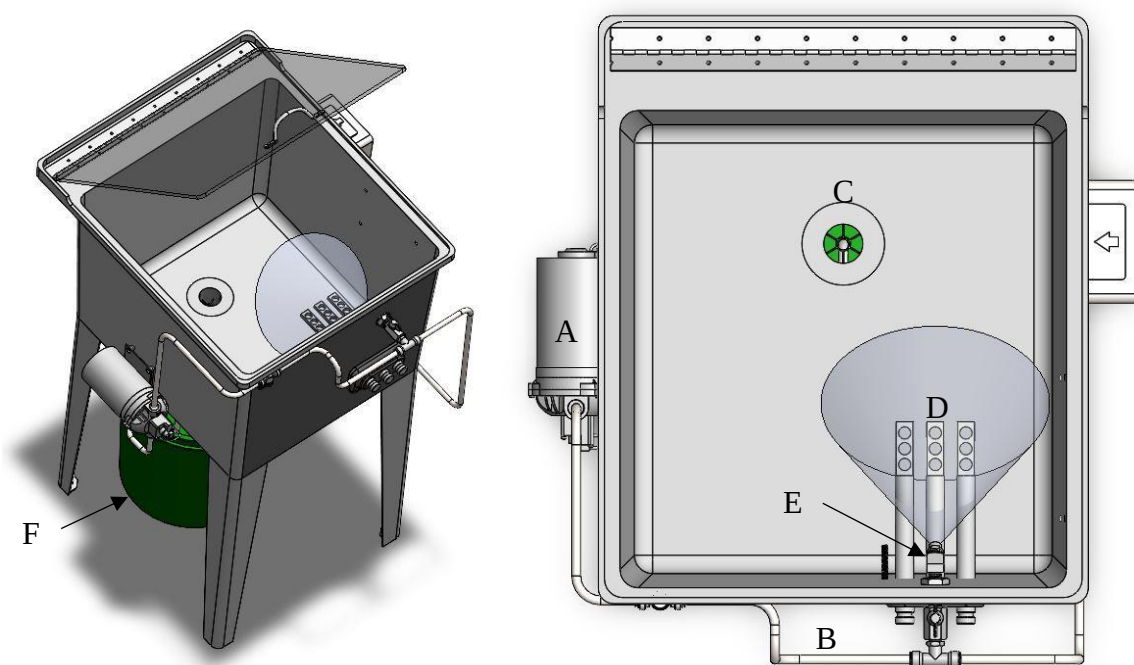


Figure 4.1 Benchtop application system, consisting of a pump (A), tubing (B), waste drain (C), rod ports (D) and spray nozzle (E). Sanitizer was pumped to the spray nozzle which evenly coated the coupons. Sanitizer then drained into a separate waste container (F).

