

THE ROLE OF EXTRACELLULAR DNA IN THE FORMATION OF BIOFILM BY

LISTERIA MONOCYTOGENES.

by

DVIJAL PATEL

(Under the Direction of Joseph Frank)

ABSTRACT

The objective of this research was to determine the importance of extracellular DNA (eDNA) in the formation of biofilms of *Listeria monocytogenes*. Five strains of *L. monocytogenes* with varying biofilm production were selected for experimentation. The strains were cultured on stainless steel coupons and exposed to the enzyme DNase I. Biofilms of *L. monocytogenes* are surrounded by a three dimensional matrix consisting of eDNA. DNase I hydrolyzes DNA, including eDNA. There was a reduction in the percent of biofilm coverage, and the number of colony forming units for all strains cultured for 12 hr and 24 hr and then treated with DNase I. On the whole, *L. monocytogenes* present on stainless steel was diminished, indicating that eDNA has an important role in the biofilm production of *L. monocytogenes*.

INDEX WORDS: *Listeria monocytogenes*, biofilms, stainless steel, DNase I

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DEDICATION

To my family and friends, you are the catalysts that enable me to reach my goals.

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is a Gram positive intracellular pathogen (3, 9) responsible for the disease listeriosis of which there are approximately 1,600 cases annually in the United States. In 2008 the Centers for Disease Control and Prevention established that there were approximately 260 fatal cases each year (5). People at risk for the disease include the elderly, pregnant women, immune compromised and newborns, although the disease has been known to affect people with no preconditions (5).

L. monocytogenes is widely distributed in the environment, and when growing in biofilms is difficult to control in the food processing environment due to resistance to treatment by chemical sanitizers and heat (1-4, 12-13). One reason for *L. monocytogenes*' importance is because of industry difficulty in controlling the bacteria (1-2, 4, 7). Biofilms are groups of cells that work together to form favorable living conditions by providing a barrier for themselves (17). This barrier reduces the influences of the outside environment from interrupting the biofilms' ability to grow and interact with each other (14, 16). The barrier is a three dimensional matrix that reduces the ability of standard sanitizing techniques to kill or remove the cells from the surface (14, 16). Sanitizers like chlorine usually work by interrupting the cells ability to function, but the 3D matrix prevents chlorine from reaching the cells making the treatment ineffective (16).

The 3D matrix of biofilms differ in the biopolymers that they contain. Biopolymers may include: proteins, polysaccharides, extracellular DNA (eDNA), and water (1, 8, 14, 16, 18).

eDNA may function to help cells adhere to surfaces (6, 11), and may also aid in the transfer of information from one cell to another, leading to a resistance to certain microbial stresses, including antibiotics (10). This syncing of cells within the biofilm may help protect it from outside influences (10).

Processing facilities hygiene systems require the removal of food residues known as soil they are deposits of food and mineral that are left from everyday operations. Soil provides nutrition and growth conditions for the biofilm. In turn, the biofilm becomes a type of soil to remove. Cleaning systems are designed to remove soils but have not been specifically designed to remove biofilms. Therefore, there is a need to design cleaning processes for the removal of eDNA within the biofilm. This concern becomes even greater when considering the biofilms resistance to sanitizing treatments. Biofilms can form on any solid surface in the processing facility, including conveyer belts and gaskets. Stainless steel was used in this experiment because it is one of the most commonly found surfaces. (13).

DNase I is an enzyme that is found in the nucleus of the cell. It is an endonuclease, whose primary function is to splice DNA (15). DNase I is active against all DNA and its' predominate scientific use is to purify solutions to extract RNA. For this study, DNase I will be used to remove eDNA from biofilms.

There is little information on the role of eDNA in the biofilm formation in *L. monocytogenes*. Therefore, the objective of this research is to determine the role of eDNA in biofilm formation by *L. monocytogenes*.

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CHAPTER 2

LITERATURE REVIEW

Growth and survival characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a Gram positive facultative intracellular pathogen (21, 33), of concern as a food-borne pathogen because it is widespread in the environment (29, 31). *L. monocytogenes* has several distinct lifestyles that allow the bacteria to survive in a multitude of environments (34). It lives as a saprophyte within the soil, outside of the host, and as a pathogen once within the body causing disease (19).

L. monocytogenes was first discovered by Murray et al (35), in 1926, observing six cases of death in rabbits (35). The genus *L. monocytogenes* contains six species. The cells are usually found individually or in connected chains that form a Y or V shape; *L. monocytogenes* grows well on almost all commonly used media, sugars within the media increase growth (10). Temperature limits for *L. monocytogenes* are between 2 and 45°C (30). The bacterium grows best at neutral pH, but can grow from pH 4.4 to pH 9.6 (7, 20, 36). Nutritional requirements for *L. monocytogenes* are mainly glucose and glutamine which are the primary source of carbon and nitrogen (37). It is a motile organism having peritrichous flagella; motility occurs between 20 and 37°C.

L. monocytogenes has been observed in many environments including water, soil, and feces of humans and animals (3). Many reservoirs enable the growth of the bacteria due to its simple nutritional requirements (37). Soil is an ideal environment because of its ability to yield objects that have been infected from contact with *L. monocytogenes* and then discarded. Feces

from an infected host may work itself into the soil and from there infect potential food products (3).

Virulence and the nature of listeriosis

L. monocytogenes is a human pathogen and a risk to food safety, causing the disease listeriosis. The bacterium is distributed throughout the environment and has the ability to contaminate multiple food products. Foods contaminated with *L. monocytogenes* enact a class I food recall. It is one of ten pathogens that are under surveillance by FoodNet, a network of The Center for Disease Control and Protection, responsible for monitoring infections and outbreaks (8, 40).

Listeriosis is a severe condition that affects the elderly, immune compromised and pregnant. It is an opportunistic disease affecting people with weak immune systems, such as HIV/AIDS patients and neonates (8). In pregnant females listeriosis causes spontaneous abortions of the fetus. Listeriosis has a fatality rate of up to 30% (33).

Once ingested by a living organism the bacteria transitions into a pathogen causing listeriosis (33). According to Barbuddhe et al (3), the gastrointestinal (GI) tract is the main portal of entry. After entering the GI tract the bacteria is able to penetrate the mucosal tissue either directly or indirectly. Acting directly the bacteria causes the invasion of enterocytes, while indirectly it infiltrates the Peyer's patches region of the gastrointestinal tract. *L. monocytogenes*'s hemolytic toxin known as listeriolysin induces diarrhea and inflammatory responses from its host (3). Natural GI microflora may compete with the *L. monocytogenes* invasion response causing a milder response and reaction (19).

The transmission of *L. monocytogenes* in the environment acts in a cyclical manner, in which animals may be infected by the bacteria present in the environment, and then continue to spread the infection throughout the environment (2).

Virulence

Virulence is an important factor in determining the potential danger of a specific bacterium. Listeriosis has the ability to cause spontaneous abortions in pregnant females in addition to causing severe meningitis in the elderly and immune-compromised, such as those infected with HIV/AIDS. There have been remote cases where people with no predispositions were infected with listeriosis, this may be due to the ingestion of large inoculums (21, 28, 33). Virulence varies depending on the strain of *L. monocytogenes* (27-28).

Survival and growth in processing facilities.

L. monocytogenes is a potential source of contamination in processing facilities because of its ability to form biofilms (13). Survival is based on the bacterium's ability to resist sanitizer treatments during the cleaning process. Planktonic cells prove to have little resistance to sanitizers because of the lack of protection individual cells have compared to clusters. Growth is based on survival and the ability to proliferate on food residues.

Foods are contaminated via many reservoirs, but the main concern regarding the control of *L. monocytogenes* is in the handling of equipment. Unhygienic practices are the main source of *L. monocytogenes* contamination (2). These practices can cause the spread of the disease to humans.

Studies by Somers et al (44), showed that biofilms on brick and conveyors are resistant to sanitizers (44). Stainless steel is a material that is widely used in the food industry because of its durability, cost and easy maintenance. There are multiple varieties of stainless steel finishes, but

the most common is grade number 4. It has small grooves created by the mechanical polishing. These grooves provide an environment for microbial attachment and the subsequent growth of biofilms. Studies by Rodriguez et al (40), suggested that electropolished stainless steel does not have an advantage over mechanically finished stainless steel, showing that biofilms can attach under both circumstances thus indicating the extensive range in which biofilms can grow (40, 47).

RAPID type 9, a subtype of *L. monocytogenes*, is thought to reside in multiple fish processing facilities. This persistent strain's presence is an issue because it heightens the probability of contamination in food products (28).

Deli meats and slicers

A food item that provides a difficult challenge to food safety regarding *L. monocytogenes* contamination is deli meats. Deli meat was the highest ranked, ready-to-eat food vehicle for *L. monocytogenes* in 2003 (14). Retail sliced meats have a higher prevalence of *L. monocytogenes* contamination than pre-packaged meats. There is a higher risk in retail sliced meats because there are fewer safety features that can be implemented as opposed to pre-packaged meats. Improper handling in addition to a food product with limited processing creates an advantageous environment where *L. monocytogenes* has the ability to survive and grow with ease (14). A potential reason for *L. monocytogenes*' high risk to this product is the use of difficult to clean slicers. Slicers cut the meat, but meat residues may be left behind. These residues are an ideal environment for bacterial growth especially if they are not cleaned properly due to human error or because they have been transferred into a part of the slicer that is difficult to clean. These residues create the potential for contamination every time the slicer is used. The drier the biofilm the higher the risk of biofilm contamination due to aerosolization. Cell to cell surface

interactions become weaker, leading to increased contamination. However, wet biofilms will support higher levels of microbial growth. Foods with a high water activity are at risk for greater contamination if they come into contact with biofilms. Among the different deli meats, some types that are susceptible are bologna and hard salami (39).

Biofilm matrix, composition and structure

Biofilms are a complex community of cells growing on an interface that functions in a harmonious fashion to maintain themselves. Multiple species of bacteria have the capability to form biofilms; some are single-species biofilms while others work together in an attempt to survive in a wide array of environments. Phenotypes may even vary within a single-species biofilm in order to optimize the maintenance of the biofilm (12). Biofilms are encased, liquid-solid structure consisting of various biopolymers. Vilain et al (48), concluded that biofilm formation by *Bacillus cereus* requires DNA as part of the extracellular polymeric matrix to function (48).

The biofilm matrix is a complex structure whose composition varies with the bacteria species and growth environment. Research by Jahn and Nielson (26), estimated that biofilms consist of 70 to 98% water. Studies show that important components that may compose the biofilm matrix are proteins, polysaccharides and extracellular DNA (49). In regards to Gram positive cells such as *L. monocytogenes*, extracellular DNA is a central component of the biofilm matrix, playing a vital role in stability (11, 24).

Jordan et al (29), stated that *L. monocytogenes*' pathogenicity is an issue because it is capable of attaching to a wide range of surfaces in the form of biofilms. Studies done on alpha-proteobacterium *Caulobacter crescentus* biofilms show that nutrients are shared and protection is

provided by the matrix that blankets the colony. The matrix may increase or decrease depending on environmental conditions (4, 29).

eDNA in the matrix

Extracellular DNA (eDNA) is a component present in the matrix of some biofilms. In Gram positive bacterium where it is present, it contributes to the early formation of the biofilm (11). eDNA is present in the matrix of *L. monocytogenes* biofilms and may provide multiple advantages for the biofilm, possibly helping it evade destruction by outside influences such as sanitizing treatments, if present in processing facilities (24).

eDNA is relevant in the study of some biofilms especially *L. monocytogenes*, because research indicates a connection between eDNA and the ability of cells to attach to surfaces (24). Structurally speaking, eDNA can be similar to intracellular DNA and is one of many substances found in the matrix of some biofilms (45). eDNA of certain species starts off as genomic DNA. Studies conducted on the biofilms of *Acinetobacter sp.* indicate that eDNA is similar to intracellular genomic DNA, except for its location and occasionally size (50). Random polymorphic DNA analysis of *Acinetobacter sp.* shows that eDNA originated from genomic DNA, but is not structurally identical throughout the entire form. These differences possibly stem from the interactions with other materials in the extracellular matrix, such as polysaccharides and proteins (50).

The amount of eDNA found in a particular biofilm depends on the species of bacteria being studied. Certain species have different amounts of genomic DNA, affecting the amount of eDNA available for use in the biofilm. According to Stienberger et al (45), eDNA is quantifiable and can be differentiated from cellular DNA (45). Lysis is the main mechanism that releases eDNA into the matrix of *Escherichia coli* biofilms. Usually lysis is initiated through

environmental factors that may cause apoptosis or cell death. Cell death initiates lysis releasing all materials that are stored within the cell out into the extracellular matrix, including genomic DNA. Other mechanisms for eDNA discharge from the cell are DNA-containing vesicles and DNA secretion. Sanchez-Torres et al (41), states that secretion aids in eDNA release from *E.coli* biofilms. Deleting RNA is also proposed to cause an increase in eDNA. The global regulator H-NS is responsible for eDNA production and restoration of eDNA from the extracellular matrix via the cell in *E. coli* (41).

Biofilms may advantageously use eDNA to stabilize the structural matrix of the cell. Martins et al (34), indicated that the addition of exogenous DNA to a biofilm matrix of *Candida albicans* increased the biomass of the biofilm, denoting that eDNA is a contributor to the stability of biofilms of this particular species. The consequence is a stable biofilm allowing for a greater risk of contamination, depending on the growth environment (33).

eDNA is important to some cells' ability to attach to surfaces, but there may also be some attachment without the presence of eDNA, just in reduced levels. Das et al (11), stated that in studies performed with thermodynamic analysis, eDNA was crucial in acid-base interactions between surfaces and cells of Gram positive bacteria. Harmsen et al (24), showed the importance of eDNA in *L. monocytogenes* by culturing cells without eDNA present in the matrix. Harmsen concluded that neither genomic DNA nor any other DNA could restore the biofilm so that it would attach to surfaces until peptidoglycan (PG) and N-acetylglucosamine (NAG) interacted with DNA added *in vitro* causing attachment to restore. Harmsen states that for the *in vitro* DNA to be effective in attachment it must exceed 500 base pairs (24).

Studies done on the biofilms of *E.coli* K-12 BW25113, *Acinetobacter baumannii* ATCC 17978, *Stenotrophomonas maltophilia*, and *Staphylococcus aureus* ATCC 25923 showed that

extracellular ATP assists in intercellular communication (51). Injured cells send ‘distress calls’ to others cells using ATP, alerting other cells to prepare for protection. In receiving the message, the cells increased biofilm formation and cell adhesion by intensifying eDNA production to the biofilms. Extra eDNA assembly created a defense system; stabilizing the biofilms from environmental factors (51).

Berne et al (4), revealed that eDNA may prevent unwanted cells from settling on an already established biofilm of *Caulobacter crescentus* by binding to the attachment site of the intruder. This mechanism within the biofilm may be a result of the excess accumulation of daughter cells within the biofilm itself. Prevention is accomplished by eDNA binding to the ‘polar headfast’ of the daughter cells flagella, thwarting attachment. Berne stated that this process also promotes the dispersal of daughter cells, leaving the established cells alone to have optimal biofilm size (4).

Biofilm attachment and formation

Initial attachment to a surface is the required first step in the biofilm formation. Araujo et al (1), stated that the initial adhesion is based on many factors, including: Van der Waals interactions, electrostatic interactions and acid-base interactions. These exchanges are influenced by physiochemical properties, such as: hydrophobicity, surface charge and electron donor/acceptor properties (1).

Attachment mechanisms

Different biofilms produced by different bacteria have various mechanisms for attachment to surfaces. Initial biofilm formation may involve the use of flagella, pili, capsules, and other attachment devices (18). *Escherichia coli* and *Pseudomonas aeruginosa* use motility

for attachment, incorporating the use of flagella and pili, respectively (18, 43). *Staphylococcus aureus* initiates surface proteins known as Bap (biofilm-associated protein) for attachment (46).

In the process of attachment, biofilms of many species are likely to encounter resident microflora, depending on their environment. Although little is known about initial biofilm attachment, Habimana et al (22), stated that *L. monocytogenes* has difficulty adhering to surfaces in which the microflora secretes exopolysaccharide (EPS).

There can be attachment of cells to surfaces without the formation of biofilms. The presence of nutritional factors that allow proliferation of the cell is a determining feature in biofilm formation. Food residues provide the nutritional requirements needed for growth. A planktonic cell can attach to a surface, but without proliferation it is not considered a biofilm.

L. monocytogenes showed limited motility at 12°C and increased motility at 22°C, while cells at 37 °C showed a decrease in movement. Di Bonaventura et al (13), stated that this diminution in movement is an indication of irreversible attachment and the beginning of biofilm formation. These experiments revealed that temperature plays an important factor in attachment of the cells (13).

The presence of NaCl increases *L. monocytogenes*' ability to adhere to plastic surfaces. This amplification of attachment is caused by NaCl's initiation of auto-aggregation in cells. Even moderate amounts of salinity initiate aggregation and biofilm formation (27-28).

Non mutant strains of *L. monocytogenes* are motile planktonic cells that have flagella. Mutants without flagella were defective in biofilm formation, suggesting that the attachment to surfaces requires flagella mediated motility by the cell before attachment (31). According to Araujo et al (1), cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* are active in the

use of flagella for initial biofilm attachment. Cellular appendages on the cell surface are also a constituent in the attachment process (1)

Transcriptional activator Prf A regulates the *L. monocytogenes* virulence genes. These genes also aid *L. monocytogenes* transition from an extracellular organism to an intracellular pathogen. If the cell lacks Prf A, then it is defective in attachment and biofilm formation (31).

A major component of *L. monocytogenes* that enhances the bacteria's ability to invade and attach is the cell wall bound protein and virulence factor Internalin A. Certain strains that carry Internalin A displayed a better biofilm-forming ability than strains without the protein, or strains that formed the protein, but not in its full length (17).

