Development of *Listeria monocytogenes* biofilms in a CDC biofilm reactor and investigation of effective strategies for biofilm control in food processing environments

by

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Abstract

Listeria monocytogenes is one of the most concerning threat for the food industry. This pathogen has shown niche adaptation and ability to form biofilms. Even if sanitation practices can minimize the risk of pathogen survival, difficult-to-clean sites remain high-risk areas. Chemical sanitizers combined with non-thermal processing technologies might represent an effective way to control *L. monocytogenes* biofilm formation.

The main objectives of this study were to: 1) understand *L. monocytogenes* biofilm formation ability under different conditions of material, temperature, and nutrients; 2) evaluate the effect of several chemical sanitizers alone or in combination with UV light to control biofilms; and 3) study the transcriptional response of *L. monocytogenes* biofilm to sublethal conditions of chemical sanitizers.

A CDC Biofilm Reactor was used to grow 4-days-old multi-strain *L. monocytogenes* biofilms on stainless steel and polycarbonate coupons under dynamic conditions using TSBYE (Tryptic Soy Broth + 0.6% Yeast Extract) or BHI (Brain Heart Infusion) as media culture at 30 °C or 37 °C incubation temperature. Biofilms grown at 30 °C in TSBYE on stainless steel reached higher cell counts (8.14 log CFU/cm^2). These conditions were selected for subsequent experiments. Biofilm survivability was investigated after 10 min exposure to lactic acid (4%), peracid (200 ppm), quaternary-ammonium (400 ppm) alone or in combination with UV light (254 nm) for 15 or 30 min. Sequential treatments effect was also evaluated. Control biofilms reached 6 log CFU/cm^2. Reductions ranging from 2.6 to 3.6 log CFU/cm^2 were observed with chemical sanitizers, while a maximum of 1.8 log CFU/cm^2 reduction was recorded after UV-C treatment alone. Combined treatments showed enhanced effect and their application sequence

was significant for lactic acid and peroxyacid ($P < 0.05$). Finally, biofilm RNA was preserved for transcriptomic analysis.

The present research represents an initial framework to develop *L. monocytogenes* biofilms under dynamic condition using the CDC Biofilm Reactor. Also, it offers a preliminary understanding of *L. monocytogenes* biofilm response to chemical sanitizers and UV light supporting the development of effective intervention strategies to control this pathogen in food processing environments.

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Dedication

I would like to dedicate my thesis to *La Morenita del Tepeyac* who has always accompanied me; to my parents and siblings that have encouraged and inspired me; and to my boyfriend Jorge that has believed in me and supported me along the way.

Chapter 1 - Literature Review

1.1 Biofilm: Definition and its characteristics

Biofilms are defined as a community of microorganisms living in a sessile form, irreversibly attached to a substratum or interface and to each other by an extrapolymeric substance (Agle, 2007; Da Silva & De Martinis, 2013). Van Leeuwenhoek was the first scientist who observed biofilms on the surface of teeth and his work was followed by many other researchers as Heukelekian and Heller (1940); they investigated that the incorporation of surfaces to marine microorganisms enhanced bacterial growth and activity. Zobell (1943) showed that the number of bacteria was higher in surfaces than in the surrounding seawater. Jones et al. (1969) utilized microscopy techniques (scanning and transmission electron) to study biofilms on trickling filters from a wastewater plant (R. M. Donlan, 2002). Nonetheless, a better description and understanding of biofilms was only possible with the invention of the electron microscope because of high-resolution images with higher magnifications than the light microscope (R. M. Donlan, 2002).

In nature, microorganisms can live in a free-floating form known as planktonic or as an anchored-sessile form known as biofilm (Moldenhauer, 2018). Inside a biofilm, bacteria are densely packed into a community enclosed by a matrix or "slime" known as the extracellular polymeric substance (EPS) that provides protection to the community against external stresses (Flemming, 2016). The biofilm can be attached to a biotic or abiotic surface, and are often formed by multiple species (e.g. *Pseudomonas aeruginosa* and *Klebsiella pneumonia*) or even multi kingdom (e.g. *Staphylococcus aureus* and *Candida albicans*) (Burmølle, Ren, Bjarnsholt, & Sørensen, 2014; Jefferson, 2004; Nadell, Xavier, & Foster, 2009).

Jefferson (2004) described four main reasons why bacteria produce biofilms: defense, favorable habitat, community, and default mode. When living as a community bacteria gain resistance to external challenges such as changes in temperature, shear force, antimicrobial compounds, and UV light damage (Borucki, Peppin, White, Loge, & Call, 2003; Bremer, Flint, Brooks, & Palmer, 2015). When living in biofilm, bacteria can also undergo horizontal gene transfer through conjugation or transformation. This process often results in a positive effect for the microorganisms because it facilitates communication, resistance genes sharing, and other characteristics that make the biofilm stronger (Agle, 2007; Molin & Tolker-Nielsen, 2003; P. Stoodley, Sauer, Davies, & Costerton, 2002).

The process of forming a biofilm starts with an initial contact and further attachment to a surface (Figure 1.1a). This contact is a reversible step and will depend on the topography of the surface, environmental conditions such as temperature, pH, nutrient availability, shear force, quorum sensing, and EPS production ability (Agle, 2007; Bremer et al., 2015). The initial attachment of the microorganism to the surface can be mediated by three mechanisms: (A) by displacing the attached cells by twitching motility through the surface; (B) by multiplying through binary division of the cells, the new daughters will grow outwards and upwards colonizing the surface; and (C) by recruiting new cells from fluids to the developing biofilm, dragging and incorporating them to the community (P. Stoodley et al., 2002). The second phase towards biofilm formation (Figure 1.1b) is the aggregation of the cells into microcolonies that are in a semi-permanent association with the surface. The rate of attachment and strength will depend on factors such as the physical and chemical properties of the surface and environmental conditions (Agle, 2007; Bremer et al., 2015). Once the community is established, bacteria start producing a polymeric substance that contains polysaccharides, proteins, genetic material, and

environmental particles such as food soils (Bremer et al., 2015). The EPS causes an irreversible attachment strengthening the surface-biofilm interaction (Agle, 2007). Finally, the microcolonies will turn into macrocolonies separated by channels in which water, nutrients, and oxygen will be distributed throughout the biofilm (Agle, 2007; Bremer et al., 2015; P. Stoodley et al., 2002).

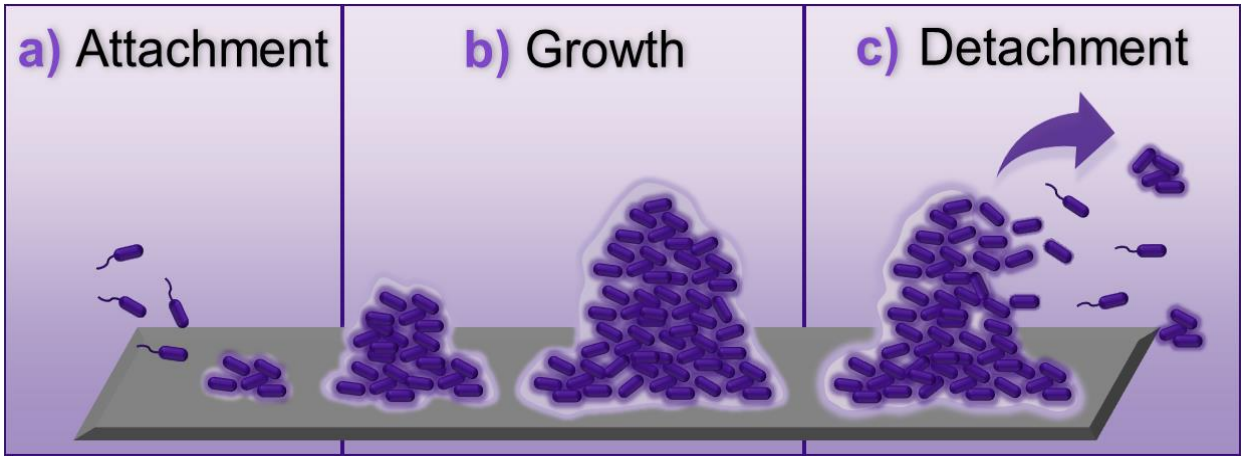


Figure 1.1. Biofilm development process (a) initial cell attachment to a surface, vegetative cells become sessile and biofilm formation is still reversible. (b) cells divide and multiply to grow the biofilm wide-wise and in a three-dimensional structure. (c) cell detachment is the last phase to colonize different locations.

Biofilms can face different environmental challenges that lead to a detachment phase (Figure 1.1c). Oxygen limitation, starvation, or reduction in the EPS production cause the dispersal of the cells. Moreover, external circumstances such as high fluid shear, abrasion, or cleaning and sanitation may also induce detachment (Bremer et al., 2015; Spormann, 2008; P. Stoodley et al., 2002). The main mechanisms through which biofilm can undergo detachment are erosion, sloughing, and seeding (Bremer et al., 2015). The regulation of this process is dependent on the physiological state of the cells and the attachment mechanism that the biofilm utilized in

its initial formation. Attachments mediated by EPS are more difficult to switch and only sloughing detachment will induce dispersal of the cells (Spormann, 2008).

1.2 Role or Extracellular Polymeric Substance (EPS)

The extracellular polymeric substance plays an essential role in the development and maturation of biofilms, this matrix can be considered the "house of biofilm cells" (Flemming, 2016). The EPS constitutes around 50-90% of the organic matter in the biofilm(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002)(R. M. Donlan, 2002), and is composed primarily by polysaccharides but also proteins, nucleic acids and fibers (Bremer et al., 2015; R. M. Donlan, 2002; Xavier & Foster, 2007). The EPS, also known as "slime", retains large amounts of water preventing the desiccation of the biofilm (R. M. Donlan, 2002; Flemming, 2016). Among its multiple functions, the EPS serves as (Da Silva & De Martinis, 2013; Flemming, 2016; Nadell et al., 2009; Rendueles & Ghigo, 2015):

- Protection against external threats such as antibiotic compounds, predator grazing, environmental changes;
- Mechanical stability;
- Resistance against physical and chemical aggressions;
- Cohesion and adhesion within cells and to a surface;
- Mediator for genetic information exchange.

The secretion of EPS varies among microorganisms and generally its density increases as the biofilm ages (R. M. Donlan, 2002). In a multi-species biofilm, the presence of EPS-producer

and non-producers can lead to different interactions. Studies have shown that EPS-null mutants are defective in forming a biofilm and EPS non-secretor did not attach to surfaces, neither they bind together efficiently (Nadell et al., 2009). Another experiment (Xavier & Foster, 2007) with computer-based simulation compared the dominance of EPS producers versus non-producers in a biofilm. Authors showed that under high densities of EPS, the non-producers dominated due to a higher rate of growth. Meanwhile, under low densities of EPS, the EPS-producers dominated due to competitive advantage and higher fitness.

1.3 Biofilm formation devices

It wasn't until the 1970s when scientists started looking for methodologies to grow biofilms under laboratory conditions and study them. Two main conditions to grow biofilms have been used; the first one is static where there is low or no shear force and cells undergo normal growth phases as in planktonic cultures. The second is dynamic (continuous flow) where the planktonic cells are being washed-out and only the cells capable of adhering to surfaces remain (Fletcher, 1977; Franklin, Chang, Akiyama, & Bothner, 2015; Kornegay & Andrews, 1968).The main biofilm-formation devices are described below.

1.3.1 Microtiter plates

This method was originally developed in 1977 by Madilyn Fletcher at the University College of North Wales in Gwynedd, UK to study bacterial attachment to polystyrene in Petri dishes (Fletcher, 1977). Today, a 96-well microtiter plate (Figure 1.2a) is used to assess the attachment of bacteria onto abiotic surface materials (Merritt, Kadouri, & O'Toole, 2005). Biofilms are formed under static conditions in the wells for a determined period that can range from hours to days depending on the goal of the study; then the planktonic cells are washed away

to leave the adhere bacteria behind (Azeredo et al., 2017; Merritt et al., 2005). Attached cells are subsequently stained and visualized under a microscope. This method allows for direct enumeration of biofilm, colony biofilm assay, air-liquid interface assay, and microscopic visualization (Merritt et al., 2005). Among the advantages of this method are its high-throughput applications, non-invasive microscopic visualization, and its relatively inexpensive cost as only a plate reader machine is needed. Some of the disadvantages include inaccurate measurement of loosely attached cells and prone to sedimentation. Early stages of biofilm formation are not suitable in this method (Azeredo et al., 2017).