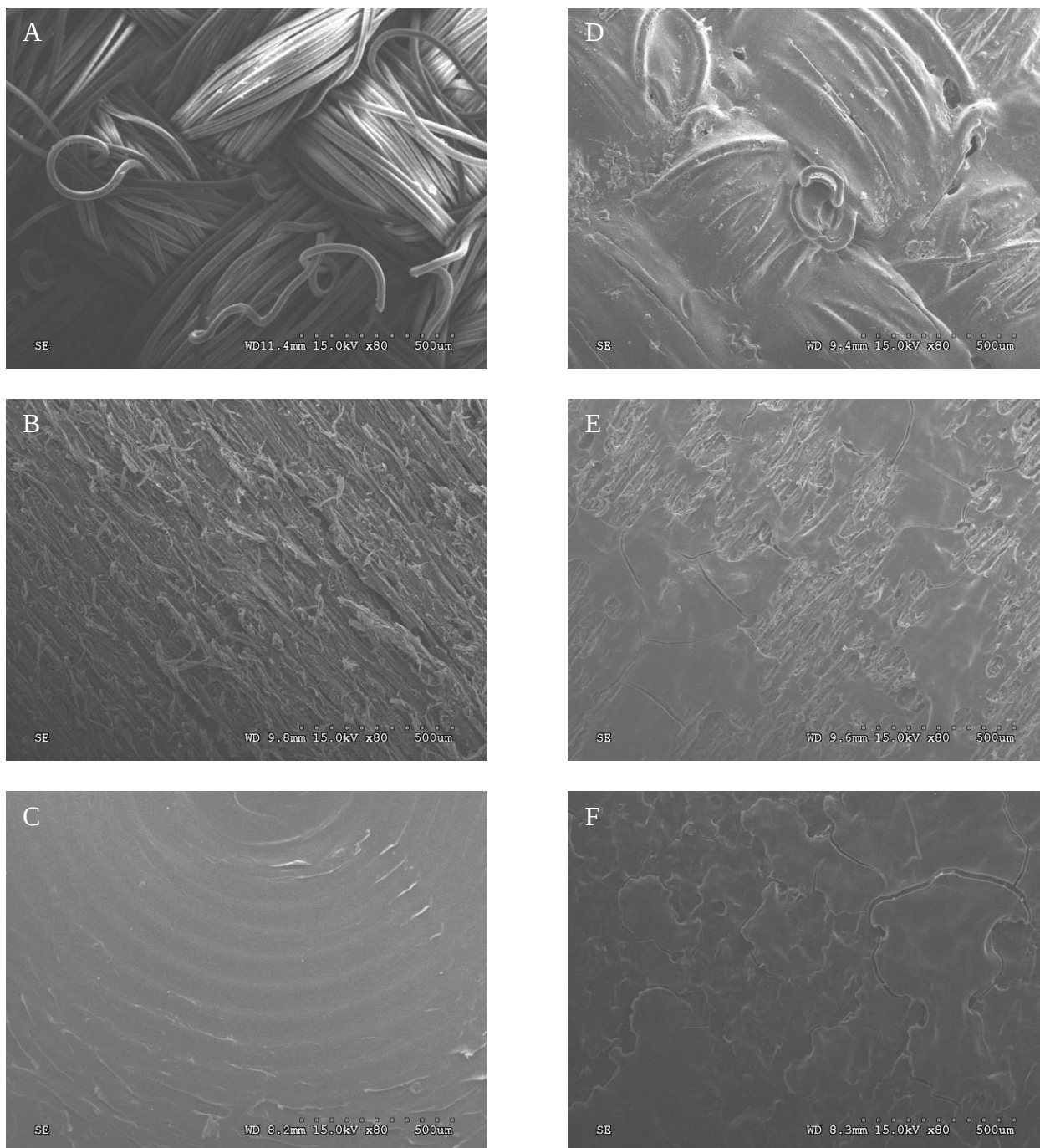


Figure 4.2 Scanning electronic microscope (SEM) images of clean uninoculated surfaces (A) nylon, (B) wood, and (C) high-density polyethylene. SEM images of 96-hour biofilm on nylon (D), wood (E), and high-density polyethylene (F).

Table 4.1 Type 3 fixed effects comparing experimental factors when *Salmonella* biofilms were exposed to 500 ppm free chlorine, 500 ppm peroxyacetic acid (PAA), 75psi steam, and 5% silver dihydrogen citrate (SDC) for 1- or 2-minute and 100 ppm chlorine dioxide gas for 24-hour.

Effect	Pr>F
Sanitizer	<.0001
Application Time	<.0001
Sanitizer*Application Time	<.0015
Surface	<.0001
Sanitizer*Surface	<.0001
Application Time*Surface	<.0023
Sanitizer*Application Time*Surface	<.0001

Table 4.2 Log CFU/coupon of recovered cells on nylon, wood, and HDPE after application of sanitizer treatments (PAA, chlorine, SDC, steam, and chlorine dioxide gas). The following counts were recovered from untreated control coupons with mature biofilms after 96 hours: 9.59±0.29, 9.34±0.40, and 8.77±0.34 log CFU/coupon on nylon, wood, and HDPE, respectively.

Sanitizer	Surface	Log CFU/coupon		
		1-minute	2-minute	24-hour
PAA	Nylon	6.09±1.29 ^{A1b}	3.00±0.97 ^{B1b}	-
	Wood	6.41±0.38 ^{A1a}	4.90±1.25 ^{B2b}	-
	HDPE	2.84±0.65 ^{A2b}	2.62±0.44 ^{A1a}	-
Chlorine	Nylon	7.44±1.15 ^{A1a}	6.54±0.77 ^{B1a}	-
	Wood	7.07±0.49 ^{A1ab}	6.91±0.72 ^{A1a}	-
	HDPE	4.14±0.66 ^{A2a}	3.18±0.74 ^{B2a}	-
SDC	Nylon	8.55±0.50 ^{A1c}	7.87±0.28 ^{B1c}	-
	Wood	7.49±0.41 ^{A2b}	7.42±0.44 ^{A1a}	-
	HDPE	5.96±0.44 ^{A3c}	4.82±0.60 ^{B2b}	-
Steam	Nylon	3.17±0.87 ^{A1d}	2.80±0.50 ^{A1b}	-
	Wood	2.73±0.64 ^{A1c}	2.52±0.22 ^{A1c}	-
	HDPE	2.72±0.77 ^{A1b}	<2.40±0 ^{A1a}	-
Chlorine dioxide gas	Nylon	-	-	<2.40±0 ¹
	Wood	-	-	<2.40±0 ¹
	HDPE	-	-	<2.40±0 ¹

Capital letters compare application time within the same surface type and sanitizer for statistical difference by row. Different numbers within the same sanitizer and application time indicate a significant difference among surfaces. Different lower letters were used to compare sanitizer efficacy for the same application time and treated surface.

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Chapter 5 - Conclusion

The ability of *Salmonella* to form biofilms in food processing environments is a significant food safety concern as biofilms have been shown to be more resistant to sanitizers than their planktonic counterparts.

My research shows:

- Surface type influences the attachment, structure, and physiochemical properties of *Salmonella* biofilms;
- Sanitizer efficacy is influenced by surface type, sanitizer, and application time.

Overall, the differences in sanitizer efficacy are probably due to the diverse biofilm structures formed on the surfaces evaluated. Therefore, produce growers should select food contact surfaces with care to minimize *Salmonella* attachment and subsequent biofilm formation; and evaluate the interactions between surface type, sanitizer, and application time when implementing cleaning and sanitation protocols.