Adherence to plastic surfaces is another ability that is important due to the amount of plastic used in industrial systems. In addition to stainless steel, plastic can be an essential part of processing facilities and adherence to this surface gives *L. monocytogenes* an advantage over other bacteria.

Strain EGDe is an example of *L. monocytogenes* that contains proteins with the ability to influence attachment to surfaces. Specifically, this strain is able to attach to abiotic surfaces that are prevalent in food processing facilities, especially among drains and conveyors. These proteins are a concern because they may contribute to surface adhesion, but they are not an essential requirement for adhesion (29).

Biofilm formation

Growth is a determining factor in regards to biofilm formation and is dependent on many variables. The availability of nutrients is essential to growth, as is the ability to survive environmental stresses that could damage the biofilm. Attachment and biofilm formation are different situations. Attachment is a process that an individual cell undergoes, while formation

and growth are something that is part of the biofilm. Before biofilms can be formed, attachment by a cell to a surface is needed. The cell has the potential to form a biofilm if proper conditions are available, otherwise it is merely a planktonic cell attached to a surface.

Little is known about biofilm formation and regulation of *L. monocytogenes*; however, it has been determined that nutrients are an important contributor. Without nutrients, biofilms have fewer layers of the three dimensional matrix and are not as structured. With nutrients, the biofilms are highly organized and form spherical colonies connected by a network very similar to knitted chains (38).

The response regulator Deg U, within *L. monocytogenes*, is required for growth at high temperatures, which is a fundamental trait of this bacterium. The ability of *L. monocytogenes* to survive and grow at high temperatures is the main reason for its growth in diverse environments. In enabling efficient biofilm formation, the *degU* gene increases the risk of contamination of a particular site. If this site is a food processing facility the biofilms expounds an elevated risk to the food being manufactured (21).

Consequences of biofilm formation

The main consequence when it comes to biofilm formation of *L. monocytogenes* is the spread of disease (33) . Biofilms allow bacteria to be more prominent in the environment (44). A direct consequence of biofilms in processing facilities is the increased risk of contamination of foods and food products, exposing the general population to the risk of listeriosis.

Each cell has its own survival characteristics, but biofilms are particularly hazardous because they are hard to eradicate. Biofilms are harder to remove than planktonic cells therefore they pose a greater threat in contamination of food.

L. monocytogenes is regularly found in retail environments as biofilms and can persist for over a year. The complex configuration of the biofilm helps harbor the risk of contamination on their inhabited surface. The protection of the three dimensional matrix that engulfs the actual cells prevents sanitizers from reaching their target. Since cells are not eliminated they persist and remain a hazard to food products (38).

Chemical sanitizer and heat resistance

L. monocytogenes is hard to eradicate with standard sanitizing techniques due to its ability to form biofilms (1, 5). Biofilm resistance is a large contributor to potential food contamination (13). The control of *L. monocytogenes* is difficult for many reasons, such as its widespread presence in the environment, its resistance to many environmental factors, adaptability to external stresses, and growth in a wide range of temperatures and environments. Because of these traits *L. monocytogenes* is able to flourish under many environmental conditions (33).

Strains of *L. monocytogenes* have different abilities when it comes to the formation of biofilms. Folsom and Frank (15), found that chlorine resistance is one distinction between strains of *L. monocytogenes*, as well as between biofilms and planktonic cells. Cells in biofilms have a higher tolerance to chlorine, particularly sodium hypochlorite. This tolerance could be partially due to the morphology of the biofilms. The matrix of the biofilm may play a part in resistance by posing as a barrier between the chlorine and the cells. In different strains of *L. monocytogenes* biofilms where chlorine resistance varied, biofilm morphology was also different, suggesting that the morphology has an influence on chlorine resistance in *L. monocytogenes*. However, biofilm cell density was not associated with chlorine resistance (15-16).

Heat treatment is sometimes used against *L. monocytogenes* at potential biofilm sites. This method could possibly be used to control *L. monocytogenes* in food processing facilities, where they pose the most risk of contamination. There is little information available on the proper methods needed for heat treatment against biofilms of *L. monocytogenes*. Chmielewski and Frank (9), study showed that hot water sanitation of stainless steel could be an effective method for inactivating *L. monocytogenes* (9).

Cell to cell transfer of information

A plausible reason that eDNA is valuable to biofilm formation is its ability to transfer information from one cell to another as demonstrated in *Streptococcus mitis*. Horizontal information transfer was shown in research by Hannan et al (23), where the transference of antibiotic resistance is demonstrated. The ability of eDNA to transfer this information also advocates the possibility that it could transfer other information, keeping the cells in the biofilm colony as efficient as possible. This network of communication would provide collective resistance to environmental factors, plausibly benefitting the biofilm and the constituent cells (23). Studies done by Xi and Wu (51), on biofilms of *E.coli* K-12 BW25113, *Acinetobacter baumannii* ATCC 17978 *Stenotrophomonas maltophilia*, and *Staphylococcus aureus* ATCC 25923 reinforces the importance of cell communication in biofilm survival, due to environmental factors that are constantly stressing biofilms. A consequence of the shared information between cells within the biofilm is resistance to antimicrobials. This is a potential risk due to the high pathogenicity of *L. monocytogenes* (23, 33).

Removal of biofilms

There are procedures that have been incorporated to counter the spread of biofilms. Ultrasonification is a preliminary method used to remove *L. monocytogenes* biofilms. This

method breaks up the three dimensional structure that surrounds the biofilm. Once this structure is broken up, normal chemical sanitizers can then be used in direct contact with *L.*

monocytogenes cells for treatment. Planktonic cells were effectively treated with chemicals such as chlorine, and responded better to treatment with hydrogen peroxide. Ultrasonification in addition to chemical treatment has shown reduction in cell numbers (5).

Treatment of stainless steel coupons with acidic EO water for 30 -120 seconds shows a reduction in biofilms of *L. monocytogenes*. This reduction occurs as a reaction to the low pH level according to Araujo et al. (1). A mixed treatment of acid and alkaline produced a greater reduction than just acidic EO water. Once again this may be a result of the acid base properties associated with adhesion. The reduction obtained by acidic EO treatment was temporary and surface growth was seen over time. The proposed mix of acid/base treatment can be used to reduce biofilm populations and increase inactivation of biofilms (2).

The combination of ozonation and ultrasound treatment showed a positive impact on the removal of biofilms of *L. monocytogenes* from stainless steel. Studies illustrated that pH contributes to the surface adhesion of biofilms, ozonation disrupts these interactions, reducing biofilm formation by a 3.8 log reduction (6).

Sanitizers are more effective on young *L. monocytogenes* biofilms (less than seven days old). However, lactic acid based sanitizers worked best for biofilms older than seven days. Quaternary ammonium compound based sanitizers worked best at pH 10.42 – 11.46. Another aspect to consider is the biofilm surface; biofilms on smooth surfaces are more susceptible to sanitizers than those on rough surfaces (25). Polysaccharides and proteolytic enzymes can also be effective against biofilms. Alkaline pH buffer removed a majority of biofilm biomass in many bacterial species (32). Chlorinated-alkaline, low-phosphate detergent, dual peracid

sanitizers were fifty percent effective. Solvated-alkaline environmental sanitation product, hypochlorite sanitizers were eighty six percent effective (44).

Planktonic cells of *L. monocytogenes* identified with electrophoresis testing showed susceptibility to DNase I. DNase I had no effect on the DNA of the planktonic cell as long as it was inside the cell. The cell wall may be a proponent in keeping DNase I out (42).

eDNA is important to the abilities of *L. monocytogenes* to attachment and stabilize the biofilm. Finding a way to diminish these abilities is beneficial in the prevention of contamination. It has been found that DNase I is effective in hydrolyzing DNA and thus reducing the optimal length of the eDNA (24). Removing eDNA from Gram positive cells reduces those cells ability to initially attach to surfaces.

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CHAPTER 3

THE ROLE OF EXTRACELLULAR DNA IN THE FORMATION OF BIOFILM BY

LISTERIA MONOCYTOGENES.¹

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Abstract

Biofilms are a potential source for contamination in processing plants, particularly because of their ability to survive treatment by certain sanitizers during cleaning. A possible reason for this resistance is the three dimensional matrix that biofilms produce to engulf the cell community, which sanitizers are unable to penetrate. The objective of this research was to investigate the overall importance of extracellular DNA (eDNA) in the ability of *Listeria monocytogenes* to attach to stainless steel and produce biofilms, in addition to determining its role in stabilizing the biofilm structure. Twenty-five strains were screened to monitor their biofilm producing ability, from this group five strains that differed in biofilm production were selected for study. Biofilms were grown on stainless steel coupons for 12 hr and 24 hr at 25°C and then rinsed gently with sterile water to remove planktonic cells before the addition of deoxyribonuclease I (DNase I). 200 µL (200 units) of DNase I was diluted 1:10 with peptone solution and added to the biofilms for 24 hr at 25°C. The strains were again rinsed and observed. Results indicate that the strains differed in their response to biofilm formation in the presence of DNase I. There was a significant reduction of biofilm percent coverage in almost all strains treated with DNase I in both 12 hr and 24 hr increments. Biofilms cultured for 12 hr showed a greater reduction than cultures grown for 24 hr, suggesting that eDNA is a factor in the cells' ability to attach and initiate biofilm formation. On the whole, *L. monocytogenes* present on stainless steel were diminished, indicating that eDNA has an important role in biofilm production by *L. monocytogenes*.

Key words: *Listeria monocytogenes*, biofilms, stainless steel, DNase I

Introduction

L. monocytogenes is a pathogen that is adaptable and widespread in the environment (7). The Centers for Disease Control and Prevention states that *L. monocytogenes* is capable of contaminating a wide array of foods, including deli meats, cheeses, smoked seafood, unpasteurized milk, and may also grow in certain refrigerated products (3). *L. monocytogenes* causes the disease listeriosis, which affects the elderly, immune compromised and pregnant. Initial symptoms are mild, but if left untreated may lead to more severe conditions such as meningitis, and spontaneous abortions of fetuses (15). *L. monocytogenes*' prevalence in the food industry may partially be due to its ability to form biofilms (5, 13).

Biofilms are a cluster of cells that form a synergistic relationship with each other (8), and are difficult to eradicate with standard cleaning and sanitizing techniques (2). Consisting of a protective 3D matrix, the components of which may be water, proteins, polysaccharides, and eDNA, the cells are sheltered from outside influences. The presence of biofilms increases the risk of contamination of food, and therefore the risk of disease.

Extracellular DNA is similar to intracellular genomic DNA except for size and location (19). eDNA is notable in the study of biofilms because there may be a connection between eDNA and the ability of the biofilms to attach to surfaces (10). eDNA found in a particular biofilm varies depending on the species of bacteria. Studies indicate eDNA is a functional part of *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *Staphylococcus aureus* biofilms (16, 20, 24). eDNA is able to transfer information from one cell to another, helping the biofilms spread information that may be useful for survival such as antimicrobial resistance (9). A major trait of eDNA may be

its capability of increasing cell attachment to surfaces (10). The main mechanism by which eDNA is formed is through lysis of genomic DNA into the 3D matrix (19).

There is little information regarding the components that compose the matrix of *L. monocytogenes* biofilms. Water is the main constituent of the biofilms, composing up to 98% of the matrix volume produced by Gram positive cells (11). Studies indicate that eDNA may be a major component of biofilms produced by various bacterial species, but knowledge is limited as to the extent of its importance (1, 10, 12). Proteins are also present in the matrix. Knowledge of components within the biofilm matrix will be beneficial in understanding *L. monocytogenes*' ability to attach and proliferate within environments posing a risk to humans. The objective of this research was to determine the role of eDNA in biofilm formation by *L. monocytogenes*.

Materials and Methods

Preparation of strains

Twenty five strains of *L. monocytogenes* were screened for biofilm production. Strains were obtained from various sources (Table 3.1). The cultures were stored on cryogenic beads (Microbank, Pro-Lab, Ontario Canada) at -80°C (Ultra Low Temperature Freezer, New Brunswick Scientific, England). The process involved growing each strain in 13 ml of tryptic soy broth (Becton, Dickinson and Co., Sparks, MD) for 24 hr and then the collection of the cells by centrifugation (Beckman Instruments Inc, Munich, Germany) at 6000 rpm, providing a G-force of 3,750, for 15 min at 25°C. The supernatant fluid was decanted and discarded. The pellet was vortexed (Mini-Vortexer, VWR Scientific Products, Batavia, IL) and placed in a container with the cryogenic beads. The cell-bead mixture was vortexed, labeled and placed in storage at -80°C for future use.

Preparation of stainless steel coupons

Stainless steel coupons (5 cm by 2 cm, University of Georgia Instrument Shop, Athens, GA) with a grade 4 finish were used in the experiment. Coupons were degreased using acetone (Sigma-Aldrich, St Louis, MO), rinsed using distilled water and then cleaned by submersion in CIP-200 cleaner (Acid-Based Process and Research Cleaner, Steris Co., St Louis, MO) for 20 min. The clean coupons were rinsed 3 times in sterile distilled water and left to air dry in a biological safety cabinet (SterilGARD II, the Baker Co., Sanford, ME). Each coupon was then placed into a 50 ml test tube (Pyrex, USA), capped and sterilized using the dry cycle of the autoclave (PRIMUS Sterilizer Company Inc., Omaha, NE).

Inoculation of coupons and growth of biofilm

To prepare the inoculum, cryo-beads containing each strain were removed from -80°C storage and cultured in 10 ml TSB for 24 hr at 25°C. The inoculum was obtained by repeating the culturing process. To inoculate the stainless steel coupons, 15 ml of TSB was added to a 50 ml test tube containing the 5 cm by 2 cm sterile coupon; approximately two thirds of the coupon was covered by the TSB. The sub-cultured strains (15 µl) were added to the individual test tubes of TSB and inoculated. All test tubes were incubated for 24 hr at 25°C. After the allotted time the inoculated coupons were rinsed with sterile distilled water to remove planktonic cells and left to dry in the biological safety cabinet.

Colony Forming Units (CFU) analysis of biofilm

CFU quantification was done simultaneously with biofilm analysis. Coupons (1.5 cm X 1.5 cm) containing biofilms cultured for 12 hr and 24 hr were gently rinsed with distilled water and submerged in a mixture of peptone (10 ml, Becton Dickinson and Company, Sparks, MD) and microscopic glass beads (425-600 µm, Sigma-Aldrich, St. Louis, MO). They were then vortexed for 3 min to shake cells free from the stainless steel. The mixture was then diluted and

plated on tryptic soy agar (Becton, Dickinson and Co. Sparks, MD) in triplicate, incubated at 37°C for 24 hr. CFU were counted and CFU/sq cm was calculated. Twelve and 24 hr CFU cell density determinations were repeated 5 to 6 times.

Microscopic analysis of biofilm

Coupons containing biofilms were prepared for microscopic analysis by being placed in Petri dishes (VWR International, Radnor, PA) and stained with Quant-IT PicoGreen dsDNA Reagent (Invitrogen, Ltd, Paisley, UK). The stain was defrosted from the -20°C freezer and diluted 1:200 with TE buffer (1X, Promega Corp. Madison, WI) and added directly to the coupons. After staining, the coupons were air dried in the dark.

Biofilms were visualized using an epifluorescent light microscope (Nikon Corp, Japan) equipped with the Nikon super high pressure mercury lamp (Nikon Corp, Tokyo, Japan), the lens used was 'Nikon Plan Fluor 10X/0/0.30', and a B-2A filter (Nikon). The Nikon 10X lens in addition to the optical lens (10X) gives the image a final magnification of 100X.

All images were taken in the dark so that optimal fluorescence was observed. Coupons were kept in the dark before microscopy. Images were taken under the 'manual exposure' and the color snap options on the MagnaFIRE 2.0 C program. All images were minimized and saved onto a portable USB data storage device (Sandisk Cruzer 8GB, Milpitas, CA). Five images were taken of each biofilm. The images were converted to grayscale via the Adobe Photoshop 6 program (Adobe Systems Inc, San Jose, CA), and the grayscale images were analyzed via the ImageTool program (Department of Dental Diagnostic Science, The University of Texas Health Science Center, San Antonio, TX). The program is designed to convert pixels from grayscale to black and white. Black pixel area was biofilm and white was background. Contrast was

manually set and a count was obtained for all black and white pixels. The count was analyzed and a percentage of pixel area, standard deviation and mean were recorded.

DNase I treatment of biofilms

The inocula were prepared as previously described. Stainless steel coupons (1.5 cm by 1.5 cm University of Georgia Instrument Shop, Athens, GA) were cleaned and sterilized as previously described and added to well plates (Corning Inc., Corning, NY). Coupon size was changed from 2 cm by 5 cm to 1.5 cm by 1.5 cm because the wells were too small to hold the larger coupons used in the initial screening process. There were 12 wells per plate, each well able to contain 6.9 ml, TSB (2 ml) was added to each well, and inoculated with 2 μ L of the subcultured strains. The coupons were incubated for 12 hr or 24 hr at 25°C. After incubation, coupons were removed from the wells, rinsed with sterile water, and placed in empty well plates. DNase I (200 μ l, Thermo Fisher Scientific, Milwaukee, WI), diluted with peptone (1:10) was added to each stainless steel coupon and incubated for 24 hr at 25°C. (Experimentation regarding different dilutions was completed indicating that 200 μ L was suitable for this study). This experiment consisted of adding undiluted DNase I to biofilms grown for 24 hr and noting the effects on the biofilm images. Once the allotted time was up, the stainless steel coupons were quantified to get a CFU count, the process involved submerging the stainless steel coupon in a mixture of peptone and acid washed glass beads (425-600 μ m Sigma-Aldrich Co., St. Louis, MO) and shaken for 3 min using a vortexer before quantification and treatment with Quant-IT PicoGreen dsDNA Reagent for image analysis.

Results

Screening of *L. monocytogenes* for biofilm formation

Strains of *L. monocytogenes* were screened to observe their degree of biofilm formation (Figure 3.1). Strains used were isolated from a variety of sources including food, poultry processing environment and clinical isolates (Table 3.1).