1.3.2 Calgary device

This device is a modified method of the microtiter plate and it was first introduced in 1999 by a group of scientists from the University of Calgary in Alberta, Canada (Ceri et al., 1999). It consists of a 96-well plate with 96 pegs in the coverlid (Figure 1.2b), where bacteria attach and form the biofilm under batch conditions. Low shear force can be provided by placing the plate on an orbital shaker (ASTM, 2017c). This device is typically used to test minimal inhibitory concentration of antimicrobial compounds. Several products and multiple concentrations can be tested at the same time (ASTM, 2017c; Azeredo et al., 2017). The Calgary device is used for the MBEC™ (Minimum Biofilm Eradication Concentration) assay which is a standard method approved by ASTM International under the designation E2799 (ASTM, 2017c). This method includes all advantages of the microtiter plate with the addition that it overcomes the problem of biofilm formed by sedimented cells at the bottom of the microtiter plate. Nevertheless, one disadvantage is the difficulty of collecting individual pegs for enumeration, since sonication may not detach strongly attached cells (Azeredo et al., 2017).

1.3.3 Biofilm ring test

The biofilm ring test was first presented in 2007 by a group of French scientists from Champanelle, France (Chavant, Gaillard-Martinie, Talon, Hébraud, & Bernardi, 2007). This device also utilizes a 96-well plate and the bacterial culture is mixed with magnetic microbeads before filling the wells. After incubation time, the microtiter plate is placed in a "Block Test" that contains 96 magnets that will attract the free beads (not enveloped by a biofilm matrix) to the center of the well creating a dark spot (Figure 1.2c) (Chavant et al., 2007). The wells that contain the biofilms will not show the dark spot due to the microbead being entangled in the biofilm matrix (Azeredo et al., 2017; Chavant et al., 2007). This method is used to study the early stages of biofilm development and therefore requires less time than other devices. Furthermore, this method does not need further staining or washing steps (Azeredo et al., 2017). The advantages of this method include the rapid high-throughput, application for early stages of biofilm formation and to loosely attached cells. The main disadvantages are the requirement for specific equipment (magnetic device and scanner) and that it is useful only for early stages of biofilm formation (Azeredo et al., 2017).

1.3.4 Drip flow biofilm reactor

This device was developed in 2009 by scientists at the Center for Biofilm Engineering at Montana State University in Montana, U.S.A. (Goeres et al., 2009). This reactor simulates different environments such as conveyor belts or pipes in food-processing plants, but also catheters in medical settings (Goeres et al., 2009). Current models contain four or six independent channels (Figure 1.3a) that hold a coupon made of different materials (e.g. stainless steel, polycarbonate, glass). The culture media and bacterial inoculum are added to each channel independently (BioSurface Technologies Corp., 2019; Goeres et al., 2009). Biofilms are formed

in the air-liquid interface under low shear conditions (BioSurface Technologies Corp., 2019). This device can be used for enumeration assays, microscopic visualization, and disinfection or antibiotics tests (Azeredo et al., 2017; Goeres et al., 2009). The drip flow biofilm reactor is a standard method approved by ASTM International under the designation E2647 (ASTM, 2013a). The advantages are that it allows several biofilms to grow from different inoculum and different culture media, compatibility of various coupon geometry, and allowance for biofilm visualization. The main disadvantage of this device is the heterogenicity of the biofilm developed (Azeredo et al., 2017).

1.3.5 Rotary biofilm reactors

The invention of the rotary biofilm reactors can be attributed to Kornegay and Andrews in 1968 in the U.S.A (Kornegay & Andrews, 1968). There are different types of rotary devices such as the rotary annular reactor, rotary disk reactor (Figure 1.3b right), or the Centers for Disease Control and Prevention (CDC) Biofilm Reactor (Figure 1.3b left) (Azeredo et al., 2017). The rotary annular reactor consists of a static outer cylinder and a rotating inner cylinder that create turbulent flows and shear forces (Azeredo et al., 2017). This device mimics water distribution systems or industrial piping systems (BioSurface Technologies Corp., 2008). The rotary disk reactor is a vessel that contains a magnetic disk that holds several coupons, the magnet allows for the rotation of the disks creating medium shear stress (Azeredo et al., 2017). This device is accepted as a standard method by ASTM International under the designation E2196 (ASTM, 2017a). The advantages of this method include availability of several sampling units, feasibility of using several surface materials at the same time, and growth under high shear forces. The disadvantages include the use of only one inoculum at a time, the fixed geometry of the coupons that depends on the reactor design, and the high cost (Azeredo et al., 2017).

The CDC Biofilm Reactor was developed by the Centers for Disease Control and Prevention to study biofilm formation of *Legionella pneumophila*; nonetheless, due to its versatility, it can be used to examine biofilms of different bacterial species (ASTM, 2017b). The CDC Biofilm Reactor provides high shear conditions and a continuous flow environment. Factors like temperature, nutrient source and concentration, shear force, material, and flow rate can be modified to simulate a specific environment (BioSurface Technologies Corp., 2018). This reactor consists of a 1 L glass vessel with an effluent outlet at ~350 ml, an inoculation port, a baffled stir bar to provide high shear force, and a polyethylene lid that holds eight independent rods. Each rod supports three removable coupons for biofilm growth surface (BioSurface Technologies Corp., 2018).

1.3.6 Flow cell chamber

The use of this device was first presented in 1989 by a group of scientists from the University of Saskatchewan in, Canada. This method allows for *in situ* examination of the biofilm, and consist of a small chamber with an upper window where a glass cover slide is placed to allow for microscopic visualization (Figure 1.3c) (Franklin et al., 2015). It contains channels through which media is continuously pumped into the chamber. This device allows for non-destructive microscopic visualization throughout the process of biofilm formation (Azeredo et al., 2017). Different coupon surfaces or even capillary tubes can be placed inside the chamber to study biofilm development (Franklin et al., 2015). This method is a direct inspection of the biofilm growth process and allows for non-destructive microscopic on-line *in situ* visualization. Disadvantages include the need for special equipment (e.g. peristaltic pumps, microscopes), and does not permit direct access to the cells (Azeredo et al., 2017).

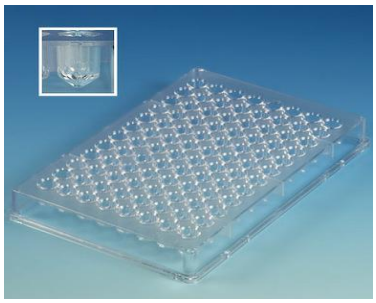
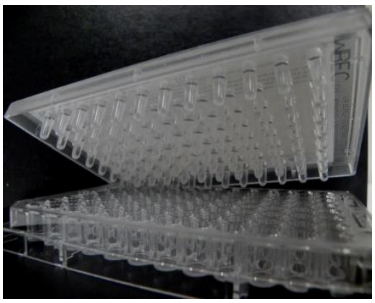
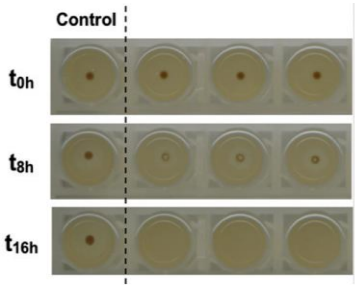
a. Microtiter plate	b. Calgary device	c. Biofilm ring test
		
Source: Stellar Scientific	Source: Innovotech	Source: Azeredo et al., 2017

Figure 1.2. Devices used to grow biofilm under static conditions in a laboratory setting.

a. Drip flow biofilm reactor	b. Rotary biofilm reactors	c. Flow cell chamber
		
Source: BioSurface Technologies Corp.	Source: BioSurface Technologies Corp.	Source: BioSurface Technologies Corp.

Figure 1.3. Devices used to grow biofilm under dynamic conditions in a laboratory setting.

1.4 Strategies to control biofilm in the food industry: cleaning and sanitation

The formation of biofilms in food processing facilities represent a hazard for food safety, since they are more difficult to eradicate as compared to planktonic cells. Most processing plants rely on effective cleaning and sanitation programs to prevent contamination of the final product. These measures are needed to guarantee the creation of safe environments where food products are processed. Cleaning involves the complete removal of any food soil from a surface using

appropriate solutions applied either manually or mechanically using the cleaning-in-place (CIP) or cleaning-out-of-place (COP) technique (Ministry of Agriculture Food and Rural Affairs, 2006; Schmidt, 2009). After cleaning, a sanitation step is performed. Sanitation requires the application of techniques to treat a clean surface creating a hygienic and healthful environment (Marriot & Gravani, 2006). An ideal sanitizer should fulfill the following properties (Marriot & Gravani, 2006):

- Have a broad and uniform spectrum for microbial destruction;
- Be resistant to different environmental stresses such as temperature and pH;
- Be non-toxic and non-irritating;
- Have an acceptable odor or no odor;
- Be stable in concentrated and final dilution form (for chemical sanitizers).

Sanitation can be achieved through different methods that can be classified in thermal, radiation, high hydrostatic pressure, or chemical sanitation (Marriot & Gravani, 2006; Schmidt, 2009). The strategy selected for controlling microbial growth will depend on the food product, facility design or accessibility. Based on their effectiveness, sanitizing agents can be classified as: (1) sterilant, any substance or method that destroy all forms of microbial life (e.g. heat or autoclaving); (2) disinfectant, any agent that destroys vegetative bacteria and infectious fungi on inert surfaces (e.g. general households disinfectants); (3) sanitizer, any substance that does not completely eliminates microbial contaminants but reduces its load to levels that are considered safe from a health point of view (Marriot & Gravani, 2006; Schmidt, 2009).

1.5 Impact of *Listeria monocytogenes* to the food industry

Listeria monocytogenes is considered one of the top food safety challenges in the food industry (Matthews, Kniel, & Montville, 2017). This pathogen is considered an ubiquitous microorganism that can be found in the soil, wastewater, decaying vegetation, silage, and inert surfaces (Da Silva & De Martinis, 2013). *L. monocytogenes* can be introduced into a food-processing plant by many routes: soil on worker's shoes, clothing or vehicles, or through contaminated raw vegetables or animal tissues (Matthews et al., 2017). Once *L. monocytogenes* has entered a processing facility it can attach to different surface materials such as stainless steel, glass or rubber (Matthews et al., 2017). It has the ability to form biofilms that might eventually become resident in the processing plant (Kathariou, 2002). Once formed, biofilms are difficult to control since they are usually formed in difficult-to-clean sites such as drains, floor, conveyor belts, or even processing equipment (Da Silva & De Martinis, 2013; Matthews et al., 2017). Tan et al. (2019) collected environmental samples from the floor under a conveyor belt that transported tree fruits that tested positive for *L. monocytogenes*. The formation of biofilms on different materials commonly used in food processing facilities has been studied, materials such as stainless steel, rubbers, polypropylene, polyurethane and other polymers were addressed by Beresford et al., 2001. Researchers have also studied many factors that affects the characteristics of *L. monocytogenes* biofilms such as the serotype and strain origin, motility, temperature, nutrient availability and others (Kadam et al., 2013; Lemon, Higgins, & Kolter, 2007; Pang, Wong, Chung, & Yuk, 2019).

From the 1980s onwards, several outbreaks of listeriosis were reported; thus, the Food Safety and Inspection Service (FSIS) and the U.S. Food and Drug Administration (FDA) developed risk assessment strategies to address the control of *L. monocytogenes* in processing

plants. In 2003, the FSIS issued the final rule: Control of *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat (RTE) Products also known as "the *Listeria* rule". This rule contains the regulations required to produce safe RTE products. According to this rule, *L. monocytogenes* is a hazard and if the final product contains the pathogen it will be considered adulterated (Food Safety and Inspection Service, 2014). The *Listeria* rule is applied to all facilities that produce RTE products that are exposed to the environment after a lethality step. The FSIS provide three alternative methods for *L. monocytogenes* control:

- Use of a post-lethality treatment (PLT) to reduce or eliminate *L. monocytogenes* AND use of an antimicrobial agent OR process (AMAP) to suppress or limit *L. monocytogenes* growth;
- Use of either PLT OR AMAP;
- Rely on sanitation program to control *L. monocytogenes* in the environment and no PLT nor AMAP are implemented.

Alternatives 1 and 2 have direct effect into the food product, instead, alternative 3 relies on sanitation alone to control the presence of *L. monocytogenes* in the environment. For this reason, facilities that follow alternative 3 require more frequent verification testing on food contact surfaces, usually once a month per line; while those following the alternative 3 must have a strong sanitation plan. Therefore it is essential to understand the susceptibility of *L. monocytogenes* to the different sanitation strategies (Food Safety and Inspection Service, 2014).