Biofilm production ranged from 0.5 to 49.7% coverage. Strains could be classified into three groups based on their ability to produce biofilms. Strains producing over 20% growth were considered high producers. Strains 18, 311, G3990, and G3982 exhibited high biofilm formation, between 18 ($20.9 \pm 6.8\%$) and G3982 ($49.8 \pm 1.8\%$). These strains were isolated from food plant environments and clinical sources (Table 3.1). Moderate biofilm-producing strains consisted of biofilms with coverage between 7 and 19.9%. Moderate biofilm producing strains ($7.1 - 9.5\%$) were Scott A, N390, 19115, K342, G257, and A232. These strains were isolated from poultry processing plants and infected humans (Table 3.1). Low biofilm producing strains had biofilm coverage of 6.9 % or lower. Low biofilm producing strains were D123, 17, YM6, B233, M370, I267, 960, H261, 302, YM3, E249, 303, 1237, 961, and L350. These strains were isolated from food plant environments, poultry processing plants, infected monkeys and the monkey environment (Table 3.1). The lowest biofilm producing strain was L350 ($0.5 \pm 0.4\%$), the highest of this subset was D123 ($5.2 \pm 1.1\%$).

Five strains representing high, moderate and low biofilm production were selected for further study. Strain G3982 ($49.8 \pm 1.8\%$) was selected because it was the highest biofilm producer. Strain 311 ($29.7 \pm 3.5\%$) produced significantly less biofilms than strain G3982, but was also selected to represent a consistently high biofilm producer. Strains 19115 ($9.1 \pm 5.0\%$) and N390 ($9.5 \pm 3.4\%$) were selected because they were moderate biofilm producers, and strain D123 ($5.2 \pm 1.1\%$) was selected to represent a consistently poor biofilm producer.

Four of the five strains selected for further study exhibited consistency (low standard deviation) in their biofilm-forming ability. An exception was G3982, selected because it was the highest biofilm producer of those studied. Figure 3.1 indicates that most of the strains tested exhibited a high standard deviation for biofilm production. Inconsistent biofilm production of some strains has been observed in other studies. The causes of this variation are unknown.

Effect of DNase I on biofilm production

DNase I was added to biofilms for 24 hr after culturing strains for 12 hr (Table 3.2), further biofilm production was reduced for all of the strains tested. High biofilm producers exhibited reductions in biofilm production of 69% for G3982 ($15.6 \pm 5.8\%$) and 51% for 311 ($14.7 \pm 2.3\%$). Low biofilm producers exhibited reductions of 16% for D123 ($4.4 \pm 1.4\%$). The moderate biofilm producers exhibited reductions in biofilm production of 59 % by 19115 ($3.7 \pm 1.5\%$) and 84 % by N390 ($1.5 \pm 0.8\%$). Table 3.2 indicates that 12 hr growth with the addition of DNase I for 24 hr produced a reduction in all cultures. N390 exhibited the highest reduction while D123 showed the least.

When DNase I was added to biofilms after 24 hr of incubation (Table 3.2), biofilm production was reduced for almost all of the strains tested. High biofilm producers exhibited reductions in biofilm production of 62% by G3982 ($18.6 \pm 5.7\%$) and 7% by 311 ($27.7 \pm 7.3\%$). Low biofilm producers did not show a significant increase or decrease in biofilm coverage after DNase I, exhibiting a percent difference of 7% for D123 ($5.6 \pm 2.3\%$). The moderate biofilm producers exhibited reductions in biofilm production of 48 % by 19115 ($4.7 \pm 2.4\%$), and 80% by N390 ($1.9 \pm 0.8\%$). Table 3.2 indicates that 24 hr cultures of biofilms with the addition of DNase I for an additional 24 hr showed greater resistance to DNase I than 12 hr cultures. N390 displayed the most reduction while D123 showed the least.

Overall data presented in Table 3.2 indicates that when DNase I was added to biofilms cultured for 12 hr, it produced a greater reduction in subsequent biofilm formation than when it was added after 24 hr of growth. Some cultures showed large differences, cultures 311-12 hr ($14.7 \pm 2.3\%$) and 311-24 hr ($27.7 \pm 7.3\%$), showed an increase of 13.0% biofilm formation between the two time periods in the presence of DNase I. G3982-12 hr ($15.6 \pm 5.8\%$) and the culture G3982-24 hr ($18.6 \pm 6.0\%$) showed a difference of only 3.0%. Other strains showed an increase in biofilm formation also, but not as significant as these high biofilm producers.

Effect of DNase I on CFU in the biofilm

Biofilm CFU quantification (Table 3.3) was obtained without the addition of DNase I. This gave us the data needed to compare the effectiveness of the DNase I treatment on biofilms. The CFU count for the biofilms was consistent with the area covered data from the initial screening (Figure 3.1). Table 3.3 reveals that all strains had similar bacterial enumeration numbers. High biofilm producing strains G3982 and 311 had a high CFU/cm² of log 9. Moderate biofilm producing strains 19115, and N390 produced a CFU/cm² of log 8.0, and strain D123 had a CFU/cm² of log 7.9. In relation to the moderate biofilm producers, strain D123 achieved greater cell density than expected, showing a moderate cell density instead of low. Data from biofilms with DNase I added after 12 hr is presented in Table 3.3. All biofilms produced a lower cell density than the biofilms that had no DNase I added. This outcome confirms the area covered data presented in Table 3.2. High biofilm producers showed a log reduction of log 3.3 CFU/cm² for G3982 and log 2.7 CFU/cm² for 311. Moderate biofilm producers N390 and 19115 showed log reductions of 3.1 CFU/cm², and 3.0 CFU/cm² respectively. Low biofilm producing strain D123 showed a log reduction of 2.5 CFU/cm². There was a reduction in all 5 strains between log 2.5 and 3.5 CFU/cm². On the whole, strain

D123 (2.5 CFU/cm²) was reduced the least, most likely because the strain was already a low biofilm producer (6). With a lower density the strain had less biofilm to lose or produces little or no eDNA and therefore was not as affected by the DNase I as the moderate or high biofilm producing strains.

Biofilms with DNase I added after 24 hr (Table 3.3) had a lower cell density than the biofilms grown for 24 hr that had no DNase I added, confirming the area covered data presented in Table 3.2. High biofilm producers showed a reduction of log 2.9 CFU/cm² for G3982 and log 2.5 CFU/cm² for 311 after DNase I treatment. Moderate biofilm producers N390 and 19115 showed log reductions of 2.9 CFU/cm², and 2.5 CFU/cm² respectively. Low biofilm producing strain D123 showed a log reduction of 2.4 CFU/cm². There was a log reduction in all 5 strains between 2.4 and 2.9 CFU/cm². The only discrepancy regarding Tables 3.2 and 3.3 is with strain D123. Table 3.2 showed no significant increase or decrease in D123-24 hr biofilm percent coverage, while data in Table 3.3 shows a decrease in cell density compared to the cell density of D123 that was untreated. The mean cell densities of 12 hr cultures with DNase I added were less, compared to the cell density of all 5 strains cultured for 24 hr with DNase I added for an additional 24 hr. The mean difference in cell density between 12 and 24 hr cultures were not significant: log 0.44 CFU/cm² for G3982 and log 0.19 CFU/cm² for 311 both high biofilm producers. Log 0.41 CFU/cm² for 19115, and log 0.4 CFU/cm² for N390, which are moderate biofilm producers and log 0.17 CFU/cm² for D123 the low biofilm producing strain. Even though these results show a reduction, they are too small to be considered significant.

Effect of eDNA treatment on biofilm morphology

Microscopy images allow comparison of morphology of treated and untreated biofilms. Images in Figure 3.2 show the comparison between biofilms of *L. monocytogenes* strain G3982

that has and has not been treated with DNase I. (A) and (B) are images of biofilms that have been grown for 12 hr without the addition of DNase I. Whereas images C and D are of *L. monocytogenes* G3982 that have been grown for 12 hr with DNase I added for an additional 24 hr. The difference between A, B and C, D, is in Table 3.2 indicating the coverage of the 5 tested strains. Strain G3982 when no DNase I was added, coverage of $49.7 \pm 1.8\%$ is represented, while 12 hr growth with DNase I added was $15.6 \pm 5.8\%$. Figure 3.3 compares images of strain G3982 cultured for 24 hr treated with DNase I for an additional 24 hr. Coverage with DNase I added was $18.6 \pm 5. \%$. The images illustrated the reduction in both 12 hr and 24 hr biofilms treated with DNase I.

Figure 3.4 shows an enlarged image of strain G3982 without any treatment which exhibited $49.7 \pm 1.8\%$ coverage (Table 3.2). The image shows the cells are connected in a linear fashion, possibly corresponding to the grooves of the stainless steel. Being a high biofilm former, the strain appears to grow in the whole groove. The grooves seem to be the preferred attachment sites, perhaps because they offer an increased surface area. The biofilm consists of confluent growth. The linear cell growth tends to be separated sporadically by black spaces. These spaces may consist of either biofilm matrices or water, which are not fluorescent because PicoGreen is a specific stain for double stranded DNA. The black spaces are observed on all the images, suggesting that they may be water channels. Many biofilms required water channels for diffusion of nutrients and waste.

Figure 3.5 is an image of biofilms produced by strain G3982 cultured for 12 hr and treated with DNase I for an additional 24 hr. Compared to Figure 3.12 there is less biofilm coverage ($15.6 \pm 5.8\%$ compared to $49.7 \pm 1.8\%$, Table 3.2). Figure 3.5 shows that after treatment with DNase I, it is apparent that primarily attachment of single cells or clumps takes

place. It is possible that the DNase I hydrolyzed the eDNA, resulting in less attachment of cells and possibly the release of cells from the biofilm. The image shows some microcolonies that do not fill out the grooves of the stainless steel.

Figure 3.6 shows biofilms produced by G3982 treated with DNase I for 24 hr after an initial 24 hr growth period. The image corresponds to coverage of $18.6 \pm 5.7\%$ (Table 3.2). The image, similar to Figure 3.5, shows clumps or single cell dispersal indicating that hydrolysis of eDNA had a large impact on the biofilm formation. The cells are covering more of the stainless steel than in Figure 3.5, but less than the DNase I-free control (Figure 3.4). There is little confluent growth, unlike Figure 3.4. Additionally, there is no association of biofilms with the stainless steel grooves. The single cells or clumps appear randomly dispersed rather than structured. The difference between Figures 3.5 and 3.6 derives from the amount of time the biofilms were grown. This difference is important because it may provide information on the function of eDNA in the biofilm. eDNA's primary function may be attachment in 12 hr cultures. Twenty-four hour cultures are better established and may use eDNA for stability perhaps in addition to attachment. This different functionality may be the reason for increased percent coverage in Figure 3.6 relative to 3.5.

Figures 3.7 and 3.8 provide images of *L. monocytogenes* 311, including 12 hr (Figure 3.7.) and 24 hr (Figure 3.8) with and without DNase I added for an additional 24 hr. Again these images are comparable with data presented in Table 3.2, representing the coverage of strain 311. Strain 311 with no DNase I added was ($29.7 \pm 3.5\%$). Twenty four hr cultured biofilms for strain 311 with DNase I added for 24 hr was ($27.7 \pm 7.3\%$). These two results are close together in percentage, displayed in Figure 3.8.

Figure 3.9 is an enlarged image of biofilms produced by strain 311 without treatment. The image corresponds to coverage of $29.7 \pm 3.5\%$ (Table 3.2). The biofilm image shows uniformity in structure and is similar to biofilms produced by strain G3982 because of the biofilm formation within the grooves of the stainless steel. The biofilms are almost confluent since all the cells seem to be connected with each other. The black spaces that surround the biofilms may again be water channels. These black spaces could also be the matrix that surrounds the cells. The structure of the biofilm indicates a well-established biofilm.

Figure 3.10 shows 12 hr growth of 311 with DNase I treatment for an additional 24 hr. The image has a percent coverage of $(14.7 \pm 2.3\%)$ shown in Table 3.2. Overall the treatment appears to have removed biofilm cells, and only dispersed single cells and small clumps remain. The center of the image shows a high density of single cells or clumps. The density is not similar to Figure 3.9, which is untreated, but clearly shows single cells or clumps attached in close proximity to each other; this lack of uniformity is commonly observed with biofilms. The single cells or clumps are often attached within grooves of the stainless steel.

Figure 3.11 shows biofilms produced by strain 311 cultured for 24 hr with DNase I treatment for an additional 24 hr. The image corresponds to coverage of $27.7 \pm 7.3\%$ (Table 3.2). The biofilm has almost the same coverage as the untreated control shown in Figure 3.9. Even though the two biofilms are similar in coverage, they are different qualitatively. Figure 3.11 shows dispersed attachment of single cells or clumps and perhaps growth, while Figure 3.9 shows confluent biofilms. The biofilm in Figure 3.11 is more uniform in its coverage, aligning with the grooves of the stainless steel coupon. The uniformity is maintained throughout the biofilm and infers that the cells could be microcolonies that are derived from growth rather than

attachment. This is only suggestive because the biofilm distribution may be influenced by the structure of the coupon.

Figures 3.12 and 3.13 shows the changes due to the addition of DNase I to strain N390 12 hr and 24 hr. Again the images represent the changes seen in Table 3.2. Twelve hour biofilms showed a percent reduction of 84% and 24 hr biofilms with DNase I added for 24 hr showed a decrease of 80%.

Figures 3.14 and 3.15 illustrate changes in biofilms produced by strain 19115. There was a decrease in visible biofilms in both 12 and 24 hr after treatment (Table 3.2). Both Figures show reduction in percent coverage of biofilms due to the addition of DNase I for 24 hr. The decreases were 59% for 12 hr and 48% for 24 hr growth.

Figures 3.16 and 3.17 display the changes in *L. monocytogenes* D123. Table 3.2 indicates that treated 12 hr biofilm of D123 showed similar results to untreated D123. This is indicative in Figure 3.16 which shows similar images of both treated and untreated coupons. Images in Figure 3.17 illustrate the similarities in biofilms of culture D123-24 hr treated and untreated. Table 3.2 shows that D123-24 hr with no DNase I added produced a percent coverage of $(5.2 \pm 1.1\%)$ while the same culture with DNase I added for 24 hr was $(5.6 \pm 2.3\%)$, illustrating similarities in growth. This is the only culture that projected similar percentages (Figure 3.17).

Figure 3.18 shows an image of biofilms produced by strain D123 without DNase I treatment. The image corresponds to coverage of $5.2 \pm 1.1\%$ (Table 3.2). The image primarily indicates single cell attachment or clumps of *L. monocytogenes* on stainless steel. The cells are not preferentially attached within grooves of the coupon, and appear to be randomly dispersed.

There is no confluent growth unlike untreated strains G3982 and 311. Therefore eDNA may not play a major factor in attachment of strain D123.

Figure 3.19 shows an image of biofilms produced by strain D123 cultured for 12 hr and treated with DNase I for 24 hr. The image corresponds to coverage of $4.4 \pm 1.4\%$ (Table 3.2). The image shows dispersed single cells or clumps and microcolonies. The cells appear randomly dispersed except for small clusters that have formed within the grooves of the coupon.

Figure 3.20 shows an image of biofilms produced by strain D123 cultured for 24 hr and treated with DNase I for an additional 24 hr. The image correspond to coverage of $5.6 \pm 2.3\%$ (Table 3.2). The biofilm image is similar to that produced by strain D123 without treatment. Single cells or clumps and microcolonies are randomly dispersed, showing no preference to grooves.

Discussion

Controls

Oliveira et al (18), studied biofilm formation of *L. monocytogenes* strain ATCC 19117 on stainless steel and its biotransfer potential. They used growth conditions, including growth medium and substratum similar to our study and measured biofilms accumulation by bacterial enumeration. The biofilms of Oliveira et al (18), had 4.08 ± 0.67 Log CFU/cm² after 48 hr at 37°C. Our untreated biofilms were denser, as they averaged 8.39 CFU/cm² after incubation for 24 hr at 25°C (18). The differences in data could be due to different strains and technique used for enumeration. While we removed the biofilms for analysis by vortexing with glass beads, Oliveira used surface swabbing. Also Oliveira grew the biofilms in media agitated with a stir bar, whereas we grew them under static conditions. The techniques used by Oliveira are similar to those we did for producing biofilms; additional steps we took that Oliveria did not was the addition of DNase I as a treatment.

Milanov et al (17), used electron microscopy to view images of *L. monocytogenes* biofilms on stainless steel that were grown for 7 days at 25°C. Seven out of 14 strains showed biofilm formation, which were classified by their 3D structure, while non-biofilm forming strains were singular monolayer cells (17). This study has many similarities to our own. The use of stainless steel and similar temperatures has given similar images in regards to biofilms. Milanov showed in detail the 3D accumulation of biofilms (17). Our research yielded similar images, but not in such detail. The images we have acquired show *L. monocytogenes* stacked atop each other; this behavior is confirmed by Milanov's images.

eDNA and *L. monocytogenes*

The purpose of this research was to determine the role of eDNA in the formation of biofilm by *L. monocytogenes*. Results of this research indicate that eDNA may have various functions when it comes to aiding *L. monocytogenes* attach and remain adhered to surfaces. Hydrolysis of eDNA decreased attachment of *L. monocytogenes* to stainless steel. eDNA is a contributing factor to cell attachment and biofilm formation for some strains (Tables 3.2, 3.3 and Figures 3.2 - 3.20). Area covered by biofilm decreased with the hydrolyzing of eDNA via DNase I, as did the number of CFU; this was all visible in fluorescent microscopic images. The ability for *L. monocytogenes* to attach is the first step in biofilm formation. The initial attachment process involves flagella (14), but the adhesive nature of the cell envelop is also important. eDNA may contribute to this adhesiveness. The adhesiveness of eDNA excreted into the biofilm matrix is also important once the biofilm is established as it may help stabilize the biofilm by keeping it attached to the surface and maintaining its structure. eDNA in the biofilm may contribute to the survival of *L. monocytogenes* in food processing facilities, providing the potential to contaminate food products. The main reason for the two time intervals of 12 and 24 hr was to observe maturity. Results for 12 hr experimentation showed eDNA's ability to attach, while the 24 hr showed eDNA's ability to stabilize the already established biofilm. This can be considered in a processing facility standpoint; the time intervals portray a situation where biofilm buildup over a weekend is similar to the 24 hr interval while daily buildup is closer to 12 hr.

Tetz et al (22), studied the effects of eDNA destruction by DNase I on the biofilm formation of *Escherichia coli* and *Staphylococcus aureus* species. Biofilms were cultured in Luria-Bertani or Mueller-Hinton media at 37°C for 24 hr on glass coverslips. DNase I addition to the biofilms was followed by 24 hr incubation, similar to our own experiments. Measuring

techniques used were CFU, OD and electrophoresis. Control CFU counts were 8.74 ± 0.76 and $8.76 \pm 0.64 \log_{10}$ CFU/well for *E. coli* and *S. aureus* respectively. Biofilms formed in the presence of DNase I showed counts of 8.32 ± 0.65 and $8.23 \pm 0.52 \log_{10}$ CFU/ well (22). Our studies of *L. monocytogenes* strains showed an average control of $\log 8.38$ CFU/cm² after 24 hr of incubation at 25°C, with an average treatment of $\log 5.74$ CFU/cm². The differences between the two studies can be attributed to the different bacteria used. Within our study we observed variability in the different strains of the same species. Both studies exhibit eDNA reduction via DNase I treatment, and establish that eDNA may be an important factor in biofilm attachment.

Martins et al (16), studied the role of eDNA in the biofilm matrix of *Candida albicans* and its contribution to biofilms. Biofilms were grown on three different types of diluted media; (1) RPMI-1640 supplemented with L-glutamine, sodium bicarbonate and buffered with 0.165 M MOPS, (2) YPD, and (3) yeast nitrogenbase (YNB) supplemented with glucose. The substrata for growth was silicone elastomer strips (1 cm x 9 cm) which were placed into a flow system at 37°C for 48 hr. The extracellular matrix was isolated from the biofilms and the extracellular DNA was isolated from the matrix. Treatment with DNase I and Exogenous DNA were done to the isolated eDNA. Measuring techniques were crystal violet assay and determining bacterial populations on Sabouraud dextrose agar. The results showed a difference between mean log CFU/ml before and after sonication: 0.00 (P = 1.00) for RPMI, -0.07 (P = 0.39) for YPD, and 0.00 (P = 1.00) for YNB which indicates that eDNA is an important component of the *C. albicans* biofilm matrix. DNase I activity produced significant detachment of mature cells from the substrata. The study also indicated that eDNA is important in maintenance and stability of the biofilm (16), confirming our own data that eDNA aids in attachment and stability.

Harmsen et al (10), conducted research on the role of eDNA during biofilm formation by *L. monocytogenes*. Biofilms were cultured in brain-heart infusion broth and Hsiang-Ning medium for 11 to 14 hr at 37°C. A coverglass was used as the substrata. DNase I, RNase I, heat inactivated DNase I, and proteinase K was added to biofilm to view effects. Biofilm amount was determined using microscopy after SYTO9 staining. Additional assays conducted were culture purification and origin of eDNA of all 41 strains using gel electrophoresis of ethanol-precipitated eDNA, microtiter biofilm assays, flow cell biofilm visualization, stimulation of attachment via the introduction of DNA to growing biofilm cultured, and the addition of adhesion factors. Microscopy results indicated that eDNA has an impact on initial attachment on cells in static chambers assays, while DNase I had the most impact on biofilms followed by Proteinase K. Addition of adhesion factors showed that the introduction solely of DNA to biofilms without eDNA was not enough for attachment properties to be activated, but an adhesion factor such as peptidoglycan helped restore adhesion (10). Even through their methodology differed in substrata, time, temperature of incubation and strains used; their results were similar to ours in that the addition of DNase I to the culture reduced the amount of biofilm, an indication that eDNA is a contributing part of biofilm attachment and stability.

Biofilm Maturity

The amount of time that biofilms are cultured is critical to how they react to outside factors such as DNase I. Results imply that the DNase I is effecting biofilms at different stages, as a 12 hr biofilm is immature relative to one that have been growing for 24 hr. Evidence of this is found in Table 3.2, 3.3 and Figures 3.2 - 3.20, all of which show that the effects of the same amount of DNase I on biofilms cultured for 12 hr and 24 hr were dissimilar.

Conover et al (4), concluded that eDNA is essential for biofilm integrity of *Bordetella bronchiseptica* and *Brodetella pertussis* on both abiotic surfaces and the upper respiratory tract of mice. Biofilms were cultured in microtitre plates overnight at 37°C before the addition of DNase I reaction buffer. *B. bronchiseptica* was grown in Stainer- Scholte (SS) broth, SS medium with supplemented heptakis was used for *B. pertussis*. DNase I treatment was for 48 hr; over this time the plates were rinsed to remove planktonic cells. The biofilms were stained with 0.1% crystal violet solution to measure absorbance. Results indicate that DNase I has no significant impact on the biofilms before 6 hr, but after showed steps to inhibit development. Microscopic analysis indicated that DNase I addition for 30 -90 min to *B. bronchispetica* cultured for 48 hr (immature) and 4 day (mature) on glass coverslips disrupted biofilms and only localized clusters were visible, compared to the fuller biofilm formation of the controls, suggesting reduction and detachment of cells from the biofilm due to the absence of eDNA in both 48 hr and 4 day old biofilms (4). This paper suggests that eDNA is critical for biofilm functionality on glass surfaces, while we used stainless steel; there is a similarity in procedure and results between both studies. Results provide evidence that eDNA plays a role in multiple processes of the biofilm.

Low biofilm producing strain

Not all strains produced results indicating that eDNA is involved in attachment. Strain D123 is a low biofilm producing strain of *L. monocytogenes* and its biofilm production was not as effected by DNase I treatment. There was no change in biofilm coverage of 24 hr cultures after the addition of DNase I (Table 3.2), indicating that this culture may produces only low levels of eDNA or that eDNA is not involved in low level attachment to stainless steel.

Stacking of cells

Images (Figure 3.12, and 3.15) indicate stacking of cells in the biofilms. This layering of cells may be made possible by the presence of the extracellular matrix. The removal of eDNA from the matrix may reduce cell interactions, reducing the cell's ability to layer. The result would be single cell attachment, clumps or single layered biofilms. This interpretation is supported by data in Figures 3.12, 3.14, 3.14, 3.17, Tables 3.2 and 3.3.

Vilain et al (23), studied the adhesive properties of eDNA in aiding *Bacillus cereus* in biofilm formation with treatment with DNase I and RNase. *B. cereus* was cultured for 24 hr in Luria-Bertani (LB) broth inoculated to a density of 10^5 CFU/ml at 37 or 28°C with a shaking plate at 200 rpm. The substrata used were acid-washed glass tubes, glass beakers or glass wool fibers. Measuring techniques included electrophoresis and microscopy. Treatment with DNase I and RNase before electrophoresis indicated a substance with high mobility suggesting that DNA removal results in less attached cells. Biofilms were stained with Styro 9 and propidium iodide and then viewed using laser scanning confocal microscopy in addition to electrophoresis. The study identified the eDNA present in the biofilms of this species using genetic alteration of the cells and staining techniques (23). This study was similar to ours in that it included the use of DNase I for treatment during culturing of the biofilms. A similar conclusion was reached in that eDNA contributed to the adhesiveness of the matrix, aiding in biofilm formation. The removal of eDNA lead to more motile cells due to the decrease in adhesiveness of the matrix. In our study, this lead to attachment of single cells or clumps.

Tetz et al (21), studied the effects of DNase I on biofilms of various Gram negative and Gram positive bacteria. The motive behind the research was to establish antibiotic susceptibility of biofilm if they have been treated prior with DNase I to cleave eDNA. The bacterial strains studied were *Escherichia coli*, *Haemophilus influenza*, *Klebsiella pneumonia*, *Pseudomonas*

aeruginosa, and *Acinetobacter baumannii*. Multiple liquid media were used; Luria-Bertani, Muekker-Hinton, Schaedler, and Heamophilus test medium. The strains were cultured for 24 hr at 37°C in static conditions on glass substrata. After the addition of DNase I the cultures were further incubated for an additional 24 hr at 37°C. The measuring techniques used were CFU counts and microscopy after staining with crystal-violet (21). Experimental similarities include culturing strains at 24 hr and further incubation of cultures after the addition of DNase I. CFU analysis and microscopy were conducted in both studies with different techniques. Tetz used scraping to remove biofilm from glass substrata while we glass bead vortexing. Antibiotic experimentation was also conducted by this study. Results show high control CFU numbers for all bacteria involved in the study (all above 8.00 log₁₀ CFU/well) and a decrease in all biofilms treated with DNase I, further reduction in CFU was observed in treatment with an antibiotics/DNase I combination. Microscopy observed biofilms as microcolonies. The study concludes that eDNA is an essential component for maintenance of the biofilm (21). The results of our study corroborates the finding of Tetz, eDNA is used as a tool for stability. Mature cells that are well attached have no need for eDNA to aid in attachment, but instead use eDNA in stability of cells and ultimately the biofilm.

Images of untreated strains preferentially adhered to the grooves of the stainless steel. This pattern of attachment has been previously observed by Milanov et al (17). The attachment to this area of the coupon is not unexpected; the grooves provide additional surface area for the cells during attachment, which may increase area for nutrient uptake. The attached cells within the grooves may possibly be microcolonies, but there is also the possibility that they attached in that manner due to the structure of the stainless steel.

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CHAPTER 4

CONCLUSION

The objective of this study was to provide insight into the effect eDNA has on the biofilm of *Listeria monocytogenes*. Maturity of biofilms was monitored, as was the variation in biofilm production by different strains.

The experiment was designed to hydrolyze eDNA of biofilms cultured at two different maturity levels (12hr and 24 hr). Cultured biofilms were subjected to DNase I treatment and effects were observed via CFU quantification and Fluorescent microscopy.

Results indicate that immature biofilms (12 hr) showed more of a reduction after treatment than mature biofilms (24 hr). Low biofilm producing strains did not show significant decreases after treatment while high biofilm producing strains did. DNase I treatment changed the biofilm landscape from a multi-dimensional structure to single-cell attachment.

In conclusion eDNA is an important component of the biofilm matrix, providing multiple function to the biofilm such as attachment of immature cells, stability of mature ones, and acting as an adhesive to allow cells to stack. eDNA production is dependent on the strains being studied and cannot be generalized over all *L. monocytogenes* species.

TABLE 3.1. Strain number, source, serotype, and lab origins of *Listeria monocytogenes* used in screening process.

strain number	source of isolation	serotype	lab origins
17	food facility environment	4b	Center for Food Safety ^a
18	food facility environment	unknown	Center for Food Safety American Type Culture Collection
19115		4b	
302	monkey clinical	1/2a	Center for Food Safety
303	monkey clinical	1/2a	Center for Food Safety
J311	food facility environment	unknown	-
960	monkey clinical	1/2a	Center for Food Safety
961	monkey clinical	1/2a	Center for Food Safety
A232	poultry processing facility	-	ARS ^c
B233	poultry processing facility	-	ARS
D123	poultry processing facility	-	ARS
E249	poultry processing facility	-	ARS
G257	poultry processing facility	-	ARS
G3982	clinical isolate Jalisco Cheese outbreak	4b	
G3990		unknown	
H261	poultry processing facility	-	ARS
I237	poultry processing facility	-	ARS
I267	poultry processing facility	-	ARS
K342	poultry processing facility	-	ARS
L350	poultry processing facility	-	ARS
M370	poultry processing facility	-	ARS
N390	poultry processing facility	-	ARS
Scott A	human isolate Scott A	4b	
YM3	monkey environmental	1/2a	Center for Food Safety
YM6	monkey environmental	1/2a	Center for Food Safety

^a Strains were obtained from the Center for Food Safety, Dept of Food Science and Technology,

Athens, GA.

^b Serotyping for (ARS) strains were not done.

^c USDA Agricultural Research Service, Richard B. Russell Research Center, Athens, GA

Highlighted strains were selected for experimentation.

TABLE 3.2. Biofilm coverage produced by *Listeria monocytogenes* on stainless steel 24 hr after addition of DNase I^a.

	Percentage covered ^b				
	G3982	311 ^c	N390	115	D123
No DNase I addition	49.7 ± 1.8 ^d	29.7 ± 3.5	9.5 ± 3.4	9.1 ± 5.0	5.2 ± 1.1
Added after 12 hr	15.6 ± 5.8	14.7 ± 2.3	1.5 ± 0.8	3.7 ± 1.5	4.4 ± 1.4
% reduction of 12 hr	69 ^e	51	84	59	16
Added after 24 hr	18.6 ± 5.7	27.7 ± 7.3	1.9 ± 0.8	4.7 ± 2.4	5.6 ± 2.3
% reduction of 24 hr	62 ^e	7	80	48	7 ^f

^a Biofilms were grown in tryptic soy broth for 12 hr and 24 hr. DNase I was added for 24 hr at 25°C after biofilms had been grown. Stainless steel coupons were stained with Quant-IT PicoGreen dsDNA reagent and images taken with epifluorescent microscope, magnification X10.

^b Images were analyzed by the ImageTool program to produce biofilm percent coverage.

^c Strain 311 biofilms cultured for 24 hr with DNase I added for additional 24hr showed recovery after treatment.

^d n = 5

^e Percent reduction was calculated by subtracting the 'Added after 12 hr' or '24 hr' from 'No DNase I addition' then dividing that answer by 'No DNase addition' X 100.

^f Strain D123 24 hr showed an increase in biofilm formation with the addition of DNase I.

TABLE 3.3. Effect of DNase I on Cell Density of biofilms produced by *Listeria monocytogenes* after treatment with DNase I. Biofilms were treated with DNase I, 12 and 24 hr after growth, and allowed to grow for an additional 24 hr ^a.

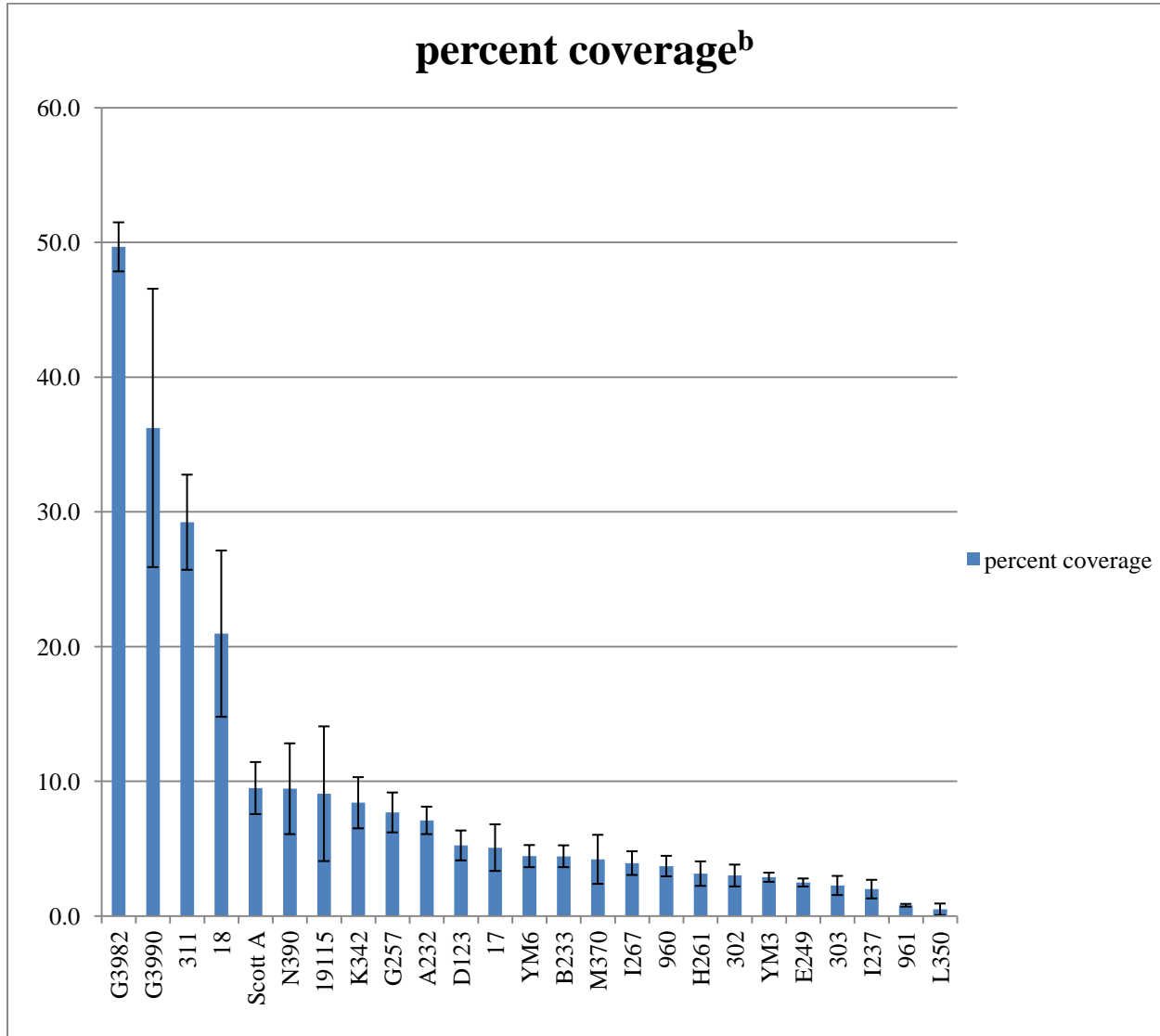
	Strain \pm SD CFU/cm ²				
	G3982	311	19115	N390	D123
Before DNase	9.0 \pm 0.01 ^b	9.0 \pm 0.1	8.0 \pm 0.04	8.00 \pm 0.4	7.9 \pm 0.04
12 hr after	5.7 \pm 0.09	6.4 \pm 0.06	5.0 \pm 0.05	4.8 \pm 0.1	5.4 \pm 0.07
log reduction	3.3 ^c	2.7	3.0	3.1	2.5
24 hr after	6.1 \pm 0.05	6.5 \pm 0.06	5.4 \pm 0.07	5.1 \pm 0.07	5.6 \pm 0.05
log reduction	2.9 ^c	2.5	2.5	2.9	2.4

^a Biofilms were grown in tryptic soy broth for 12 hr and 24 hr at 25°C before DNase I was added for 24 hr at 25°C.