1.6 Chemical sanitizers

In the food industry, the use of chemical sanitizers is one of the most frequent practices to reduce microbial load. Any type of sanitizer intended to use in food processing facilities must be

registered and approved by the Environmental Protection Agency (EPA). These sanitizers must reduce at least 99.9% (3 logs) of bacterial load on food-contact surfaces and at least 99.999% (5 logs) on food contact surfaces (Sanders, 2003). The acceptable levels of sanitizers residues are determined by the FDA (Sanders, 2003). Chemical sanitizers are classified according to the active ingredient that kills microorganisms, among the most commonly used are: chlorine compounds, iodine compounds, quaternary ammonium compounds, acid sanitizers and peroxyacid sanitizers (Marriot & Gravani, 2006). Table 1.1 summarizes the physical, chemical and biological characteristics of these sanitizers (Schmidt, 2009). Several researchers, have studied the effect of sanitizers such as quaternary ammonium (Belessi, Gounadaki, Psomas, & Skandamis, 2011; Korany et al., 2018; Pang et al., 2019), peroxy acids (Belessi et al., 2011; Fatemi & Frank, 1999; Korany et al., 2018), and lactic acid (Ban, Park, Kim, Ryu, & Kang, 2012; Yang, Kendall, Medeiros, & Sofos, 2009) against *L. monocytogenes* biofilms. These sanitizers are discussed below.

1.6.1 Lactic acid as sanitizer

Lactic acid is an organic acid-type sanitizer, usually colorless and odorless. This acid is considered, along with other acid sanitizers, to be biologically active and toxicologically safe (Boomsma, Bikker, Lansdaal, & Stuut, 2015; Marriot & Gravani, 2006). Lactic acid is effective against both Gram positive and Gram negative bacteria; although, Gram positive are often more susceptible to this compound due to the absence of outer cell wall (Stanojević-Nikolić et al., 2016; Wang, Chang, Yang, & Cui, 2015). The mode of action of this acid is related to the disruption of the cell membrane and leakage of proteins out of the cell (Wang et al., 2015). Lactic acid enters the microbial cell perturbing the cytoplasmic membrane and acidifies the cytoplasm. The sudden change of pH affects the proton motive force decreasing the available

energy for the cells to grow (Boomsma et al., 2015; Hanna & Wang, 2003; Reis, Paula, Casarotti, & Penna, 2012). Wang et al. (2015) showed the impact of lactic acid against cells of *L. monocytogenes* which displayed leakage of proteins mostly in the first 2 h after exposure and a disruption of the cell membrane. Lactic acid has been proved to be more effective than other organic acids such as propionic acid, malic acid and citric acid in controlling *L. monocytogenes* in food products (Park et al., 2011). Reductions of 0.15-0.92 logs were observed against *L. monocytogenes* 6-day-old biofilms on stainless steel after exposure to 2% lactic acid for 30 s (Ban et al., 2012). Another study obtained reductions of 3.6 or 2.3 logs of biofilm cells on polyethylene after exposure to lactic acid at 0.18% (Yang et al., 2009).

1.6.2 Quaternary ammonium sanitizers

Quaternary ammonium compounds (QACs) are surface-active agents (surfactants) commonly used to disinfectant walls, floors, and furnishing (Gerba, 2015; Marriot & Gravani, 2006). QACs are effective mostly against Gram positive bacteria and molds but no antimicrobial activity has been reported against spores or bacteriophages (Cramer, 2013). Due to its rapid activity, QACs are not recommended for use in dairy facilities because they could inactivated lactic acid starter cultures used for cheese and other dairy products (Marriot & Gravani, 2006). The mechanism of action of quaternary ammonium is still not fully understood. When a QAC penetrates the bacterial cells, it reacts with the cytoplasmic membrane causing a disruption and leakage of proteins and nucleic acids inducing cells lyse by autolytic enzymes (Gerba, 2015; Marriot & Gravani, 2006). The use of quaternary ammonium as strategy to control biofilms of *L. monocytogenes* have been previously studied. Concentrations between 100-500ppm of QACs have being used to control biofilms grown on different surfaces (i.e. stainless steel, polystyrene) and under different conditions such as temperature or biofilm age. Reductions of 3.0 – 4.0 log

CFU/cm^2 were observed in biofilms treated with quaternary ammonium (Korany et al., 2018; Pang et al., 2019; Poimenidou et al., 2016).

1.6.3 Peroxy acid sanitizers

The peroxy acids (also known as peracids) are a class of chemical compounds that contains an atomic group -OOH and are excellent broad-spectrum sanitizers against spore-former microorganisms, pathogenic bacteria and yeasts (Azanza, 2004; Mohr, 2004). Examples are peroxyacetic acid (POAA) and peroxyoctanoic acid (POOA) which combined have shown a synergistic effect as antimicrobial agent (Azanza, 2004; Oakes, Stanley, & Keller, 1998). Commercial sanitizers combine POAA, POOA and hydrogen peroxide to maximize biocide action of these compounds. POAA and hydrogen peroxide are strong oxidizing agents while POOA breaks down into octanoic acids which acts as a surfactant and modify surface properties (Azanza, 2004). Peroxyacid-based sanitizers have been considered as one of the most effective against biofilms (Fatemi & Frank, 1999; Marriot & Gravani, 2006). Researchers have used POAA at concentrations of 80-160 ppm against biofilms of *L. monocytogenes* achieving reductions of 2.8-4.8 log CFU/cm^2 and POOA at concentrations of 80 ppm reaching 2.5 log CFU/cm^2 reductions (Fatemi & Frank, 1999; Korany et al., 2018; Poimenidou et al., 2016).

Although the use of chemical sanitizers is a common practice in food processing facilities, research in novel strategies and use of hurdle technologies are being carried out to control biofilm formation. The use of steam, ozone, hot water, UV irradiation in combination with chemical sanitizers are some of the alternatives under investigation (Ban et al., 2012; Belessi et al., 2011; M. Kim, Park, & Ha, 2016; Korany et al., 2018).

Table 1.1. Most commonly used chemical sanitizers in the food processing environments and its characteristics.

Properties	Chlorine	Iodophors	Quaternary ammonium	Acid anionic	Peroxyacetic acid
Surface characteristics	<————Needs to be clean before being sanitized————>				
Temperature sensitivity	none	high	moderate	moderate	none
Concentration without rinse	200 ppm	25 ppm	200 ppm	varies	100-200 ppm
Soil affects	yes	moderately	moderately	moderately	partially
pH	not affected	neutral to acid	In most cases is not affected	3.0-3.5	neutral to acid
Hardness of water affects	no	slightly	yes	slightly	slightly
Mode of action	Inhibit glucose oxidation	Inhibit protein synthesis by disrupting bonds	Causes a bacterial cell wall failure	Disrupt cellular functions	Alter bacterial cell membrane and damage DNA

1.7 UV Irradiation

The use of irradiation has been used in food products for controlling microbial pathogens and spoilage microorganisms (Lado & Yousef, 2002). Ultraviolet (UV) light is a type of non-ionizing radiation with an electromagnetic spectrum wavelength between 100 and 400 nm that is subdivided into UV-A (315-400 nm), UV-B (280-315 nm), UV-C (200-280 nm) and vacuum UV (100-200 nm) (Gómez-López, 2012). UV-A is the responsible for tanning human skin, UV-B is causative of skin burns and might eventually lead to skin cancer, UV-C is considered the germicidal range because it effectively inactivates bacteria and viruses (Koutchma, 2014). Several researchers have effectively demonstrated the effect of UV light against bacteria (Gabriel, Ballesteros, Rosario, Tumlos, & Ramos, 2018; M. Kim et al., 2016; Sommers, Sites, &

Musgrove, 2010). The antimicrobial properties of UV-C light are related to the absorption of radiation by the carbon-carbon double bonds in proteins and DNA that leads to electronic excitation of atoms and molecules. These events damage the bacterial DNA by creating dimmers in adjacent pyrimidines, the distortion in the DNA helix prevent its replication, cause mutation and eventually lead to cell death (Gómez-López, 2012; Koutchma, 2014; Snyder, Peters, Henkin, & Champness, 2013; Woodling & Moraru, 2005). The use of UV light as an intervention technique for decontamination of liquid foods, water, food and food-contact surfaces has been approved by the FDA in the Code of Federal Regulations (CFR) Title 21 part 179 section 179.39 (Code of Federal Regulations, 2011). The use of UV light brings many advantages since it is easy to use, waterless, relatively inexpensive, chemical-free and it is considered ecologically friendly (Gómez-López, 2012).

Several researchers have already demonstrated the efficacy of UV-C irradiation against pathogenic bacteria on meats, produce, egg shell surfaces or food-contact surfaces such as stainless steel (Gabriel et al., 2018; M. Kim et al., 2016; Sommers et al., 2010). The use of UV light at 500 $\mu W/cm^2$ against foodborne pathogens such as *L. monocytogenes, S.* Typhimurium, and *E. coli* on stainless steel was reported to achieved reductions of 2.91, 4.4, and 4.7 log CFU/cm^2 respectively (T. Kim, Silva, & Chen, 2002). Biofilms of *Salmonella* spp., *L. monocytogenes* and *S. aureus* on stainless steel surfaces showed reductions > 5 logs after exposure to 0.4 J/cm^2 of UV-C light (Sommers et al., 2010). In a different study, exposure of *L. monocytogenes* biofilms to UV-C light doses of 1800 mW/cm^2 achieved 1.4 log CFU/cm^2 on stainless steel and 1.1 log CFU/cm^2 on egg shells surfaces (M. Kim et al., 2016).

1.8 Hurdle technologies

The concept of "hurdle technologies" was introduced in food preservation with the objective of enhancing the effect of different antimicrobial techniques while preserving the overall quality of food products. In 1995, Leistner and Gorris first used this concept where several factors were combined in order to extend the shelf life of a food product and at the same time preserve its characteristics (Fellows, 2009; Leistner & Gorris, 1995). When using this technique, each hurdle is based on a specific factor and requires a certain amount of energy for the microorganism to overcome each of them. These hurdles may combine principles of temperature, water activity, pH, redox potential, and others. The hurdles used are applied at a low intensity level to preserve the quality of the product and often a synergistic effect toward inhibition or inactivation of microorganisms is displayed (Fellows, 2009; Khan, Tango, Miskeen, Lee, & Oh, 2017). Hurdle technologies can be divided into four groups: (a) physical hurdles that include temperature, radiation, pressure, modified or controlled atmosphere, ultrasonication, and others; (b) physic-chemical hurdles which include factors such as water activity, pH, salt, organic acids, redox potential, etc; (c) biological hurdles such as competitive flora, bacteriocins, starter cultures and antibiotics; and (d) miscellaneous hurdles like free fatty acids, chitosan, and chlorine (European Commission, 1997; Fellows, 2009).

Several researchers have investigated the application of hurdle technologies against biofilms. For example, Ban et al. (2012) combined steam and lactic acid to treat biofilms of *L. monocytogenes*, *E. coli* and *S.* Typhimurium. Berrang, Frank, & Meinersmann (2008) treated *L. monocytogenes* biofilms with chemical sanitizers combined with ultrasonication. Chavant, Gaillard-Martinie, & Hébraud (2004) tested the combination of different sanitizers against planktonic cells and biofilms of *L. monocytogenes.*

The use of UV light as hurdle intervention has also been addressed. In a study by Kim et al. (2016) the use of sodium hypochlorite in combination with UV-C light showed a synergistic effect against biofilms of *L. monocytogenes.* Ha and Ha (2010) observed a synergistic effect of an ethanol-based sanitizer in combination with UV radiation against *B. cereus, S.* Typhimurium, *S. aureus,* and *E. coli*. The use of peracetic acids sanitizers in combination with UV radiation also enhanced the antimicrobial properties of each single intervention (Koivunen & Heinonen-Tanski, 2005). Bacteria sub-lethally injured by the first hurdle (i.e. POOA), do not have time to recover due to the immediate application of the second treatment (i.e. UV), thus leading to a much higher level of reduction as compared to the application of a single intervention (Woodling and Moraru, 2005). Therefore, the use of UV-C irradiation in combination with different chemical sanitizers might represent an efficient alternative to control *L. monocytogenes* biofilms in food processing facilities.

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Chapter 2 - Research importance

It is widely known that *L. monocytogenes* is one of the most concerning foodborne pathogens in food safety. Its ability to grow at refrigeration temperature, tolerate a wide range of pH and salt concentrations gives this bacterium an advantage over other microorganisms. Microbial biofilm-formation ability has gained interest in the scientific community and researchers have been focusing on the enhanced resistance of microorganisms and biofilms to common sanitation practices, especially within the food industry. Understanding the ability of *L. monocytogenes* to form biofilms is a first step towards the development of effective methods to control and prevent contamination of food products. Nowadays there are several approaches to grow biofilms. Factors like temperature, material, nutrient source or shear force directly impact the characteristics of the formed biofilm, and subsequently the effectiveness of the control strategy employed. The most common practice used in food processing facilities to overcome microbial contamination is the use of chemical sanitizers. Nevertheless, due to the increase of antimicrobial resistance in foodborne pathogens the concept of hurdle technologies has been introduced. This approach includes the combination of several interventions to prevent the proliferation of microorganisms. Investigating the possible synergistic effects between different antimicrobial strategies might be the suitable solution to prevent and control biofilms in food processing environments.