^b n = 5

^c Log reduction compared to Before DNase values.

FIGURE 3.1. Percentage area of stainless steel coupons covered by biofilms produced by various strains of *Listeria monocytogenes*^a.



^a Biofilms were grown in tryptic soy broth for 24 hr. Stainless steel coupons were stained with Quant-IT PicoGreen dsDNA reagent and images were taken with a fluorescent microscope, magnification X10.

^b Images were analyzed by the ImageTool program to produce biofilm percent coverage.

FIGURE 3.2. Fluorescent micrographs of *Listeria monocytogenes* G3982 grown in TSB for 12 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

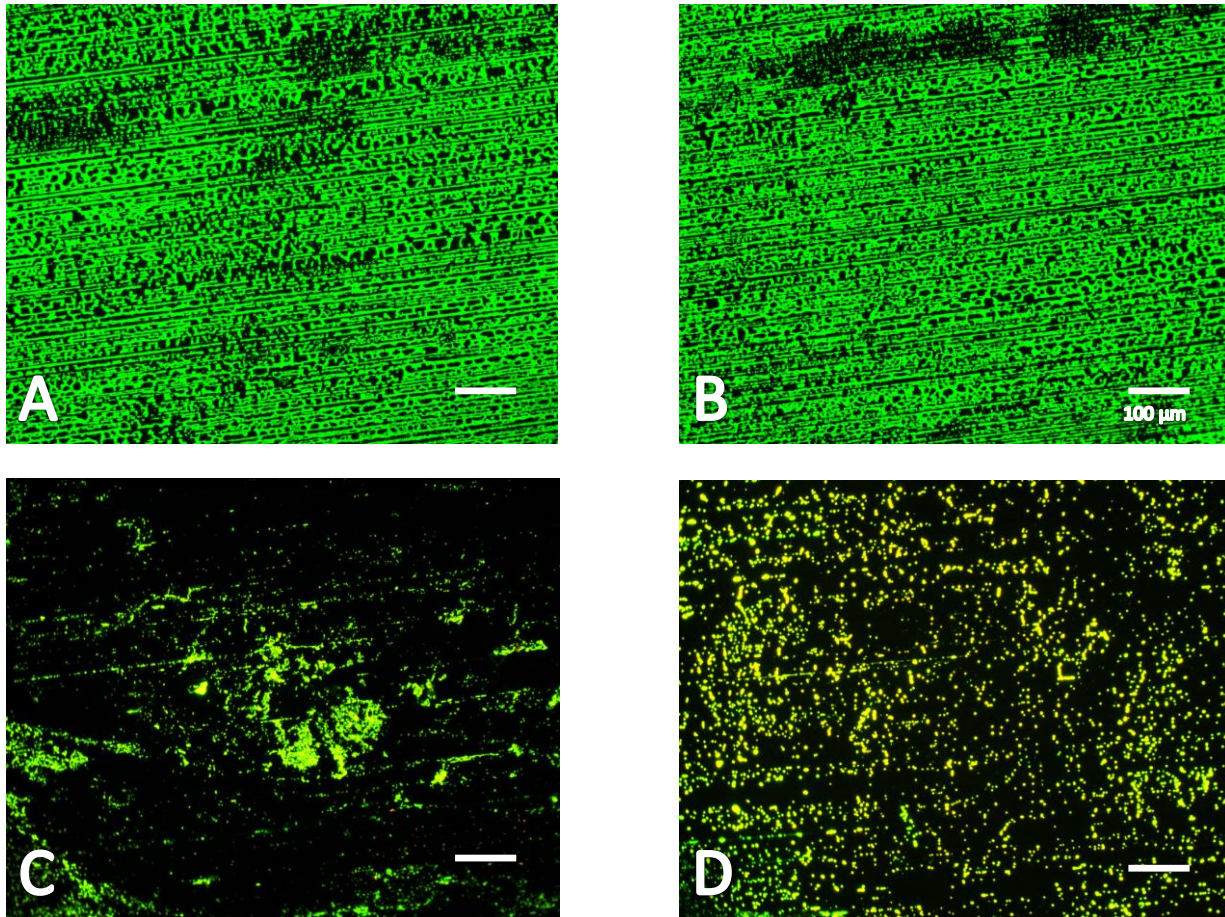


FIGURE 3.3. Fluorescent micrographs of *Listeria monocytogenes* G3982 grown in TSB for 24 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) were not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

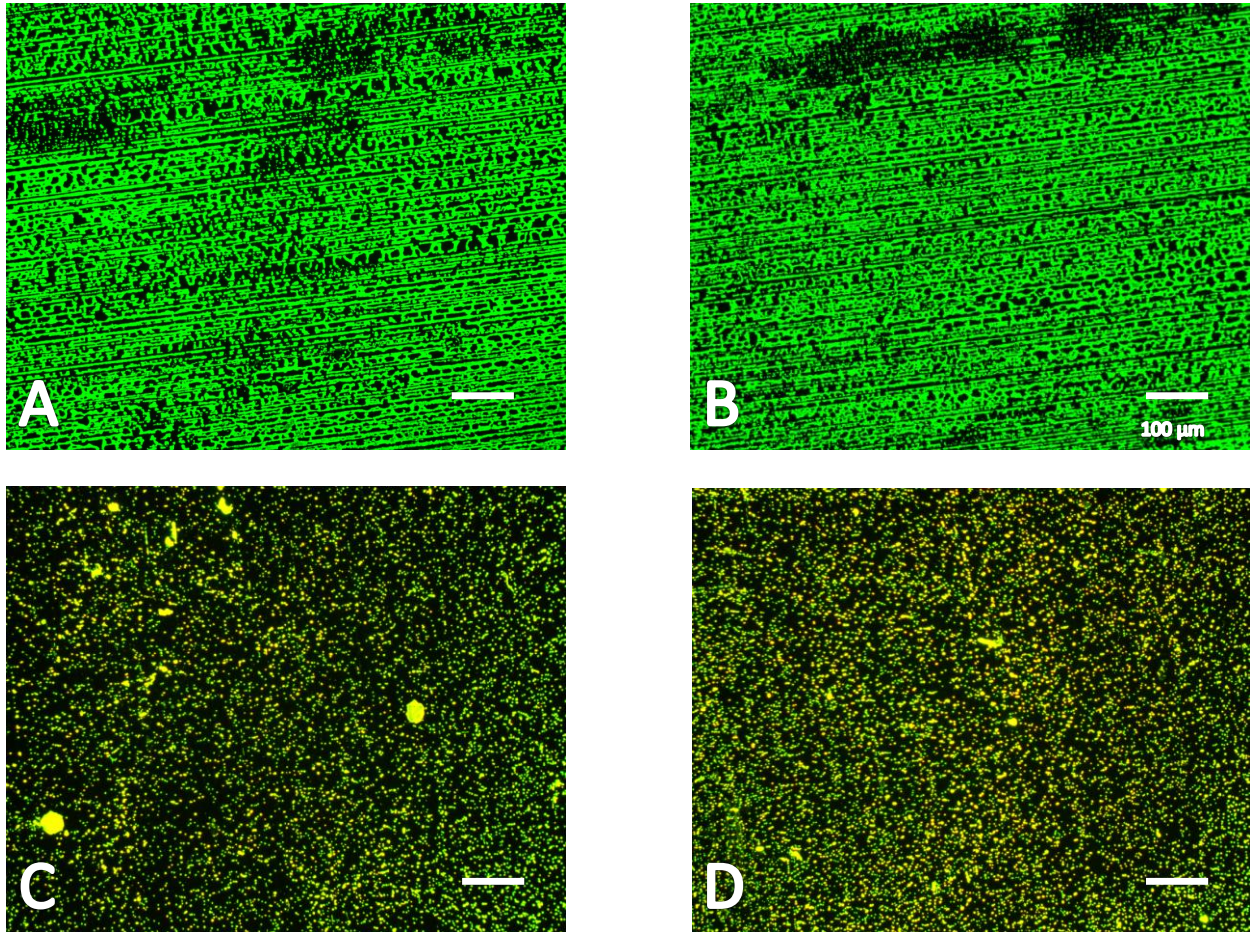


FIGURE 3.4. Fluorescent micrograph of *Listeria monocytogenes* G3982, grown in TSB for 24 hr. Biofilm cultured on stainless steel, stained with Quant-IT PicoGreen dsDNA reagent, and viewed under epifluorescent light microscope.

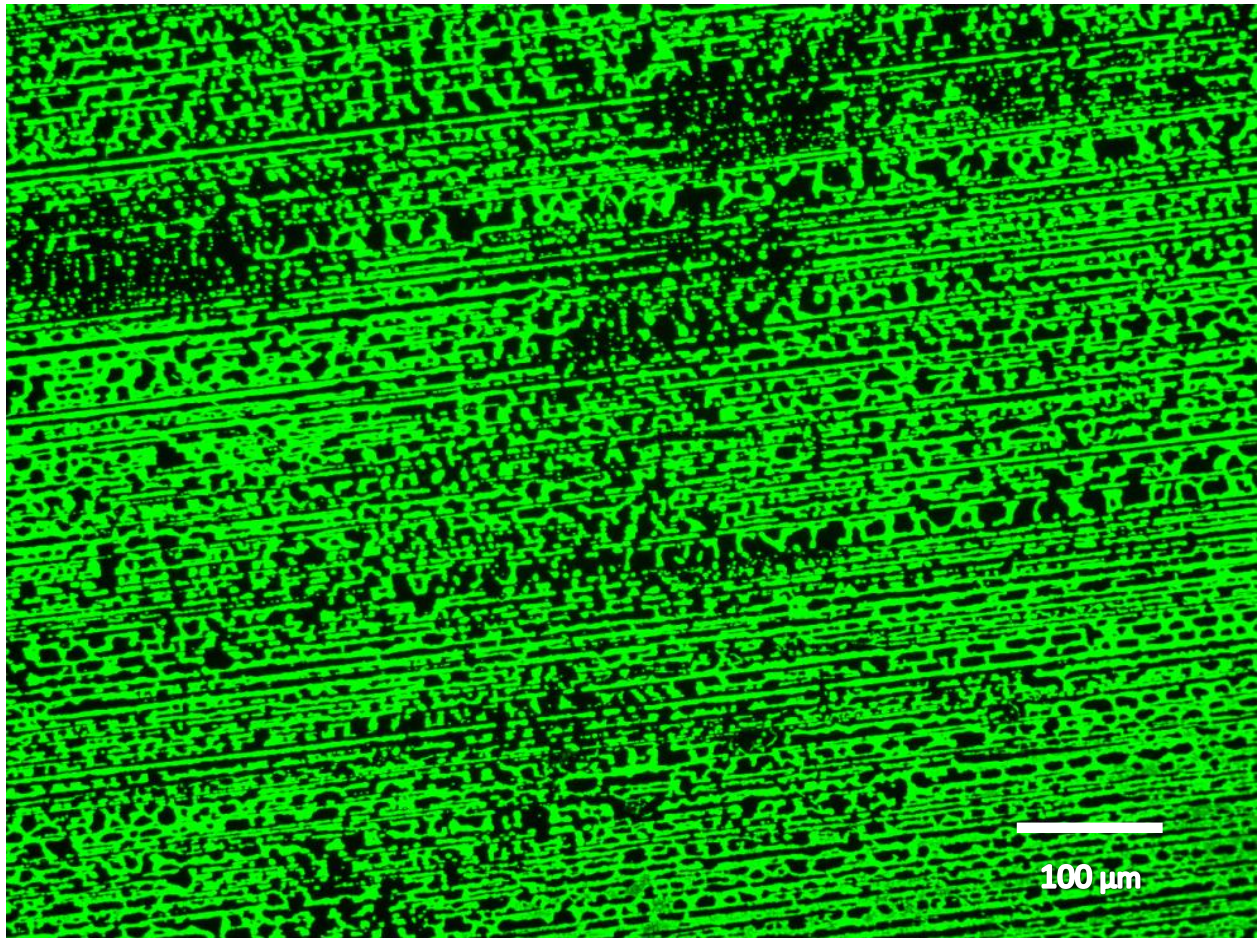


FIGURE 3.5. Fluorescent micrograph of *Listeria monocytogenes* G3982, grown in TSB for 12 hr. Biofilm cultured on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Biofilm stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

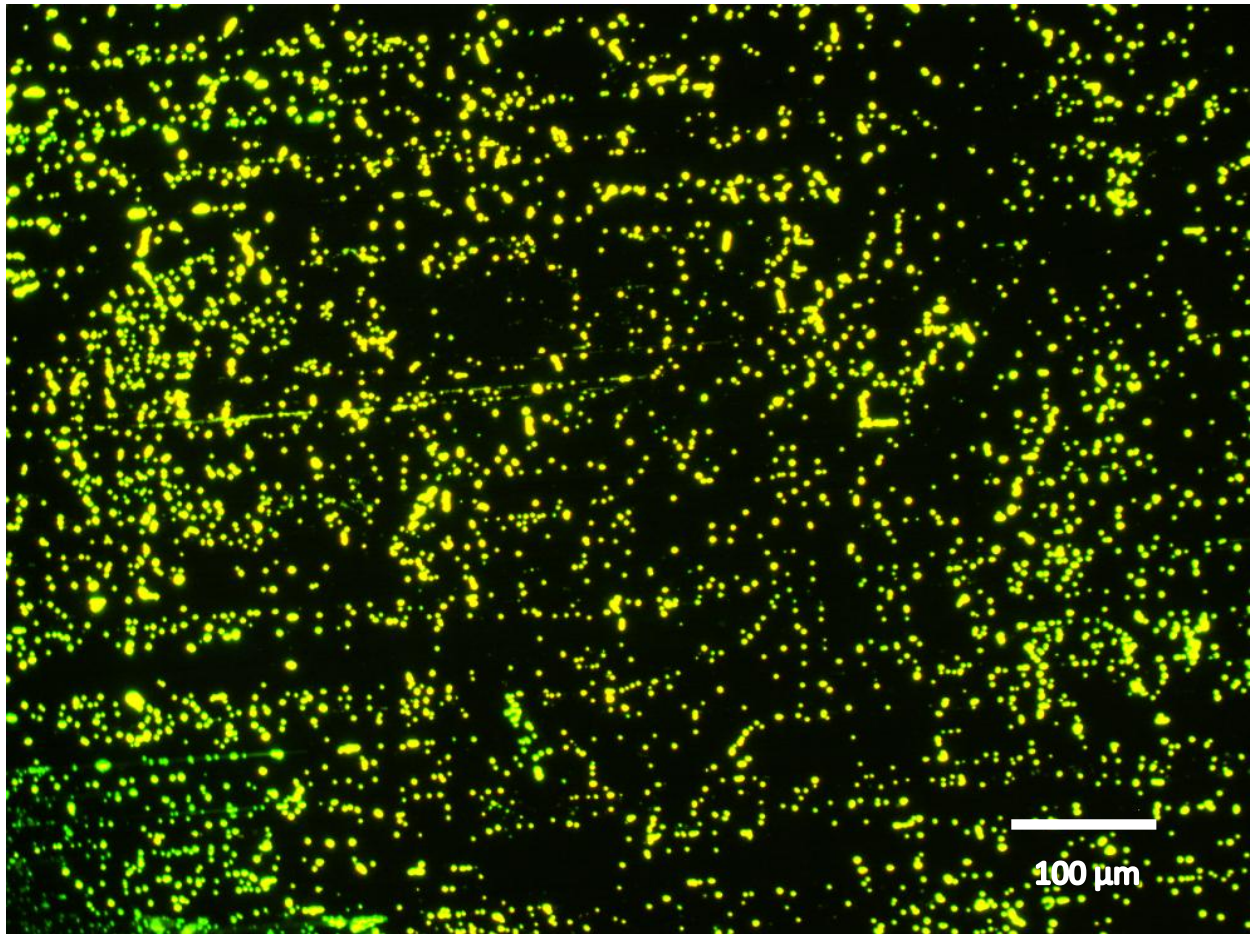


FIGURE 3.6. Fluorescent micrograph of *Listeria monocytogenes* G3982, grown in TSB for 24 hr. Micrograph was on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