For these reasons, the present research aims to:

- Investigate the effect of several growth parameters (i.e. temperature, surface material, and nutrients) on *L. monocytogenes* biofilm-forming ability using a CDC Biofilm Reactor that mimics the environmental conditions of a food processing facility;

- Understand *L. monocytogenes* biofilms susceptibility to different technologies such as chemical sanitizers (quaternary ammonium, lactic acid, peroxy acid) and UV-C light (254 nm) alone or in combination;
- Evaluate the effect of the sequence of treatment application on *L. monocytogenes* biofilm survivability.

Chapter 3 - The Use of a CDC Biofilm Reactor to Grow Multi-Strain *Listeria monocytogenes* Biofilm

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Abstract

Listeria monocytogenes is one of the most concerning pathogens for the food industry due to its ability to form biofilms, particularly in difficult-to-clean sites of processing facilities. There is a current industry-wide lack of data to refer to when selecting a strategy to control *L. monocytogenes* biofilms in the food premises. Many strategies have been developed to study biofilm formation of bacteria; however, few have targeted *L. monocytogenes* biofilms under dynamic conditions. This study addresses the biofilm formation ability of *L. monocytogenes* on stainless steel and polycarbonate under dynamic conditions using TSBYE or BHI as media culture at 30 °C or 37 °C. Higher cell counts were recovered at 30 °C in TSBYE on polycarbonate while lower counts were obtained at 37 °C in BHI on stainless steel ($P < 0.05$). Nonetheless, all factors (temperature, media and material) were statistically significant ($P < 0.05$) and an interaction between temperature and media was observed ($P < 0.05$). To our knowledge, this work represents an initial framework to develop *L. monocytogenes* biofilms under different dynamic conditions. The use of CDC Biofilm Reactor is not widely used yet in the food industry and represent a novel approach to help sanitary control strategies implementation.

3.1 Introduction

In the food industry, inadequately cleaned equipment presents a potential source for *Listeria monocytogenes* contamination. This pathogen has shown an aptitude for niche adaptation to different food processing facilities and its ability to form biofilm is a challenge for food safety (Djordjevic, Wiedmann, & Mclandsborough, 2002). A biofilm is defined as a microbial community that can form on abiotic surfaces (e.g. stainless steel, polycarbonate, glass) and sometimes on biotic surfaces (e.g. plant-based foods, carcasses) (Da Silva & De Martinis, 2013; Domínguez-Manzano et al., 2012). These communities are usually an aggregate of micro-colonies in single or multiple layers with channels that allow the dispersion of nutrients into the microbial mass. Biofilms in food processing facilities represent a possible source of product contamination and a reservoir for spoilage and foodborne pathogen bacteria (Djordjevic et al., 2002). Once a biofilm is established, this microbial mass has greater resistance to external stress such as dehydration, predator grazing, radiation, antimicrobial compounds and many others, as compared to planktonic cells (Y. Pan, Breidt, & Kathariou, 2006; Ren, Madsen, Sørensen, & Burmølle, 2015; Xavier & Foster, 2007). Several research studies showed that *L. monocytogenes* biofilm formation depends on different factors, such as bacteria growth phase, temperatures, media, substrates' physical and chemical characteristics and presence of other microorganisms (Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002; Kadam et al., 2013). Furthermore, in environments, such as the food industry, biofilms are usually composed by multiple strains of the same species (multi-strain biofilms) or by multiple bacterial species (mixed-species biofilms). Multi strain and mixed-species biofilms have been found to be more resistant to disinfectants and sanitizers than mono-species and pure strain biofilms (Carpentier & Cerf, 2011). The majority of *L. monocytogenes* isolates recovered from food facilities are strains of serogroup 1/2, especially

serotypes 1/2a and 1/2b. However, serotype 4b strains cause the majority of human listeriosis outbreaks (Pan et al., 2009). These serotypes were selected for the present study.

A broader knowledge of biofilm interactions within microbial communities and with the environment might enhance the effectiveness of control methods. Biofilms present distinctive characteristics when grown under different environments and several approaches have been developed to grow and study biofilms under conditions that mimics the environment of interest. Some of the devices and platforms developed are the microtiter plate (Fletcher, 1977), Calgary device (Ceri et al., 1999), biofilm ring test device (Chavant et al., 2007), and different types of rotary biofilm reactors (Azeredo et al., 2017). Among the available technologies, the CDC Biofilm Reactor®, developed by researchers at the Centers for Disease Control and Prevention (R. Donlan et al., 2002) has been broadly used to study biofilm growth and resistance to sanitation. This high shear reactor is a valid, robust and highly repeatable device to grow biofilms; it is composed of eight rods containing removable coupons (circular-shape chips), where the biofilm is being formed. They are continuously exposed to shear stress and renewable nutrients to mimic the conditions in natural environments or industrial systems (Williams et al., 2019). Several coupon materials can be tested at the same time: stainless steel and polycarbonate are available materials to simulate food processing environments. Additionally, this reactor can hold a large number of growth surfaces (24 coupons), providing multiple biofilm samples that can be analyzed over time or treated individually to investigate susceptibility to different sanitation procedures (Franklin et al., 2015; Perez-Conesa, Cao, Chem, McLandsborough, & Weiss, 2011). Coupons can be examined to study biofilm's physiology, morphology and growth dynamics (Williams et al., 2019). As mentioned previously there are several options for studying biofilms and currently there is no accepted standard method for assessing *L. monocytogenes* biofilms growth available. Therefore,

the objective of this study was to evaluate the effects of temperature, culture media and type of surface material on the growth of multi-strain *L. monocytogenes* biofilm in a CDC Biofilm Reactor.

3.2 Materials and methods

3.2.1 Design of experiments and statistical analysis

Using a randomized split-plot design, the effect of temperature (30 ± 2 °C; 37 ± 2 °C), culture media (Tryptic Soy Broth (TSB), BD Difco, Sparks, MD + 0.6% Yeast Extract (YE), Hardy Diagnostic, Santa Monica, CA; Brain Heart Infusion (BHI), BD Difco, Sparks, MD), and material (stainless steel; polycarbonate) were evaluated on the growth of multi-strain *L. monocytogenes* biofilm. Combination of temperature and culture media served as whole plot while material was the subplot defined. Experiments were repeated three times and data were analyzed using SAS (Statistical Analysis System) version 9.4. A level of significance of $P < 0.05$ was considered for the analysis of variance (ANOVA) tests.

3.2.2 Bacterial strains

Four strains of *L. monocytogenes* were used for this study: FSL B2-323 (serotype 4b) (Texas Tech University) isolated from a dairy processing environment (Bergholz, Bowen, Wiedmann, & Boor, 2012), ATCC 7644 (serotype 1/2c) from a clinical case of human meningitis, NRRL B-33043 (serotype 1/2a) and NRRL B-33260 (serotype 1/2c) (CA, U.S.A. ,USDA ARS) obtained from a meat slaughter facility (Ward et al., 2004). Each strain was kept in a CryoCare Organism Preservative System (Key Scientific, Stamford, TX) and stored at -80 °C until experiments.

3.2.3 Preparation of the inoculum

Frozen beads were transferred into 10 ml TSB, incubated for 24 h, streaked for isolation on Tryptic Soy Agar (TSA, BD Difco, Sparks, MD), and kept at 4 °C ± 1 °C for 48 h maximum before starting experiments. For each strain, one isolated colony was used to inoculate 10 ml of fresh growth medium (TSBYE or BHI). Cultures were incubated for 24 h at 37 °C with 100 rpm shaking. Equal amounts of bacteria (1 ml) were combined to create a multi-strain *L. monocytogenes* cocktail. Equal amounts of bacteria (1 ml) were combined to create a multi-strain *L. monocytogenes* cocktail. Initial population from each strain and from the cocktail were verified by serially diluting, spread plating on TSA and enumerating after 18-24 h of incubation at 37 °C ± 1 °C. Overnight cultures reached 9.2, 9.0, 8.7, 8.8 CFU/ml for FSL B2-323, ATTC 7644, NRRL B-33043, NRRL B-33260 respectively, and 8.96 CFU/ml for the cocktail.

3.2.4 CDC Biofilm Reactor

A CDC Biofilm Reactor (Figure 3.1) was used to grow 4-day-old biofilms. This reactor consists of a 1 L glass vessel with polyethylene top, a stir blade assembly, eight polypropylene rods that hold three coupons each, for a total of 24 coupons (1.27 cm of diameter). A tubing system composed by two sizes of silicone tubes (MasterFlex 6424-16 and ColeParmer 6424-72) carries a nutrient flow from the nutrient source (20 L carboy) to the reactor and connects the vessel outlet to a waste collection carboy (BioSurface Technologies Corp., 2018).

3.2.5 Biofilm growth

A modified method from ASTM E 2562 (ASTM, 2017b) was used for reactor parameters, while culture media and temperature conditions were chosen based on previous research for *L. monocytogenes* biofilm growth (Kadam et al., 2013; Pan et al., 2010; Perez-Conesa et al., 2011). Optimal growth conditions in term of culture media and temperatures were

chosen in the present study. Our overall objective was to offer a protocol to develop *L. monocytogenes* biofilm under laboratory conditions and help the food industry towards the development of pathogens' control strategies. One ml of *L. monocytogenes* inoculum cocktail, obtained as previously described, was injected into a fully assembled sterile reactor containing 350 ml of TSBYE or BHI at a concentration of 20 g/l. The reactor was maintained in batch mode (i.e. baffle stirring and effluent line clamped) for 24 h at 30 ± 2 °C or 37 ± 2 °C. After 24 h, the effluent line was unclamped, and a continuous flow diluted to 1 g/l was pumped to the reactor. The stirring plate was set at 60 rpm and the flow at 11 ml/min, based on an operational reactor volume of 330 ml. Based on preliminary experiments (data not shown) a better biofilm recovery was observed with this rpm. After 72 h under continuous flow, the biofilm was considered matured and ready to be enumerated.

3.2.6 Microbial enumeration

After four days, each coupon was aseptically removed and transferred into 10 ml of PBS (Phosphate Buffer Saline, VWR, Solon, OH). Samples were sonicated for 30 s and then vortexed for 30 s; this process was repeated 3 times to assure complete detachment of the biofilm from the coupon (ASTM, 2013b). Serial dilutions were made in 0.1% peptone water (BD Difco, Sparks, MD) and spread-plated in duplicates on TSA. Plates were incubated at 37 ± 2 °C for 24 h, colonies were manually counted, and results recorded and expressed in log CFU/cm^2.

3.2.7 Laser Scanning Confocal Microscope Imaging (LSCM)

L. monocytogenes biofilm were observed under a LSCM (LSM-5 Pascal associated with a Zeiss Axioplan 2) available at the Microscopy Facility of Biology Department at Kansas State University. Biofilms grown at 30 °C, in TSBYE and on stainless steel were selected for microscope imaging due to the higher cell counts observed as compared to BHI at 37 °C and to its widespread

use in food processing environments. Two coupons were removed for visualization at each sampling time (3, 24, 72 and 96 h). Samples were stained using SYTO 9 (ThermoFisher Scientific, Eugene, OR) and SYTOX red (ThermoFisher Scientific, Eugene, OR) for differentiation between living and dead/compromised cells following a modified protocol described by Pang et al., 2019. Staining solution was prepared adding 3 µl of each stain to 1 ml of filtered-sterilized deionized water. Subsequently 200 µl of the prepared staining solution was added to the coupon, where biofilms were forming, for 5 min in dark conditions. Coupons were then rinsed with filtered-sterilized deionized water and all excess was drained. For fixing purposes (preserve cellular components and morphology), formalin 10% (500 µl) was added for 10 min in dark conditions. Coupons were then washed with filtered-sterilized deionized water and samples were observed under the LSCM at a 100x objective lens and 0.5 magnification. For each sample, three representative sites were visualized. Images were processed using ImageJ1 program (NIH, U.S.A.), the green channel (living cells) was split and the six z-slices were merged and converted to a 3D image for better visualization.

3.3 Results

3.3.1 Biofilm growth under different parameters

Based on previous research investigating *L. monocytogenes* biofilms, the growth and survival of one strains of serotype were not inhibited by strain of the other serotype in biofilm (Carpentier and Cerf, 2011). Therefore, in the present study, no further investigation on dominant strain within biofilms was performed. The effects of temperature, media and surface material were evaluated on *L. monocytogenes* biofilm growth after 4 days. Results are shown in Figure 3.2. Overall, when comparing the effect of temperature in the formation of the biofilm, higher counts

were observed at 30 °C as compared to 37 °C ($P < 0.05$). Biofilms grown at 30 °C and in TSBYE reached a population of 8.19 log CFU/cm^2 on polycarbonate and 8.14 log CFU/cm^2 on stainless steel. When BHI was used as culture media, biofilm counts were 7.76 log CFU/cm^2 on polycarbonate and 7.19 log CFU/cm^2 on stainless steel. Biofilms were also grown at 37 °C. When TSBYE was used, biofilm population reached 7.68 log CFU/cm^2 on polycarbonate and 7.55 log CFU/cm^2 on stainless steel. At the same temperature of 37 °C, cell counts of 7.45 log CFU/cm^2 on polycarbonate and 7.15 log CFU/cm^2 on stainless steel were observed when biofilms were cultured in BHI. The media used for biofilm formation was found to be a significant factor ($P < 0.05$) (Table 3.1). Overall, higher counts were obtained when TSBYE was used as compared to BHI. A significant effect was also observed for the material surface used to grow biofilms ($P < 0.05$). Higher cell counts were recovered from polycarbonate as compared to stainless steel. Significant interactions were only observed between temperature and media ($P < 0.05$) (Table 3.1); the highest cell count of 7.64 log CFU/cm^2 was recovered at 30 °C in TSBYE. No statistical effects ($P > 0.05$) were observed when all three factors were analyzed together (Table 3.1). Biofilm grown at 30 °C in TSBYE on polycarbonate reached the highest cell counts (8.19 log CFU/cm^2), while the combination of 37 °C in BHI and on stainless steel provided the lowest cell counts (7.15 log CFU/cm^2).

3.3.2 Microscope Imaging Analysis

Coupons were observed under a LSCM where the live cells appeared green due the SYTO 9 stain (Figure 3.3). The first sampling time, 3 hours after inoculation and under batch phase in the reactor, showed some single rod-shaped cells typical of *L. monocytogenes*. morphology attached to the stainless-steel coupon and spread throughout the coupon (Figure 3.3a). After 24 h, some clusters were visible on the stainless-steel coupon, indicating aggregation and recruitment of new

cells into the community, along with some single cells (Figure 3.3b). Seventy-two hour later, the clusters were larger and thicker and most of the cells were part of a macrocolony (Figure 3.3c). At 96 h, the biofilm was considered mature and thickly developed on the coupon surface (Figure 3.3d).

3.4 Discussion

The prevalence of *L. monocytogenes* in food processing plants is a constant concern for the food industry. Previous research have shown the presence and prevalence of *L. monocytogenes* in food processing environments that do not follow appropriate sanitary practices (Tan et al., 2019). Some of the reservoirs occupied by this foodborne pathogen includes unpolished welds, hollow parts, gaskets, cracks and other difficult-to-clean sites (Carpentier & Cerf, 2011). Furthermore, this pathogen has the ability to form biofilms (Doijad et al., 2015; Kadam et al., 2013; Lemon et al., 2007; Youwen Pan et al., 2010). Heretofore researches have utilized microtiter plate assays to grow biofilm under static conditions (Djordjevic et al., 2002; Doijad et al., 2015; Kadam et al., 2013), and staining with crystal violet to measure cells density. Stainless steel surfaces under static conditions were used to study biofilm formation of *L. monocytogenes* and a population of 10^5-10^6 CFU/cm^2 was observed after 2, 4 or 7 days (Moltz & Martin, 2005; Y. Pan et al., 2006; Pang et al., 2019). Few researchers have investigated the formation of *L. monocytogenes* biofilm on polycarbonate. Beresford et al., 2001 observed 5.8 log CFU/cm^2 after 2 h of growth under static conditions. Additionally, very few studies have been reported on *L. monocytogenes* biofilm formation under dynamic conditions and more specifically under fluid flows conditions (Perez-Conesa et al., 2011). In nature or industrial systems, factors like flow and shear forces influence biofilm properties such as attachment strength and bacterial adhesion to the surface (Goller &

Romeo, 2008). Previous researchers suggest that biofilms were more strongly attached to a surface when high shear conditions were used as compared to low shear conditions (Paul Stoodley, Cargo, Rupp, Wilson, & Klapper, 2002). (Paul Stoodley et al., 2002). Lapointe et al. (2019) used a CDC Biofilm Reactor to grow multi-species biofilm with spoilage microorganisms. Microorganisms were grown in a meat-based medium and followed for one week to provide or limit nutrients as in a simulated meat plant. They obtained biofilms cell counts of approximately 6.0, 6.5 and 7.5 log CFU/cm^2 for *L. plantarum, P. fluorescens,* and *L. mesenteroides,* respectively. In our study, we also utilized a CDC Biofilm Reactor to provide conditions where *L. monocytogenes* was capable to attach and form biofilms under a dynamic scenario. To our knowledge, this study reports a protocol for the greatest log density of *L. monocytogenes* biofilm under dynamic conditions, numbers that may be more representative of certain locations within food processing facilities (Dygico, Gahan, Grogan, & Burgess, 2019). Other microorganisms such as *Pseudomonas, Salmonella,* and *Staphylococcus* have shown differences in biofilm formation when grown under static and dynamic conditions. These microorganisms had lower biofilm density when formed under dynamic conditions, although biofilms were not exposed to flows of fluids but to constant agitation (Ramsey & Whiteley, 2004; Stepanović, Ćirković, Mijač, & Švabić-Vlahović, 2003; Stepanović, Vuković, Ježek, Pavlović, & Švabic-Vlahović, 2001). In an experiment conducted by Buckingham-Meyer et al., 2007, *P. aeruginosa* and *S. aureus* showed higher biofilm density when grown in the CDC Biofilm Reactor as compared to a static biofilm method. In the present study, we were able to reach 10^7 CFU/cm^2 under high shear and a continuous flow environment. Although this study did not compare different rates of flows and shear forces, we reported higher cell counts than compared to the literature (Moltz & Martin, 2005; Y. Pan et al., 2006; Pang et al., 2019).

Temperature has also been proven to influence the ability of *L. monocytogenes* to form biofilm by affecting its flagellum-mediated motility capability (Lemon et al., 2007). At 30 °C the expression of flagellum is active and therefore the microorganism is motile. However, at 37 °C the expression of a negative regulator (MogR) of the motility gene prevents flagellar motility. Consequently the initial attachment to surfaces as first step for biofilm formation is more difficult (Gründling, Burrack, Bouwer, & Higgins, 2004; Lemon et al., 2007). Research carried out by Lemon et al., 2007 proved the importance of this flagellum-mediated motility by mutating strains of *L. monocytogenes* and making them non-motile. Non-motile strains were defective in forming biofilms while their wild types (motile) were capable of forming biofilms. Flagellum-paralyzed strains (mutation of the *motB* gene) also showed deficiency in biofilm formation; the presence of the flagellum was not sufficient to enhance biofilm-forming ability. A different study on *Salmonella* spp. ability to form biofilm reports results similar to ours: higher biofilm densities were observed at 30 °C as compared to 37 °C after 24 and 48 h (Stepanović et al., 2003). In our study, we observed that culture media had an impact on the growth of *L. monocytogenes* biofilm. Comparable results were obtained by Kadam et al., 2013, when studying the effect of nutrients level on *L. monocytogenes* biofilm formation. These researchers observed higher biofilm formation with "nutrient-poor media" (Nutrient Broth and Hsiang-Ning Tsai medium) as compared to "nutrient-rich media" (BHI and TSB). In this study, we use TSBYE and BHI, media with high nutrient content at a diluted concentration of 20g/l for the batch phase and at 1g/l for the continuous flow phase in order to trigger bacteria to form biofilm. Other researches (Perez-Conesa et al., 2011) compared the effect of diluted nutrient-rich media (TSB) and minimal media, such as Modified Welshimeri's Broth, finding that minimal media provided higher biofilm formation. This can be due to the rapid consumption of available nutrients in a low-nutrient environment and

subsequent entering into a stress mode that enhances the attachment level of *L. monocytogenes* (K. Y. Kim & Frank, 1994). Different studies (Lemon et al., 2007; Youwen Pan et al., 2010; Perez-Conesa et al., 2011) have use TSBYE as nutrient source to grow *L. monocytogenes* biofilm. However, the impact of this media on *L. monocytogenes* biofilm growth has not been reported to our knowledge. Kadam et al., 2013 reported that the overall effect of temperature was more important than the effect of media when the biofilm was formed under static conditions. In our study, an interaction between temperature and media was observed meaning that the combination of these two factors had a significant influence on our results.

Finally, only one study (Beresford et al., 2001) has reported the growth of biofilm on polycarbonate, while the majority of research have been focused on stainless steel (Chavant et al., 2002; Moltz & Martin, 2005; Perez-Conesa et al., 2011), since this material is the most commonly used in the food industry. Nevertheless, further research is necessary to understand which materials enhance and/or facilitate the formation of biofilms in order to control their growth in different types of food facility environments.

3.5 Conclusion

There is a current industry-wide lack of data that researchers and operators can refer to when selecting a strategy to control *L. monocytogenes* biofilms in the food premises. Many parameters affect the formation of *L. monocytogenes* biofilms. Temperature, culture media and surface material were the three factors evaluated in this study. All these parameters were found significant ($P < 0.05$) for biofilm formation at the conditions studied in the present research and the interaction between media and temperature seemed to enhance *L. monocytogenes* biofilm formation. To our knowledge, this work presents an initial framework to develop *L.*

monocytogenes biofilms under different dynamic conditions. The use of CDC Biofilm Reactor is not generally used in the food industry and represent a novel approach to help sanitary control strategies implementation. Nevertheless, our experiments represent a preliminary study about multi-strain *L. monocytogenes* biofilm in a CDC Biofilm Reactor. Further experiments are needed to understand in more detail the ability of this pathogen to form biofilm in different velocity of shear force and different rate of fluid flow.

3.6 Acknowledgement

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3.7 Tables and Figures

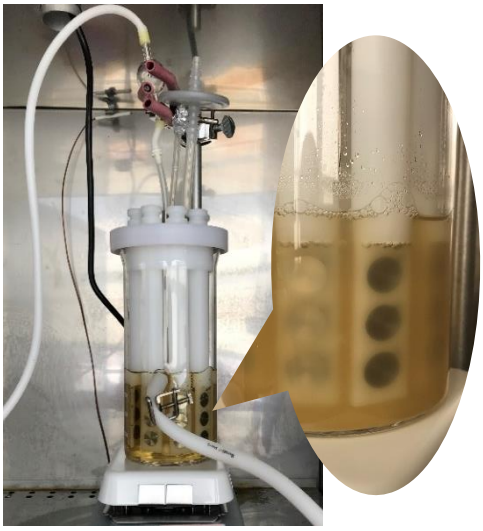

Figure 3.1. CDC Biofilm Reactor® used in this study to grow multi-strain *L. monocytogenes* biofilms under high shear conditions (coupons were zoomed in).

Table 3.1. Statistical analysis (ANOVA) of different parameters evaluated for the growth of *L. monocytogenes* biofilm in a CDC Biofilm Reactor®.

Parameters	P-value
Surface material (Stainless steel or Polycarbonate)	0.0094[#]
Temperature (30 °C or 37 °C)	0.0082[#]
Media (TSBYE or BHI)[†]	0.0010[#]
Temperature*Material	0.8725
Media*Material	0.6326
Temperature*Media	0.0460[#]
Temperature*Media*Materials	0.9762

[†]TSBYE: Tryptic Soy Broth + 0.6% Yeast Extract. BHI: Brain Heart Infusion.

[#]Denotes statistical significance with $P < 0.05$.