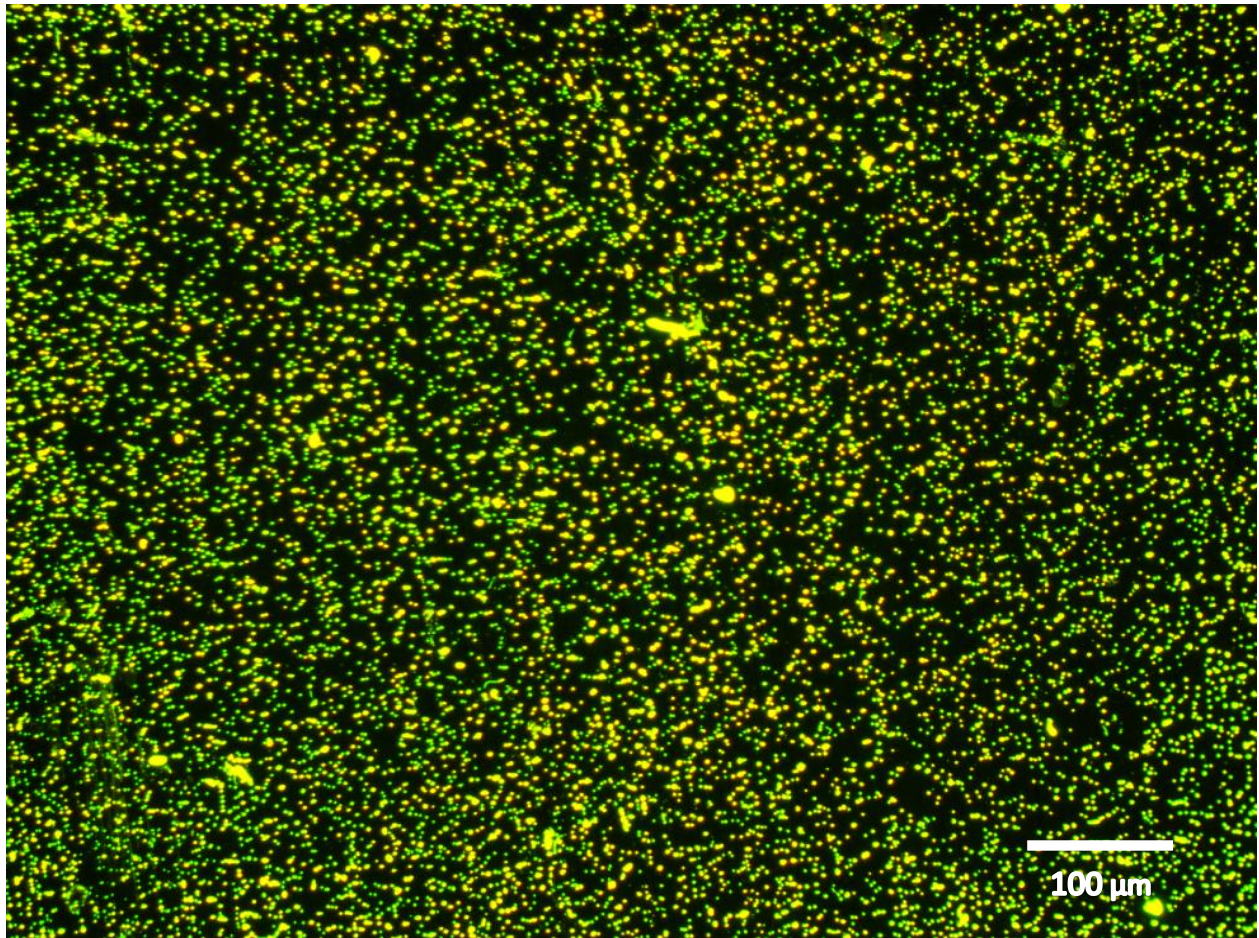


FIGURE 3.7. Fluorescent micrographs of *Listeria monocytogenes* 311 grown in TSB for 12 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) were not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

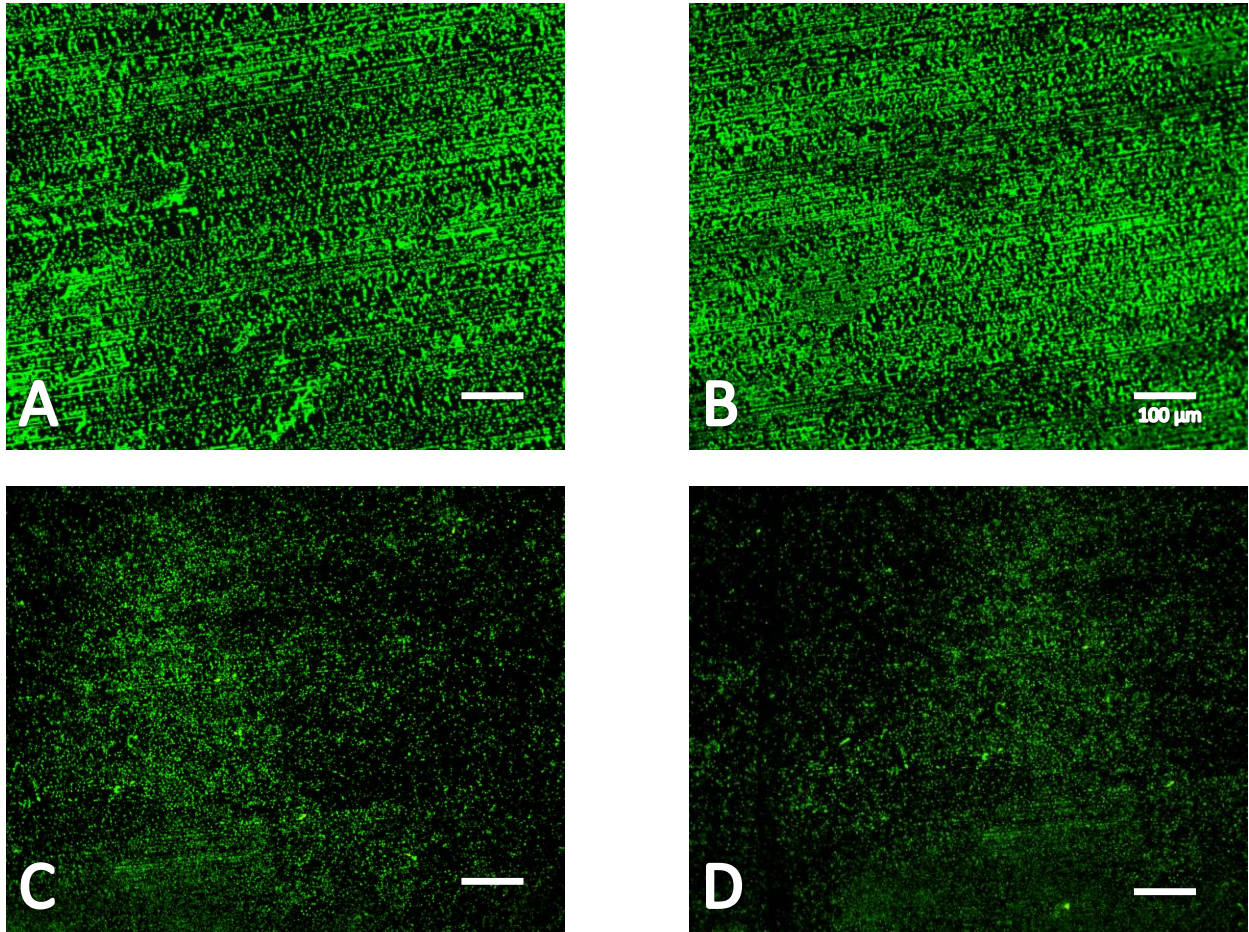


FIGURE 3.8. Fluorescent micrographs of *Listeria monocytogenes* 311 grown in TSB for 24 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

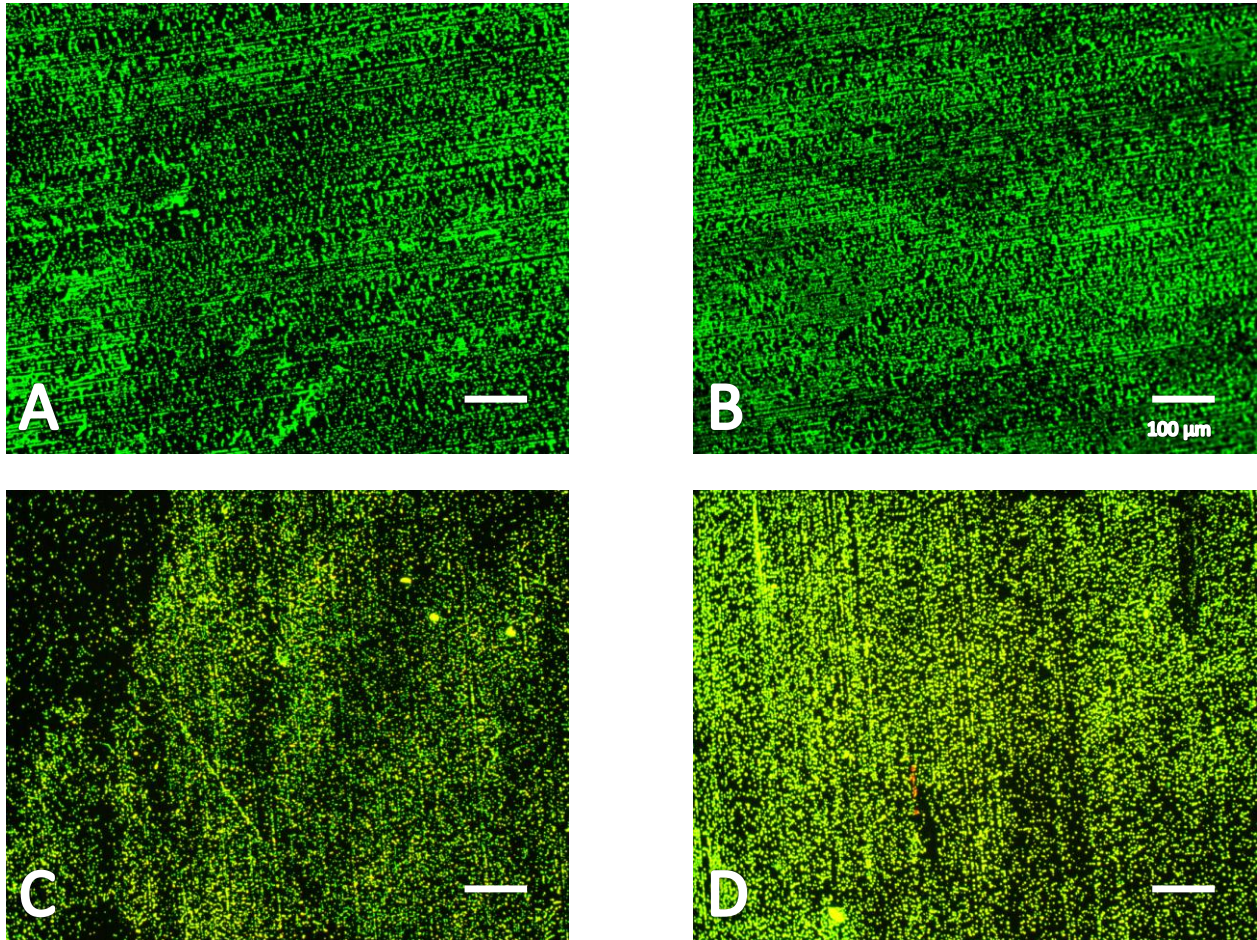


FIGURE 3.9. Fluorescent micrograph of *Listeria monocytogenes* 311, grown in TSB for 24 hr. Biofilms cultured on stainless steel, stained with Quant-IT PicoGreen dsDNA reagent, and viewed under epifluorescent light microscope.

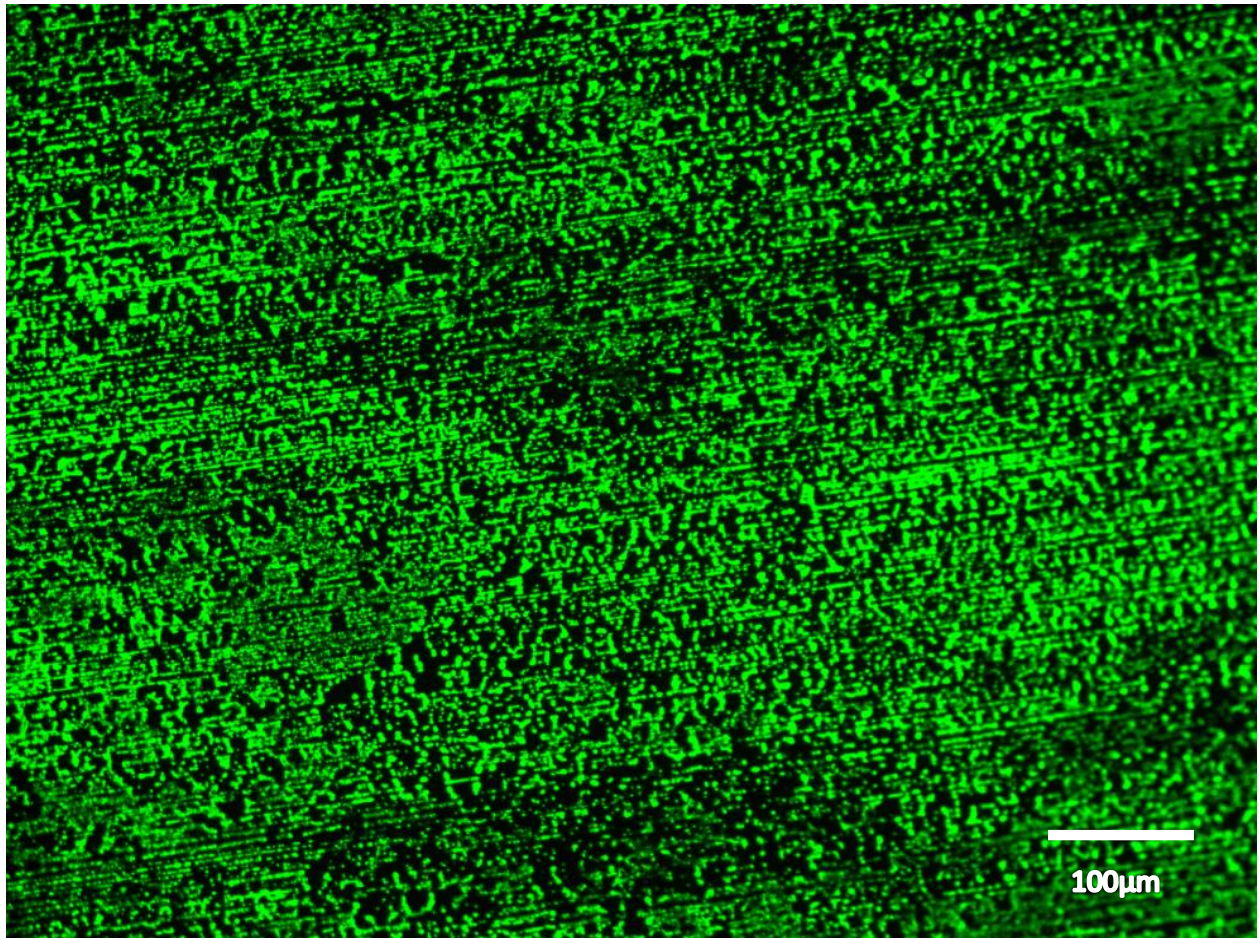


FIGURE 3.10. Fluorescent micrograph of *Listeria monocytogenes* 311, grown in TSB for 12 hr. Biofilm cultured on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Biofilm stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

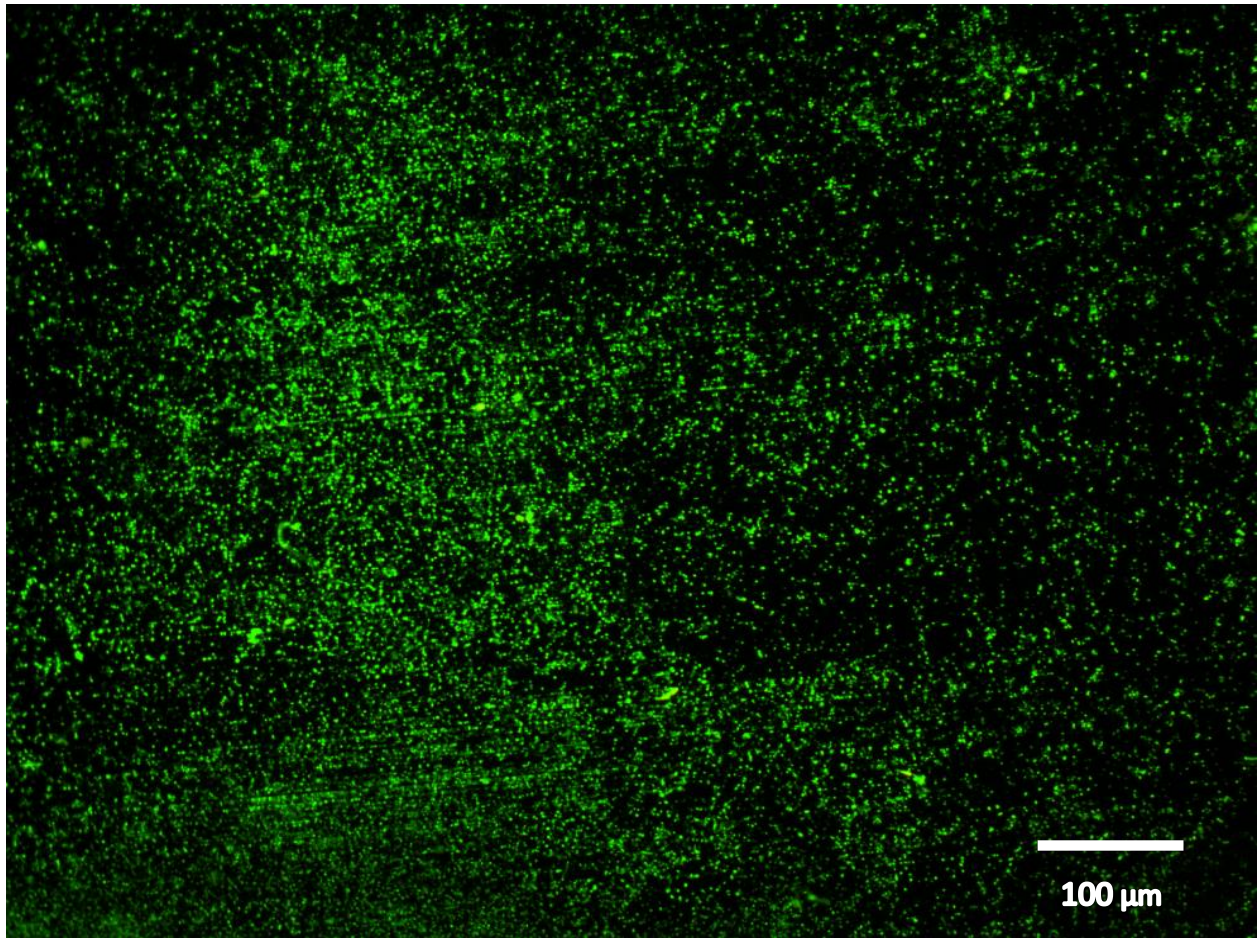


FIGURE 3.11. Fluorescent micrograph of *Listeria monocytogenes* 311, grown in TSB for 24 hr. Biofilm cultured on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Biofilm stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