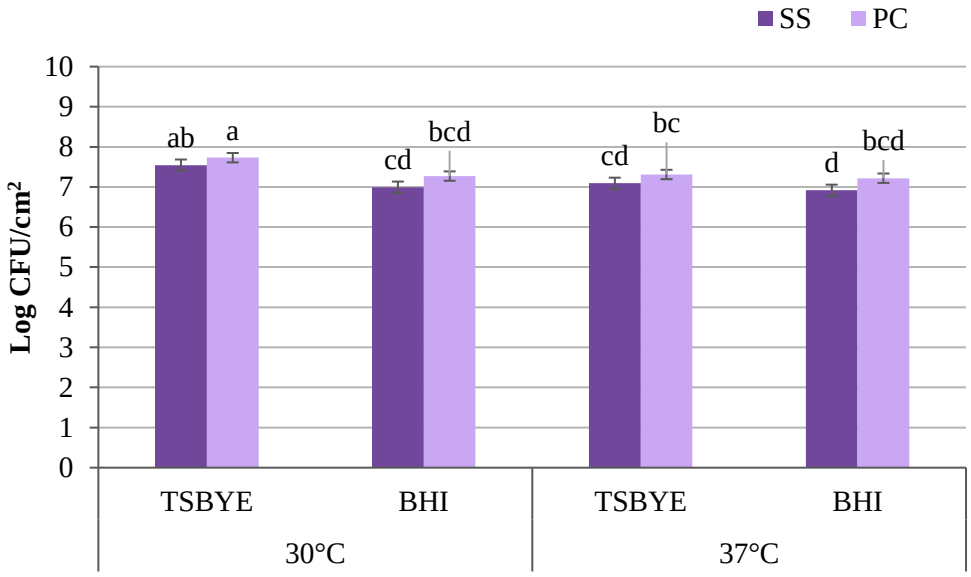

Figure 3.2. Multi-strain *L. monocytogenes* biofilm (Log CFU/cm^2) in a CDC Biofilm Reactor® under different conditions of nutrient media (TSBYE: Tryptic Soy Broth + 0.6% Yeast Extract; BHI: Brain Heart Infusion), material (SS: stainless steel coupons; PC: polycarbonate coupons) and temperature (30 and 37 °C). Different letters indicate statistical difference between conditions ($P < 0.05$).

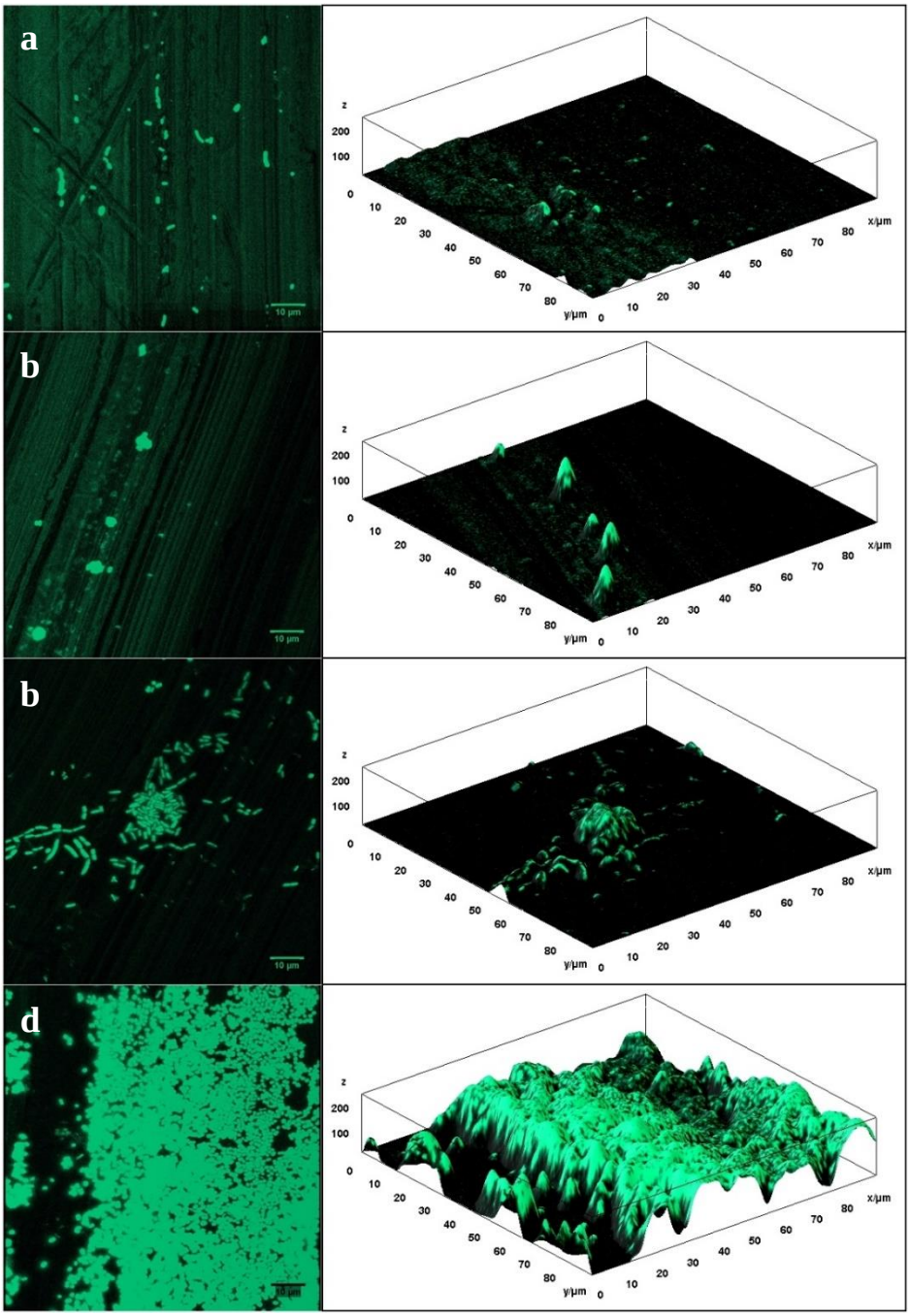

Figure 3.3. *L. monocytogenes* biofilm development on stainless steel coupons in a CDC Biofilm Reactor® at 30 °C in TSBYE (Tryptic Soy Broth + 0.6% Yeast Extract) after (a) 3 h, (b) 24 h, (c) 72 h, (d) 96 h under a Laser Scanning Microscope. 2D projection made under a laser scanning microscope and 3D projection built in Image J program. Bars in 2D images represents 10 µm.

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Chapter 4 - The Effect of Chemical Sanitizers and UV-C light on *Listeria monocytogenes* Biofilm Survivability

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Abstract

Listeria monocytogenes has shown niche adaptation and its ability to form biofilms is a challenge for food safety. After the biofilm formed it becomes difficult to disrupt. Chemical sanitizers combined with non-thermal technologies might represent an effective way to control *L. monocytogenes* biofilms. This study aims to investigate *L. monocytogenes* biofilm survivability after treatments with chemical sanitizers and UV-C light alone or in combination. A CDC Biofilm Reactor was used to grow 4-days-old multi-strain *L. monocytogenes* biofilms on stainless steel. Biofilm survivability was investigated after 10 min exposure to lactic acid (4%), peroxyacid (100 ppm), quaternary-ammonium (400 ppm) alone or in combination with UV-C light (254 nm) for 15 or 30 min. Sequential treatment effect was also evaluated. Control biofilms reached 6 log CFU/cm^2 after 4 days, reductions of 2.6-3.6 log were observed with chemical sanitizers while a maximum of 1.8 log reduction was recorded after UV-C treatment. Combined treatments showed enhanced effect and the sequence of antimicrobial treatment was significant for lactic acid and peroxyacid ($P < 0.05$). The results obtained in this research offer an initial understanding of *L. monocytogenes* biofilm response to chemical sanitizers and contribute to intervention development for effective strategies to control this pathogen in food processing environment.

4.1 Introduction

Listeria monocytogenes can adapt and proliferate in a variety of environments. This pathogen has shown niche adaptation and its ability to form biofilm represents a challenge for food safety. A biofilm is defined as a community of microorganisms that live in a sessile form attached to a substratum or interface (Da Silva & De Martinis, 2013). Once *L. monocytogenes* has entered a processing facility it can attach to different surfaces such as stainless steel, glass, plastic or rubber and form biofilms (Kathariou, 2002; Matthews et al., 2017). These biofilms are difficult to control, and they usually develop in hard-to-clean sites (Da Silva & De Martinis, 2013; Matthews et al., 2017). Drains, floors, conveyor belts, scratches and joints, porous or rough surfaces, provide ideal niches for cell adhesion and biofilm formation as well as protection from mechanical and chemical disruption (Matthews et al., 2017). The process of forming a biofilm includes three main steps: *1*) attachment to the surface; *2*) aggregation into microcolonies in a semi-permanent association; and *3*) growth and maturation of macrocolonies. At the end of the development state, cells are irreversibly attached to the surface, embedded in the matrix, and the biofilm is now mature (Agle, 2007; Bremer et al., 2015; P. Stoodley et al., 2002). Biofilm offers protection to microorganisms against external challenges such as temperature, pH or antimicrobial solutions (Bremer et al., 2015). The susceptibility of *L. monocytogenes* biofilms to sanitation strategies such as chemical sanitizers is reduced with biofilm age and the presence of various strains (Ban et al., 2012; Korany et al., 2018). The acquired resistance of mature biofilms to sanitizers is due to the protection provided by the EPS and the multiple layers of bacterial cells formed in the biofilm (Yang et al., 2009).

Food processing facilities follow sanitation standard operating procedures (SSOPs) to ensure sanitary conditions in the processing plant (Mekonen, Muhie, & Melaku, 2014). The use of chemical sanitizers is one of the most common practice for sanitation to overcome contamination, bacterial adhesion, and biofilm formation (Schmidt, 2009). However, novel technologies, such as the use of ultraviolet (UV) light, are being implemented to enhance the effectiveness of the sanitation methods and ensure the safety of the products (Khan et al., 2017). Among the most used sanitizers are quaternary ammonium compounds, organic acids and peroxyacetic acid and their antimicrobial activity against biofilms have been previously investigated. However, results indicate that the use of chemical sanitizer as single strategy is not always sufficient to control biofilms in food processing environments (Ban et al., 2012; Korany et al., 2018; Pang et al., 2019). Therefore, some SSOPs recommend sanitizers rotation or the implementation of hurdle technologies (i.e. combine intervention strategies) to achieve a higher antimicrobial effect. Among alternative germicidal technologies, non-ionizing UV irradiation has shown to be effective against bacterial biofilms (Gabriel et al., 2018; M. Kim et al., 2016; Sommers et al., 2010). When biofilms of *L. monocytogenes* were treated with chemical sanitizer, reductions between 1-3 log CFU/cm^2 were observed (Ban et al., 2012, Korany et al., 2018). The combination of UV light with other interventions, such as chemical sanitizers, has been investigated by several researchers. A UV-C light lamp was ceiling-mounted in a fish smoke house after daily cleaning and disinfection procedures: a significant decrease of *Listeria* positive environmental samples was reported after irradiation exposure for 48 hours (Bernbom, Vogel, & Gram, 2011). The effective combination of sodium hypochlorite and UV light was observed against *L. monocytogenes* biofilms in industrial kitchens, facilities, and restaurants (M. Kim et al., 2016). In this type of combination approach, bacteria sub-lethally injured by the first

treatment (i.e. chemical sanitizer) activates the defense mechanism to address the damage caused by this stress. Then, due to the immediate application of the second treatment (i.e. UV light), the bacterial defense mechanism do not have time to recover, thus leading to a much higher level of reduction as compared to the application of a single intervention (Woodling & Moraru, 2005). The response mechanism of *L. monocytogenes* to these interventions are different, while the chemical sanitizers cause oxidative or acid stress conditions and thus activate the SOS response mechanism; the damage caused by the UV light activates the photoreactivation repair system (Snyder et al., 2013; van der Veen et al., 2010). Therefore, combining these techniques might represent a good strategy to overwhelm the bacterial response and achieve a higher reduction in biofilms of *L. monocytogenes.*

Therefore, the objectives of this study were to: *i*) investigate *L. monocytogenes* biofilm survival ability after treatments with chemical sanitizers (i.e. quaternary ammonium, lactic acid and peroxy acid) and UV-C light (254 nm) alone or in combination; and *ii*) understand the effect of sequence of treatments to elucidate possible enhancement of overall antimicrobial activity.

4.2 Materials and Methods

4.2.1 Bacterial strains

The same strains of *L. monocytogenes* investigated in previous researches (Mendez, Walker, Vipham, & Trinetta, in press) were used for this study. FSL B2-323 (serotype 4b) (Texas Tech University) was isolated from a dairy processing environment (Bergholz et al., 2012), ATCC 7644 (serotype 1/2c) from a clinical case of human meningitis, NRRL B-33043 (serotype 1/2a) and NRRL B-33260 (serotype 1/2c) (CA, U.S.A. ,USDA ARS) were obtained from a meat

slaughter facility (Ward et al., 2004). Each strain was kept in a CryoCare Organism Preservative System (Key Scientific, Stamford, TX) and stored at -80 °C until experiments.

4.2.2 Biofilm formation

A Centers for Disease Control and Prevention (CDC) Biofilm Reactor was used to develop 4-days old biofilms on stainless steel surfaces. A protocol developed in our laboratory was used (Mendez, Walker, Vipham, & Trinetta, in press). Briefly, each *L. monocytogenes* strain was grown overnight at 37 ± 2 °C in 10 ml of Tryptic Soy Broth (TSB, BD Difco, Sparks, MD) with 0.6% of Yeast Extract (Hardy Diagnostic, Santa Monica, CA) (TSBYE), equal amounts of bacteria were combined to create a cocktail from where 1-ml was used to inoculate the reactor containing 350 ml of TSBYE. The initial cocktail population was verified by spread plating on Tryptic Soy Agar (TSA) (BD Difco, Sparks, MD) and enumerating after 24 h of incubation at 37 ± 2 °C. Biofilms were grown at 30 ± 2 °C on stainless steel round-shaped coupons (1.27 cm^2 of diameter) following a 24-hour batch phase and a 72-hour continuous flow stirring phase.

4.2.3 Chemical sanitizers exposure

Coupons with mature biofilms were removed from the reactor after 4 days. Three chemical sanitizers were used with an exposure time of 10 min: 4% Lactic acid solution (Purac® Corbion, Blair, NE), a peroxy acid-based sanitizer diluted to 100 ppm (SYNERGEX, EcoLab, St Paul, MN) and a quaternary ammonium-based sanitizer diluted to 400 ppm following manufacturer recommendations (STER-BAC, EcoLab, St Paul, MN). After treatment, solutions were neutralized by transferring the coupons into 10 ml D/E Neutralizer Broth (BD Difco, Sparks, MD).

4.2.4 UV-C light treatments

Experiments to evaluate the effect of UV-C light to inactivate biofilms were conducted using a small chamber (Figure 4.1), in which UV irradiance could be measured and controlled. UV-C light (254 nm) was applied for 15 min (0.2 J/cm^2) or 30 min (0.45 J/cm^2). A germicidal UV lamp (Lumalier, Memphis, TN) was the emission source and an ILT1700 research radiometer (International Light Technologies, Peabody, MA) was used to monitor the intensity (W/cm^2) of the UV irradiation. Coupons with 4-day mature biofilms were placed at a distance of 20 cm and treated for 0, 15 or 30 min. Coupons were flipped half time through the total exposure and then aseptically transferred to 10 ml of Phosphate Buffered Saline (PBS) (VWR, Radnor, PA).

4.2.5 Combined treatment application

With the intent of observing a possible increase of antimicrobial activity, the combined application of chemical sanitizers and UV-C light was evaluated. Surfaces with mature biofilms were exposed for 10 min to chemical sanitizers followed by UV-C light treatment, as previously described, for 0, 15 and 30 min. Treatment sequence effect was also evaluated to understand if UV-C application before the sanitizer step could enhance antimicrobial effectiveness and/or vice versa, hence the application of UV-C light followed by chemical sanitizer was also evaluated. After the different treatments, coupons were aseptically placed in 10 ml of D/E neutralizer broth. Control treatments were evaluated by placing a coupon with untreated biofilm in 10 ml of PBS or in 10 ml of D/E neutralizer broth.

4.2.6 Cells recovery and microbial counts

After exposure to single or combined treatments, coupons were sonicated for 30 s and then vortexed for 30 s. This process was repeated 3 times to assure complete detachment of

biofilm cells (ASTM, 2019). Subsequently, serial dilutions were performed in 0.1% peptone water (BD Difco, Sparks, MD) and spread-plated in duplicates on TSA. Plates were incubated at 37 ± 2 ^{o}C for 24 h. Colonies were manually counted, and results recorded as log CFU/cm^2.

4.2.7 Statistical analysis

Treatments were randomized across coupons and experiments were run six times to reduce experimental error. Statistical significance was determined at $P < 0.05$. An analysis of variance (ANOVA) and a multiple comparison of means test were used to evaluate significant difference among the results. Data were analyzed using the GLM procedure of SAS 9.4 (Statistical Analysis System Inc, Cary, NC).

4.3 Results

4.3.1 Effect of single treatment application on the reduction of L. monocytogenes biofilms

The effects of UV-C light, lactic acid, peroxy acid and quaternary ammonium treatments alone on *L. monocytogenes* biofilms are shown in table 4.1. After 4 days at 30 °C in TSBYE, control biofilms reached a population of 6.04 ± 0.49 log CFU/cm^2. When mature biofilms were exposed to UV-C light for 15 or 30 min, a significant reduction ($P < 0.05$) of 1.73 ± 0.79 and 1.68 ± 0.97 log CFU/cm^2 was observed, respectively. After 10 min exposure to 4% lactic acid a reduction of 3.06 ± 0.85 log CFU/cm^2 was obtained, exposure to 400 ppm quaternary ammonium resulted in 2.61 ± 0.91 log CFU/cm^2 reduction, and an exposure to 100 ppm peroxy acid achieved 3.66 ± 0.90 log CFU/cm^2 reduction. The use of peroxy acid was the most effective among the single treatment application ($P < 0.05$). Overall all the chemical sanitizers

investigated in this study significantly reduced *L. monocytogenes* biofilms as compared to the untreated controls ($P < 0.05$).

4.3.2 Effect of combined treatment application on the reduction of L. monocytogenes biofilms

Table 4.2 shows *L. monocytogenes* biofilm reductions obtained after the exposure to the combination of chemical sanitizers and UV-C light treatments.

Lactic acid. The order of treatment application showed a significant effect ($P < 0.05$) for the combination of lactic acid and UV-C light. When lactic acid was followed by 15 or 30 min UV-C light, biofilms were reduced by 5.11 ± 0.66 and 4.78 ± 1.02 log CFU/cm^2 respectively as compared to the control. Conversely, when lactic acid was preceded by 15 min exposure to UV-C light, no statistical significance ($P > 0.05$) in biofilm reduction (3.26 ± 0.62 log CFU/cm^2) was observed as compared to the use of lactic acid alone (3.06 ± 0.85 log CFU/cm^2). However, a significant effect ($P < 0.05$) was observed when UV-C exposure time was extended to 30 min: 4.02 ± 0.67 log CFU/cm^2.

Quaternary ammonium. The order of treatment application showed no significant effect ($P > 0.05$) for quaternary ammonium and UV-C light. Nevertheless, an enhanced log reduction was observed by using the antimicrobial treatments together as compared to the single application. When quaternary ammonium was applied before UV-C light, a significant difference was reported ($P < 0.05$) between 15 and 30 min treatments: 3.28 ± 1.32 and 4.02 ± 1.19 log CFU/cm^2 were the reductions observed respectively. Notwithstanding, when UV-C light was used first, no difference ($P > 0.05$) was reported between 15 and 30 min: 3.45 ± 0.93 log CFU/cm^2 and 3.85 ± 0.84 log CFU/cm^2 reductions respectively.

Peroxy acid. The order of treatment application showed a significant effect ($P < 0.05$) for peroxy acid and UV-C light. Greater reduction was observed when peroxy acid was applied before the UV-C light. However, no statistical difference ($P > 0.05$) was observed between the biofilm reduction achieved by 15 and 30 min UV-C light exposure times: 4.66 ± 0.66 and 4.38 ± 0.91 Log CFU/cm^2 respectively. Likewise, no difference ($P > 0.05$) was reported between 15 and 30 min of UV-C exposure when UV-C light treatments preceded the sanitizer (4.00 ± 0.81 and 4.51 ± 0.92 Log CFU/cm^2).

4.4 Discussion

In the food industry, *L. monocytogenes* is widely known for forming biofilms in difficult-to-clean sites such as floors, waste water pipes, conveyor belts, and stainless steel surfaces (Colagiorgi et al., 2017). In the present study, we demonstrated that the use of chemical sanitizers against 4-days-old multi-strain biofilms of *L. monocytogenes* was able to achieve reductions of 3.06, 2.61, and 3.66 log CFU/cm^2 when using lactic acid (4%), quaternary ammonium (400 ppm), or peroxy acid (100 ppm) respectively.

The use of lactic acid as strategy to control microbial biofilms has been previously studied (Ban et al., 2012; Yang et al., 2009). Yang et al. (2009) observed a reduction of 4.21 log CFU/cm^2 in a 7-days-old *L. monocytogenes* biofilm on polyethylene surfaces when lactic acid was applied at 0.18%. In their study, the sanitizer reached a higher log reduction as compared to our results, probably due to low pH. Furthermore, Wang et al. (2015) demonstrated that lactic acid can cause a leakage of proteins through the membrane especially in the first 2 h of sanitizer exposure, and inhibit the synthesis of bacterial cellular soluble proteins. In their research, *L. monocytogenes* in planktonic state was completely inactivated after exposure to 0.5% lactic acid

for 2 h (Wang et al., 2015). However, since biofilms provide bacteria with increased protection, a higher concentration of antimicrobial solutions or the combination with other intervention strategies might offer a more effective way to control biofilm formation. Ban et al. (2012) achieved 0.92 log CFU/coupon reduction in *L. monocytogenes* biofilms on stainless steel after exposure to lactic acid (2%) for 30 s. Conversely, a 4.5 log CFU/coupon reduction was observed when the sanitizer was combined with steam for 20 s. In our study, when lactic acid (4%) was combined with UV-C light, a 5.11 log CFU/cm^2 reduction was observed.

Quaternary ammonium compounds (QACs) sanitizers have been investigated by many researchers to control microbial biofilms on different surfaces (Belessi et al., 2011; Korany et al., 2018; Pang et al., 2019; Poimenidou et al., 2016). Concentrations of 200 ppm of QACs achieved 1.35 log CFU/well and 3.9 log CFU/cm^2 reductions in polystyrene and stainless steel surfaces in the research carried out by Korany et al. (2018) and Pang et al. (2019). The effectiveness of QACs compounds have been proven to diminish as biofilms mature. Chavant et al. (2004) observed a decrease in biofilm cells mortality from 98% after 6 h to 45% after 7 days of biofilm maturation when samples were exposed to 20 ppm of quaternary ammonium compounds. A different study reported that solutions such as hydrogen peroxide and sodium hypochlorite were more effective in controlling *S. aureus* and *P. aeruginosa* biofilms than QACs (Lineback et al., 2018). Nonetheless, the use of quaternary ammonium compounds is among the most common strategy used in the food industry (Gerba, 2015). Very few studies have investigated the combination of QACs with other control strategies against biofilms (Berrang et al., 2008; Blenkinsopp, Khoury, & Costerton, 1992). Berrang et al. (2008) showed an enhanced bactericidal effect when quaternary ammonium (400 ppm) was combined with ultrasonication. A 3.5 log CFU/cm^2 reduction was observed in *L. monocytogenes* biofilms. Similar results in log

reduction were observed in our study when QACs was combined with UV-C light (3.28 – 4.02 log CFU/cm^2).

The control of biofilms with strong oxidizing properties such as peroxyacetic acid and peroxyoctanoic acid has shown to be effective (Azanza, 2004; Schmidt, 2009). Some studies have tested peroxyacetic acid at 80 and 160 ppm against *L. monocytogenes* biofilms on polystyrene achieving reductions of 3.29 and 4.34 log CFU/well, respectively (Korany et al., 2018). Fatemi and Frank (1999) utilized peracetic acid and peroctanoic acid (80 ppm) to inactivate *L. monocytogenes* biofilms on stainless steel reporting 3.2 and 3.9 log CFU/cm^2 reductions after 5 min of exposure. The sanitizer used in this study was a mixture of hydrogen peroxide, peroxyoctanoic acid and peroxyacetic acid. Hydrogen peroxide and peroxyacetic acid are strong oxidizing agents while peroxyoctanoic acts as a surface-active component retaining active antimicrobial agents (Azanza, 2004). In this study, peroxy acid followed by UV-C light showed a high biofilm reduction (4.4 – 4.7 log CFU/cm^2) demonstrating enhancement of antimicrobial activity when treatments were combined. Conversely, peroxyacetic acid treatments followed by ultrasonication showed no enhancement in biofilms reduction in the study by Berrang et al. (2008). The use of UV light at 0.3 and 0.6 J/cm^2 against biofilms of *L. monocytogenes* on stainless steel achieved reductions of 0.26 and 0.42 log CFU/cm^2. A synergistic effect was observed when UV light was combined with sodium hypochlorite (200 ppm): 3.1 and 3.8 log CFU/cm^2 (M. Kim et al., 2016).

When combining intervention strategies (e.g. as in this study), bacteria sub-lethally injured by the first treatment (i.e. peroxy acid) do not have time to recover due to the immediate application of the second treatment (i.e. UV light). A much higher level of reduction as compared to the application of a single intervention is usually achieved, since interventions

usually have different mechanism of action against bacteria (Woodling & Moraru, 2005). UV irradiations damage nucleic acids while chemical sanitizers weaken the cell wall and membranes, therefore, a much higher level of reduction as compared to applying a single treatment. Microbial repair mechanisms are overloaded, unable to repair the injuries and subsequently bacterial cells die (Koivunen & Heinonen-Tanski, 2005). When the interventions were applied following the sequence of chemical sanitizer first and UV-light second, the sanitizer was able to disrupt the membrane and protection layer of the biofilms and thus, allowing the UV rays to reach the nucleic acids inside the cell. This combination achieved a higher reduction in biofilms by overwhelming the SOS response mechanisms and photoreactivation repair system, thus resulting in a bacterial inability to recover and eventually leading to the cell death.