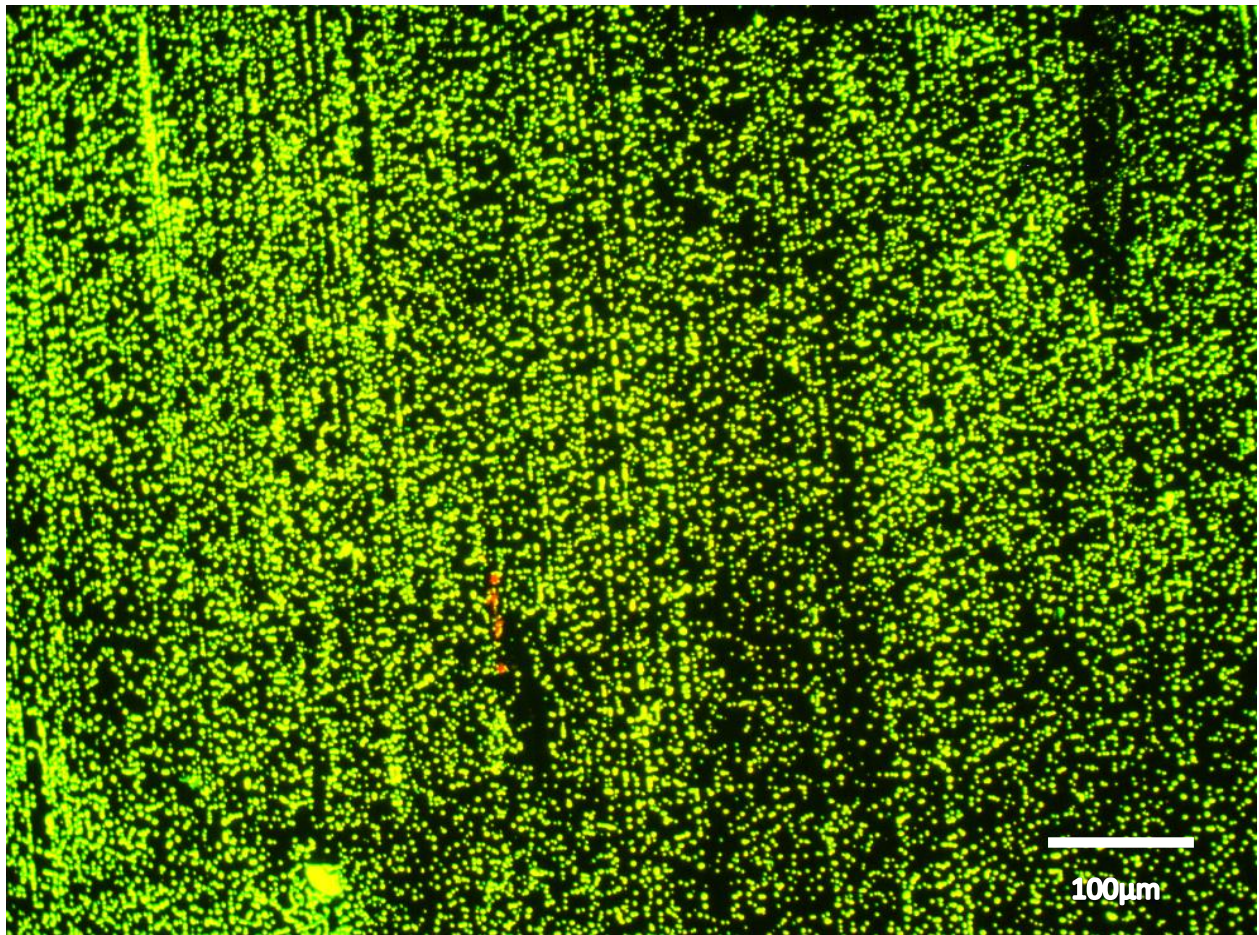


FIGURE 3.12. Fluorescent micrographs of *Listeria monocytogenes* N390 grown in TSB for 12 hr for 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

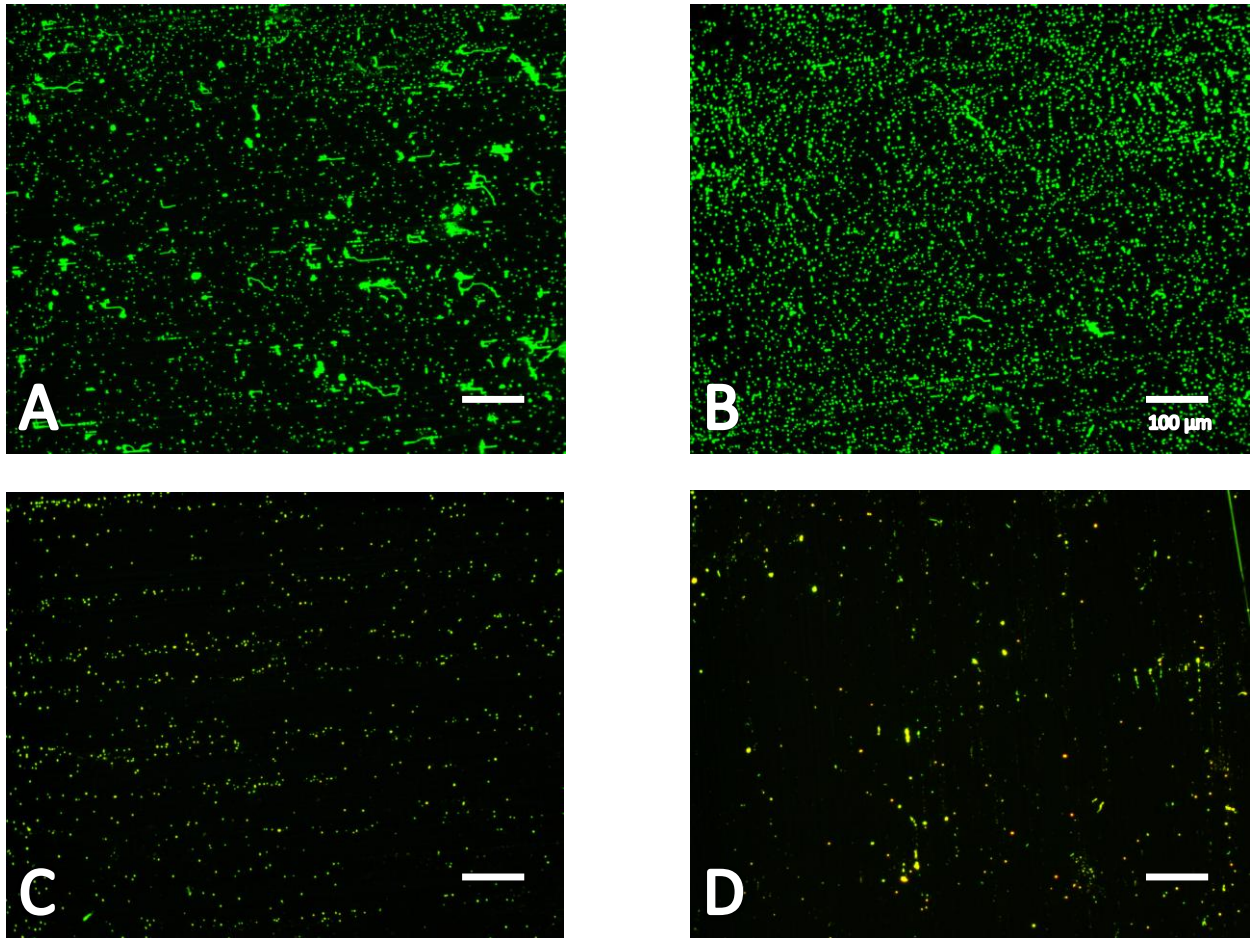


FIGURE 3.13. Fluorescent micrographs of *Listeria monocytogenes* N390 grown in TSB for 24 hr for 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

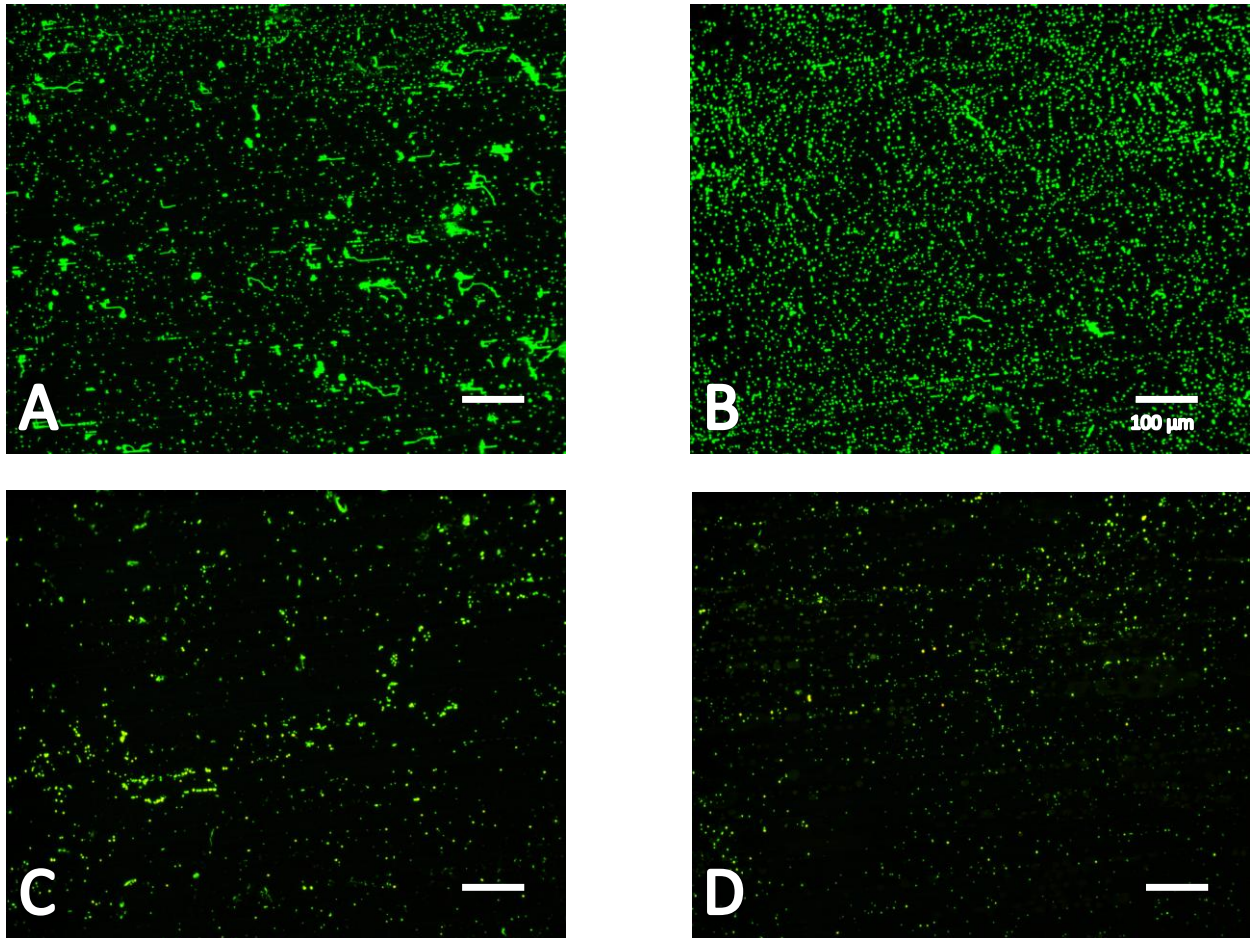


FIGURE 3.14. Fluorescent micrographs of *Listeria monocytogenes* 19115 grown in TSB for 12 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

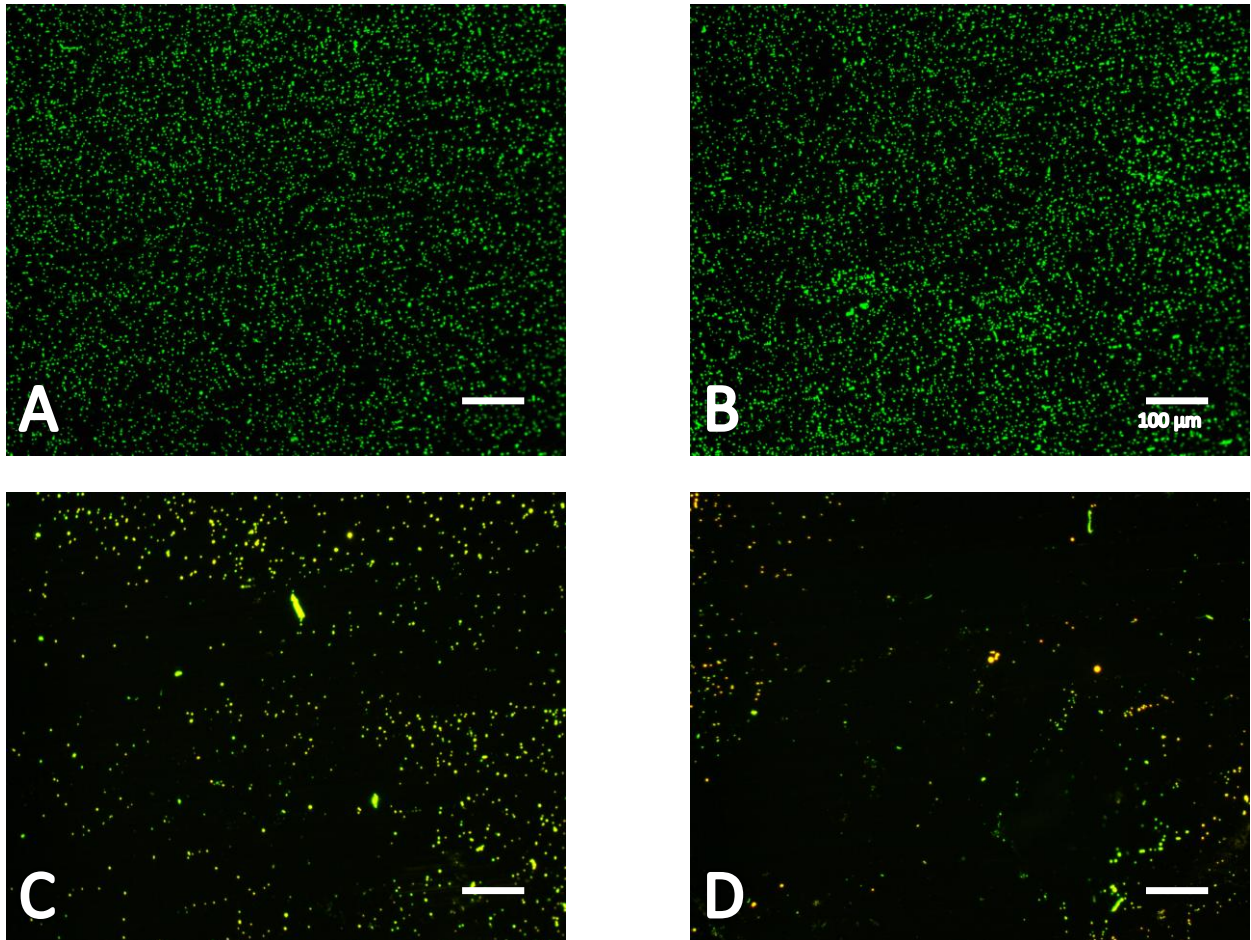


FIGURE 3.15. Fluorescent micrographs of *Listeria monocytogenes* 19115 grown in TSB for 24 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

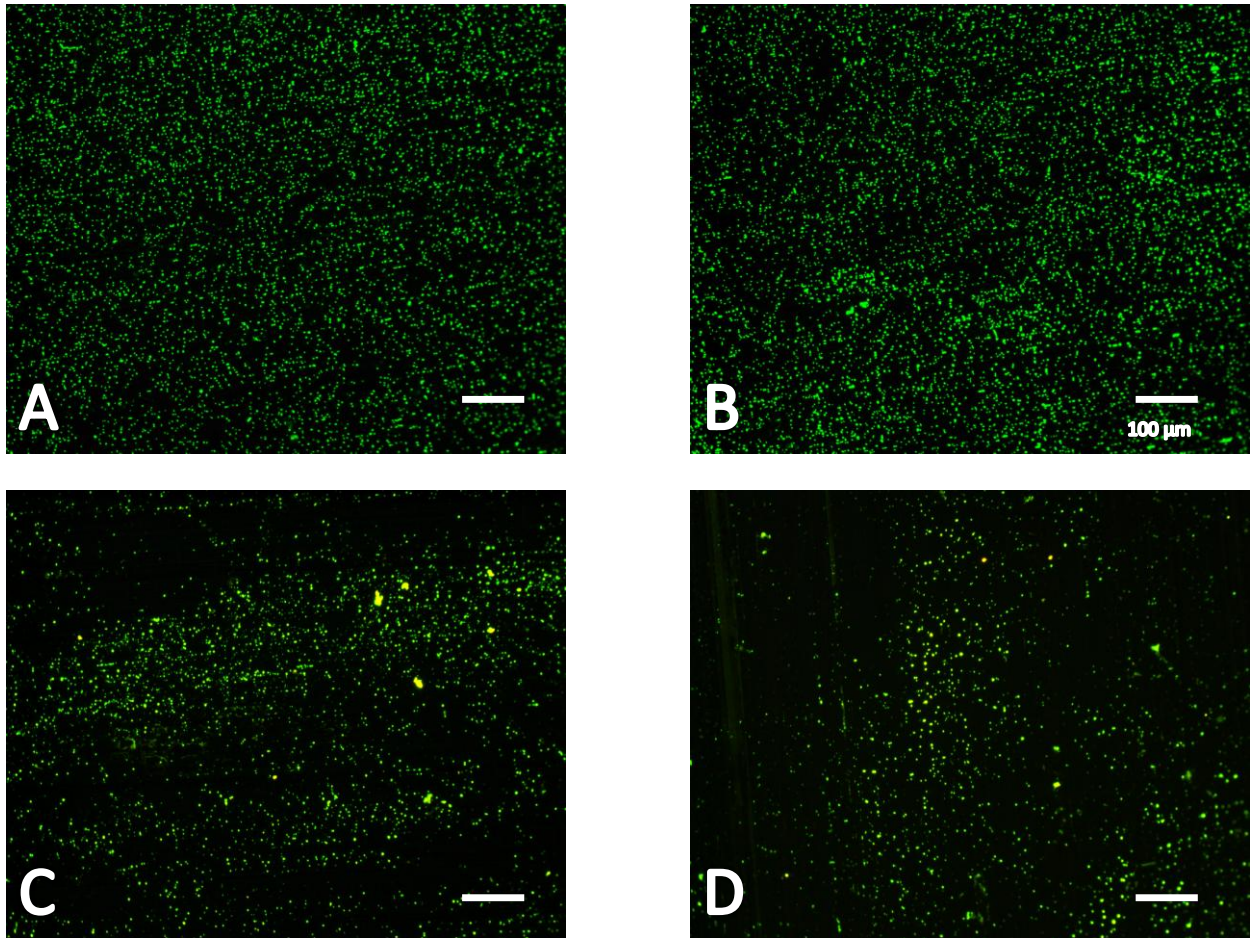


FIGURE 3.16. Fluorescent micrographs of *Listeria monocytogenes* D123 grown in TSB for 12 hr at 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