In summary, in the present study we showed the effect of three chemical sanitizers (lactic acid, quaternary ammonium and peroxy acid) alone or in combination with UV-C light to control *L. monocytogenes* biofilms on stainless steel. The use of lactic acid followed by UV-C light was found to be the most effective, while quaternary ammonium followed by UV-C light reached the lowest log reduction. Further research is needed to benchmark the proposed treatments in food processing environments. Nevertheless, the results obtained in this investigation support the use of combined treatments as effective strategies to control biofilms of *L. monocytogenes* on stainless steel.

4.5 Tables and Figures

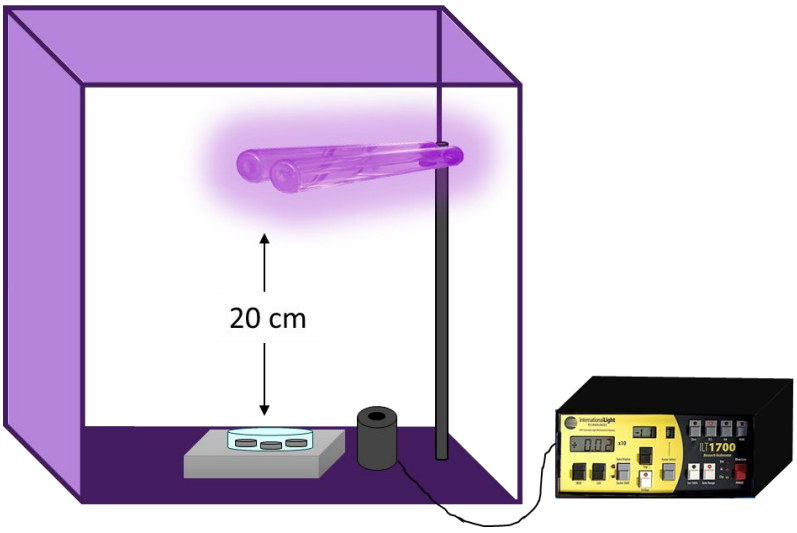

Figure 4.1. Chamber used to irradiate UV-C light (254 nm) on biofilms of *L. monocytogenes* grown on stainless steel. An ILT1700 research radiometer was used to monitor the intensity (W/cm^2).

Table 4.1. Effect of single treatment application on the reduction (mean Log CFU/cm^2 ± Standard Deviation) of *L. monocytogenes* biofilms.

Treatment*	Biofilm cells reduction (Log CFU/cm^2)†	Confidence interval (95%)
15-UV	1.73 ± 0.79^a	1.39 - 2.06
30-UV	1.68 ± 0.97^a	1.27 - 2.09
Lac	3.06 ± 0.85^b	2.70 - 3.42
Qua	2.61 ± 0.91^b	2.22 - 2.99
Poa	3.66 ± 0.90^c	3.28 - 4.04

* 15-UV: 15 min exposure to UV-C light, 30-UV: 30 min exposure to UV-C light, Lac: 4% Lactic acid sanitizer, Qua: 400 ppm quaternary ammonium-based sanitizer, Poa: 100 ppm peroxy acid based sanitizer.

† The population in control biofilms was 6.04 ±0.49 log CFU/cm2.

Different letters within the same column indicate statistical significance ($P < 0.05$).

Table 4.2. Effect of combined treatment application on the reduction (mean Log CFU/cm^2 ± Standard Deviation) of *L. monocytogenes* biofilms

Treatments[*]		Biofilm cells reduction (Log CFU/cm^2)[†]	Confidence interval (95%)
Lac	-	3.06 ± 0.85^{a}	2.70 - 3.42
Lac	15-UV	5.11 ± 0.66^{c}	4.69 - 5.53
Lac	30-UV	4.78 ± 1.02^{c}	4.13 - 5.42
15-UV	Lac	3.26 ± 0.62^{a}	2.87 - 3.66
30-UV	Lac	4.02 ± 0.67^{b}	3.59 - 4.45
Qua	-	2.61 ± 0.91^{a}	2.22 - 2.99
Qua	15-UV	3.28 ± 1.32^{ab}	2.45 - 4.12
Qua	30-UV	4.02 ± 1.19^{c}	3.26 - 4.78
15-UV	Qua	3.45 ± 0.93^{bc}	2.86 - 4.04
30-UV	Qua	3.85 ± 0.84^{bc}	3.32 - 4.38
Poa	-	3.66 ± 0.90^{a}	3.28 - 4.04
Poa	15-UV	4.66 ± 0.66^{c}	4.24 - 5.08
Poa	30-UV	4.38 ± 0.91^{bc}	3.73 - 5.04
15-UV	Poa	4.00 ± 0.81^{ab}	3.48 - 4.51
30-UV	Poa	4.51 ± 0.92^{bc}	3.92 - 5.10

[*]15-UV: 15 min exposure to UV-C light, 30-UV: 30 min exposure to UV-C light, Lac: 4% Lactic acid sanitizer, Qua: 400 ppm quaternary ammonium-based sanitizer, Poa: 100 ppm peroxy acid based sanitizer.

[†]The population in control biofilms was 6.04 ±0.49 log CFU/cm^2.

Different letters within the same column indicate statistical significance (P < 0.05).

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Chapter 5 - Transcriptional Response of *L. monocytogenes* Biofilm to Chemical Sanitizers

5.1 Introduction

The incidence of illnesses caused by *L. monocytogenes* contamination in food products have increased over time. In recent years, this pathogen have been isolated from foods and food sources that were not traditionally recognized as *Listeria* transmission vehicles such as asparagus, mashed potatoes, sprouts, and salad mixes (Desai, Anyoha, Madoff, & Lassmann, 2019). The food industry has been challenged to develop new strategies to control this pathogen in processing facilities because *L. monocytogenes* forms strong biofilms that are hard to eliminate. Common practices such as the use of chemical sanitizers might not be effective in eliminating biofilm communities since this lifestyle enhances resistance to several stress and antimicrobial compounds. *L. monocytogenes* has several well-characterized stress response regulators that allow rapid, adaptive responses to intrinsic factors such as salt, pH, and external factors like low and high temperatures (Casey et al., 2014; Pleitner, Trinetta, Morgan, Linton, & Oliver, 2014). The role of sigma factors in gene regulation and stress response have been well documented. Pleitner et al. (2014) studied the transcriptional response of *L. monocytogenes* to chlorine dioxide and observed a ClO_2 concentration-dependent effect on bacteria survivability. Differentially expressed genes were identified with functions linked to cell envelop, cellular processes, energy metabolism, and protein synthesis. Understanding bacterial response to stresses at transcriptomic level can lead to the development of more effective control strategies (Kang, Burall, Mammel, & Datta, 2019). Transcriptomic analysis characterizes transcriptional activity focusing on relevant genes and RNA transcripts produced under specific conditions. The differences encountered are typically induced by a change in the external environment, as

mentioned above (Blumenberg, 2019; Illumina, 2019). To determine the differences in the transcriptional responses, upregulated (over expressed) or downregulated (under expressed) genes are identified (Illumina, 2019). Obtaining high quality mRNA from biofilms is critical for downstream transcriptome analysis associated with *L. monocytogenes* survival and resistance mechanisms to treatments. Therefore, the objective of this study was to analyze the transcriptomic response of *L. monocytogenes* biofilms to three chemical sanitizers by identifying differentially expressed genes.

5.2 Materials and methods

5.2.1 Biofilm formation

The same strains and procedure previously described in chapter 4 parts 4.2.1 and 4.2.2 were used to grow *L. monocytogenes* biofilms.

5.2.2 Chemical sanitizer exposure

Coupons with mature biofilms were removed from the reactor after 4 days. Three chemical sanitizers were used under sublethal conditions to allow enough cells recovery for transcriptional analysis. Biofilms were exposed for 30 s to 0.5% Lactic acid solution (Purac® Corbion, Blair, NE), 10 ppm of peroxy acid-based sanitizer (SYNERGEX, EcoLab, St Paul, MN), or 10 ppm of quaternary ammonium-based sanitizer (STER-BAC, EcoLab, St Paul, MN). After treatment, solutions were neutralized by transferring the coupons to centrifuge tubes containing 30 ml of Dey-Engley (D/E) Neutralizer Broth (BD Difco, Sparks, MD), three coupons were put together to achieve higher cell recovery in the following step.

5.2.3 Cells recovery and RNA stabilization

After treatments, biofilms cells were detached by sonicating 30 s and then vortexing 30 s. This process was repeated 3 times to assure complete biofilm removal (ASTM, 2019). One ml of the solution was used to enumerate biofilms. The remaining cells were centrifuged for 8 min at 4,000 × g and coupons were then aseptically removed. Cells were centrifuged again for 5 min at 4,000 × g and only 1 ml of supernatant was kept in the tubes. Biofilm cells obtained from two tubes within the same treatment during the previous steps were combined to enhance RNA quantity and quality. Then, cells were centrifuged for 2 min at 13,000 × g and supernatant was removed leaving 50 µl to resuspend cells. One ml of RNAlater (ThermoFisher, Vilnius, Lithuania) was added. The solution was vortexed for 30 s and snap freezed in dry ice. Samples were kept at -80 °C until shipped to the Center of Food Safety and Applied Nutrition (CFSAN) College Park, MD for analysis

5.2.4 RNA extraction and purification

For each condition tested, samples' total RNA was extracted using the TRIzol® Max™ Bacterial RNA isolation kit (Life Technologies, Carlsbad, CA) following manufacturer instructions. Briefly, samples were thawed overnight at 4 °C, and 1.5 ml of the sample was transferred to a pre-chilled microcentrifuge tube and centrifuged for 5 min at 6,000 × g at 4 °C. Supernatant was removed and cells resuspended in 200 µl of Max Bacterial Enhancement Reagent preheated to 95 °C. The solution was mixed by pipetting and then incubated at 95 °C for 4 min. One ml of TRIzol® Reagent was also added and mixed. Solution was incubated at room temperature for 5 min. Then, a phase separation was perform using 0.2 ml of cold chloroform and centrifuging for 15 min at 12,000 × g at 4 °C. Afterwards, an RNA precipitation step was conducted by transferring the colorless phase containing RNA to a fresh tube, adding 0.5 ml of

cold isopropanol, incubating at room temperature for 10 min and centrifuging again for 10 min at 15,000 × g at 4 °C. Supernatant was discarded and RNA pellet was resuspended in 1 ml of 75 % ethanol, then centrifuged for 5 min at 7,500 × g at 4 °C. Supernatant was again discarded and RNA pellet air-dried. RNA was suspended in 50 µl of RNase-free water and treated with DNase I from RiboPure^{TM} RNA Purification Kit (Ambion, Inc, Austin, TX) to remove any contaminating DNA. Concentration of total RNA was measured using Qubit RNA HS Assay with Qubit 4 fluorometer (Invitrogen, Carlsbad, CA). The integrity of total RNA was assessed by the Bioanalyzer RNA 6000 pico assay (Agilent Technologies, Santa Clara, CA).

5.3 Justification for the study

The persistence of *L. monocytogenes* in food processing plants has been attributed to the formation of biofilms. Microbial communities show higher resistance to stresses (e.g. antimicrobial treatments) as compared to planktonic cells by upregulating genes linked to energy metabolism, adaptation, cellular processes, and protein synthesis (Bridier, Briandet, Thomas, & Dubois-Brissonnet, 2011; Colagiorgi et al., 2017). Currently, no much information is available on the tolerance mechanisms of *L. monocytogenes* biofilms to sanitation strategies used in food processing facilities. Previous research have investigated *Listeria* planktonic cells during treatments with nisin, chlorine dioxide (ClO_2), or benzethonium chloride (BZT) (Casey et al., 2014; Pleitner et al., 2014; Wu, Yu, Wheeler, & Flint, 2018).

Modern molecular tools like RNA-seq analysis can be utilize to investigate mechanisms linked to microbial resistance (Colagiorgi et al., 2017). This approach allows the identification of genes differentially expressed as response to external challenges and stresses. In order to develop effective control strategies in the food industry, it is necessary understand the physiological and

transcriptional response of biofilms to these interventions. Quaternary ammonium, peroxy acid and lactic acid are among the most commonly used sanitizers in processing facilities. Investigating *L. monocytogenes* biofilm response to these compounds will enhance current knowledge and contribute to the development of effective practices to control the presence of *L. monocytogenes* in processing plants.

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