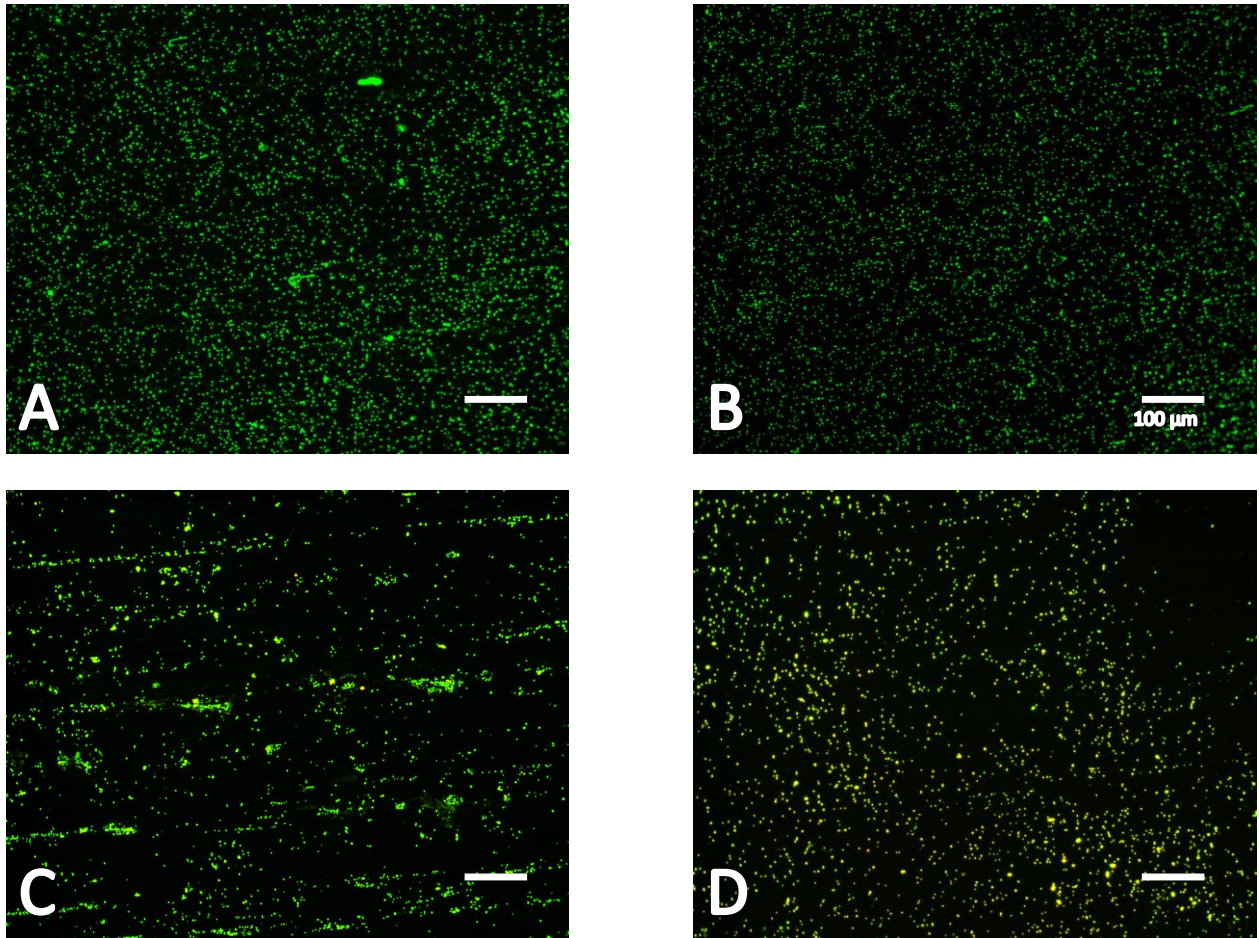


FIGURE 3.17. Fluorescent micrographs of *Listeria monocytogenes* D123 grown in TSB for 24 hr for 25°C. Biofilms cultured on stainless steel. (A) and (B) not treated with DNase I. (C) and (D) treated with DNase I for 24 hr at 25°C. All biofilms stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

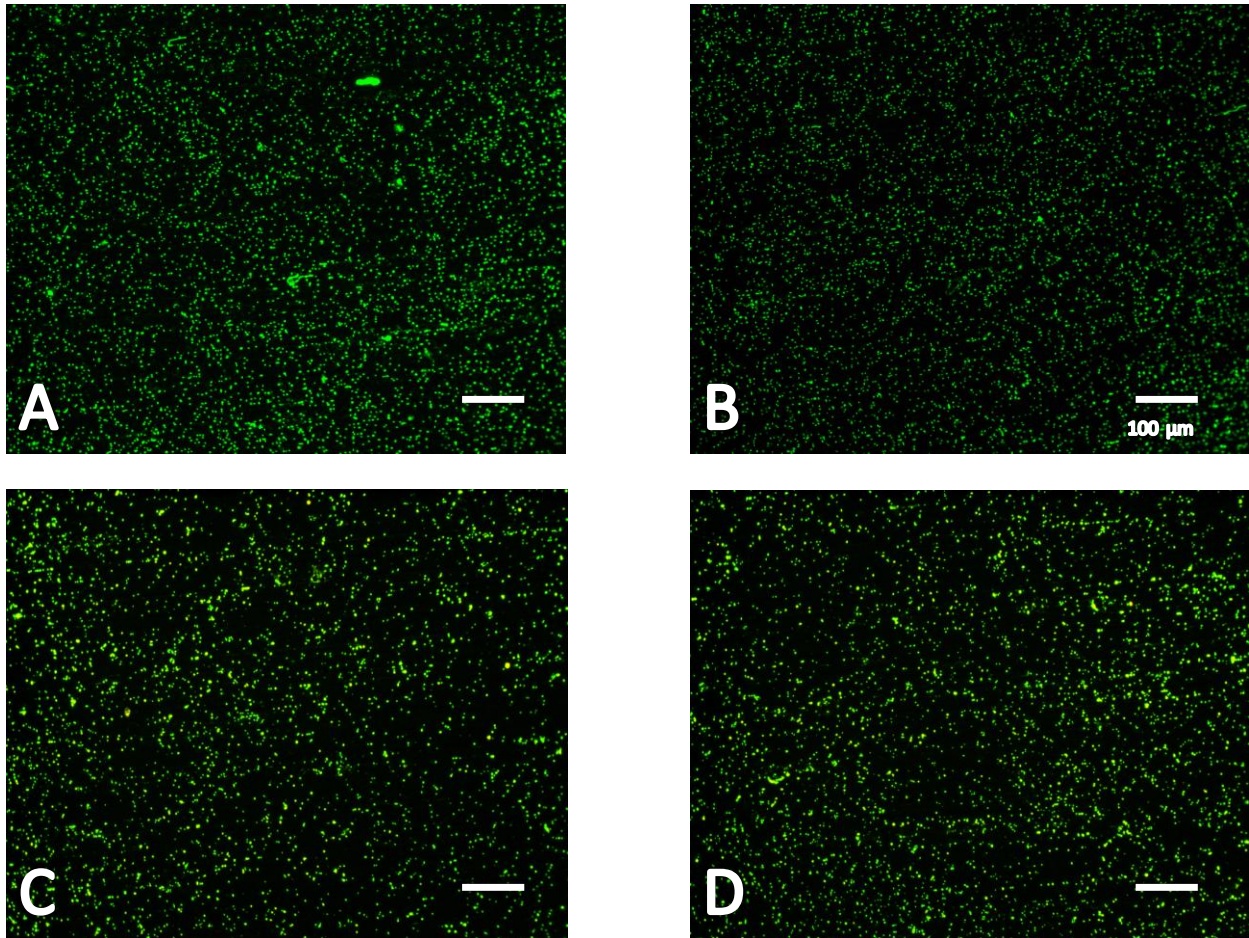


FIGURE 3.18. Fluorescent micrograph of *Listeria monocytogenes* D123, grown in TSB for 24 hr. Biofilm cultured on stainless steel. Stained with Quant-IT PicoGreen dsDNA reagent, and viewed under epifluorescent light microscope.

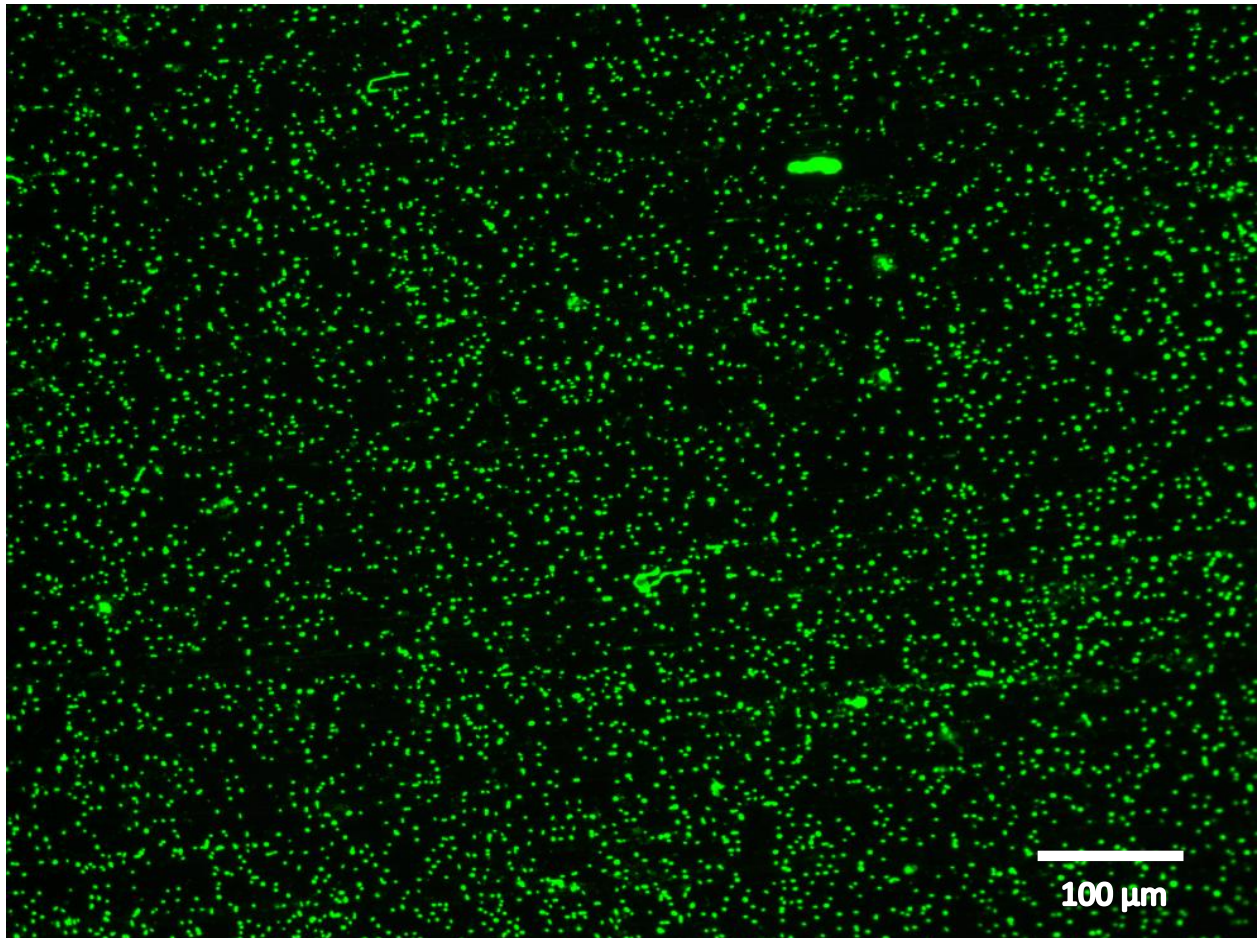


FIGURE 3.19. Fluorescent micrograph of *Listeria monocytogenes* D123, grown in TSB for 12 hr. Biofilm cultured on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Biofilm stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

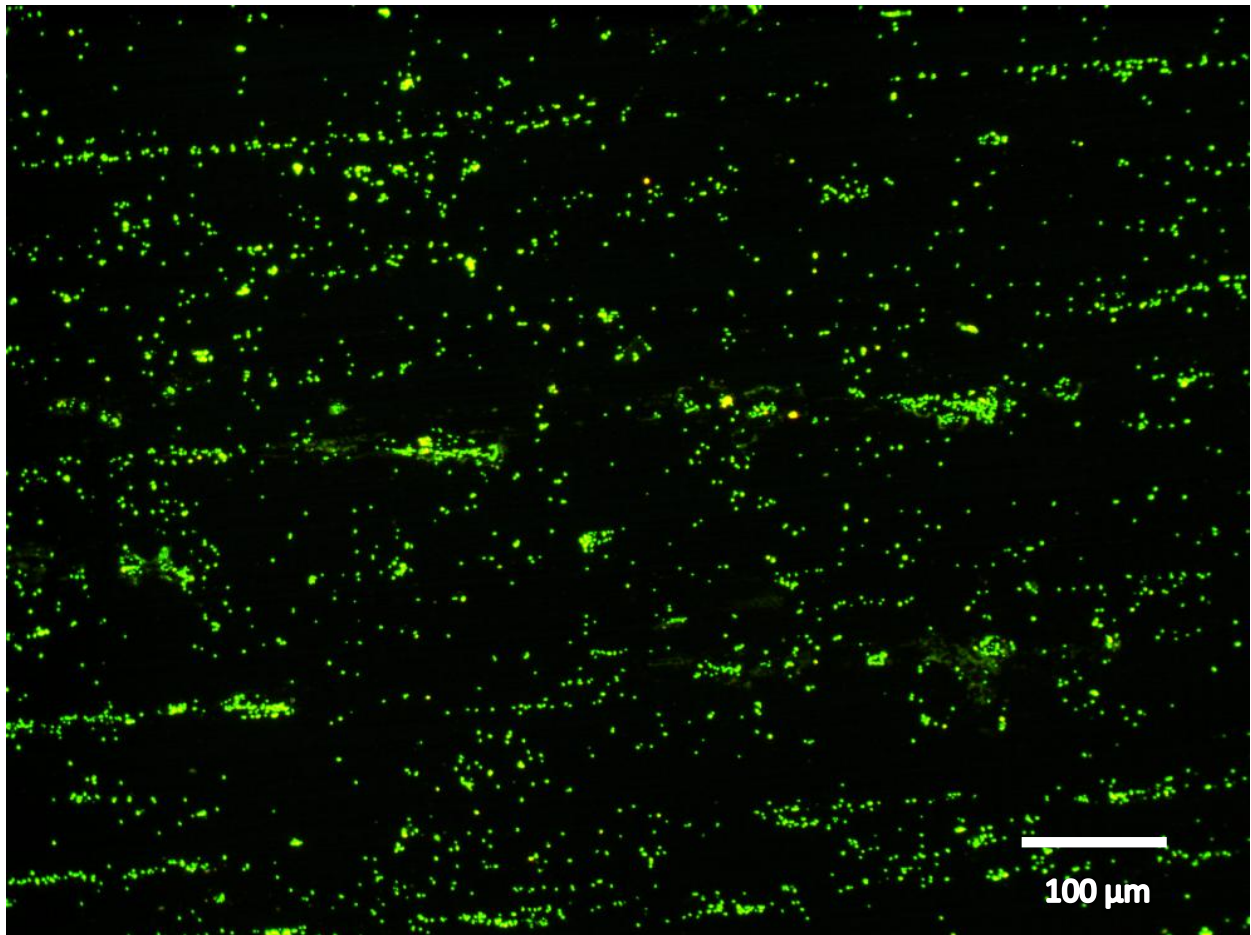


FIGURE 3.20. Fluorescent micrograph of *Listeria monocytogenes* D123, grown in TSB for 24 hr. Biofilm cultured on stainless steel. Coupon treated with DNase I for 24 hr at 25°C. Biofilm stained with Quant-IT PicoGreen dsDNA reagent, viewed under epifluorescent light microscope.

