Investigating Natural and Induced Biofilm Dispersion in Listeria monocytogenes

INVESTIGATING NATURAL AND INDUCED BIOFILM DISPERSION IN *LISTERIA MONOCYTOGENES*

A Thesis Presented

By

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Food Science

DEDICATION

To my parents, whose hard work and sacrifice put me in the position I find myself today.

ABSTRACT

INVESTIGATING NATURAL AND INDUCED BIOFILM DISPERSION IN *LISTERIA MONOCYTOGENES*

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Dispersion is a natural part of a biofilm life cycle in many bacterial species. Dispersion occurs when bacteria revert from a stationary, sessile state to a free-swimming, planktonic state and are freed from a biofilm. Bacterial biofilms consist of proteins, polysaccharides, and extracellular DNA that together make up the extracellular polymeric substances. Surrounded by this mucus-like substance, sessile cells can be extremely difficult to eradicate as compared to the planktonic form of *Listeria monocytogenes*. Biofilms are robust due to increased surface adherence, inhibition of diffusion of harmful compounds, and increased genetic diversity that exists within a biofilm. As a result, traditional biofilm removal methods are often inadequate; and a novel method for the eradication of *Listeria monocytogenes* biofilms is needed. Here it is shown that two known biofilm dispersal agents, nitric oxide and *cis*-2-Decenoic acid, do not induce dispersion in *Listeria monocytogenes* strain LM23. Nitric oxide and *cis*-2-Decenoic acid do not influence planktonic cell numbers or biofilm biomass. Ten carbohydrates were screened for their influence on biofilm biomass for use in investigation into natural biofilm dispersion in *Listeria monocytogenes* strain LM23. Carbohydrate source can significantly increase or decrease biofilm biomass as compared

to glucose. Natural biofilm dispersion in *Listeria monocytogenes* remains inconclusive, yet warrants further investigation. Changes in planktonic cells numbers, sessile cell numbers, and biofilm biomass were tracked under static growth conditions, and suggested a possible dispersion event. However, treatment of biofilms with spent media and observation using scanning electron microscopy did not clarify the results obtained. This research deems the nitric oxide donors, molsidomine (N- (ethoxycarbonyl)-3-(4-morpholinyl)-sydnone imine) and MAHMA NONOate (6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine), as well as *cis*-2-Decenoic acid as ineffective in inducing biofilm dispersion. It also brings about new research questions into natural biofilm dispersion in *Listeria monocytogenes*.

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CHAPTER 1

INTRODUCTION

In the United States, there is a zero-tolerance policy for *Listeria monocytogenes* (*L. monocytogenes*) in food products. *L. monocytogenes* is a dangerous foodborne pathogen capable of forming biofilms. The ability to form biofilms makes this organism of particular concern in food processing facilities. Once *L. monocytogenes* has established a biofilm in a food processing facility, it can be extremely difficult to eradicate. When in a biofilm, cells are far more resistant to chemical sanitizers than when alone or in their planktonic state. Also, the ability to adhere to a surface makes mechanical removal of biofilms difficult (Frank and Koffi 1990; Pan and others 2006). As a result, *L. monocytogenes* can persist in food production facilities for years and has the potential to continually contaminate foods (Fonnesbech Vogel and others 2001).

A need exists for novel methods for biofilm removal and eradication. The main object of this research is to determine if *L. monocytogenes* cells disperse from their biofilms either naturally, or when known biofilm dispersal compounds are used. Biofilm dispersion is characterized by the breakdown of the biofilm matrix itself and a transition of cells to their more vulnerable planktonic state (Monroe 2007). If biofilm dispersion can be harnessed in *L. monocytogenes*, it could offer a much more effective method for the destruction and removal of their biofilms.

CHAPTER 2

LITERATURE REVIEW

2.1 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive rod shaped bacteria that is ubiquitous in the environment, and is generally found in soil and plant materials (Weis and Seeliger 1975). It can also be associated with farm animals, such as cattle and sheep, making it a common organism in both animal and plant agriculture (Gray and Killinger 1966). It is a facultative anaerobe, meaning that it can grow in both aerobic and anaerobic conditions, if required. *L. monocytogenes* is unique in that it has the ability to grow at refrigeration temperatures. Growth at temperatures as low as -0.4°C have been recorded (Walker 1990). At 30°C, *L. monocytogenes* is capable of growing in the pH range of 4.5-7.0. However, at lower temperatures, this organism has a more narrow pH range at which it can grow (Parish and Higgins 1989). Growth at a water activity of 0.90 has been reported for *L. monocytogenes.* Yet, adverse environmental factors, such as temperature and solute concentrations can make *L. monocytogenes* more susceptible to lower water activities (De Daza and others 1991).

2.2 Listeriosis

L. monocytogenes is of particular importance in food due to its human virulence. The infection caused by *L. monocytogenes* is called listeriosis. Between the years 1998-2015, there have been over 60 foodborne outbreaks caused by *L. monocytogenes* in the United States alone. These outbreaks resulted in over 818 reported cases of listeriosis. Of

these reported cases of listeriosis, 70.7% resulted in hospitalization and 14.8% resulted in death of the infected individual (CDC 2016).

Thirteen different serovars of *L. monocytogenes* exist and all are capable of infecting humans. Despite this, servors 1/2a, 1/2b, and 4b are responsible for a majority of infections (Farber and Peterkin 1991). Symptoms of listeriosis can include: central nervous system infections, diarrhea, fever, bacteremia, and even death (Goulet and Marchetti 1996). Newborns, the elderly, pregnant women, and immunocompromised individuals are at a greater risk for contracting listeriosis. For a pregnant woman, an *L. monocytogenes* infection can be devastating, since it can lead to spontaneous abortions, stillbirth, and premature birth (CDC 2017). It is also possible for healthy adults to contract this infection as well (Rocourt and World Health Organization 1991). Symptoms for healthy individuals are generally much less severe than high-risk groups, however (CDC 2017).

2.3 Biofilm Attachment in *Listeria monocytogenes*

Attachment is the first step in the development of a bacterial biofilm. Cells must migrate to a surface to which they can attach. In *L. monocytogenes*, flagellar motility plays a major part in the initial stages of surface attachment. More specifically, flagella allow the cells to move to a surface rather than actually attach to the surface itself (Lemon and others 2007). Once at the interface of the surface, the cells can reversibly attach. If external forces do not remove the cell, it can irreversibly attach to the surface (Monroe 2007). For *L. monocytogenes*, adherence ability to the surface itself can be characterized by the amount of extracellular fibrils produced. A study by Kalmakoff and

others found that *L. monocytogenes* strains differed in their ability to adhere to a stainless steel surface. It was found that the strains that had a lesser ability to adhere to the surface either did not have extracellular fibrils, or expressed them to a lesser degree (Kalmokoff and others 2001).

It has also been shown that extracellular DNA (eDNA) in conjunction with *N*-acetylglucosamine plays a role in initial cell attachment and the early stages of biofilm formation. In order for DNA to be effective in aiding cell attachment, it must be high in molecular weight. It was found that when DNA fragments less than 500 base pairs long was added to an eDNA free culture, adhesion did not occur. Unlike with the extracellular fibrils, strain did not impact attachment facilitated by eDNA (Harmsen and others 2010).

The type of surface can also affect the ability for bacterial cells to attach. As a general rule, attachment is greater when the surface is rough as opposed to smooth (Characklis 1973). Surface hydrophobicity also plays an influence on cell attachment. *L. monocytogenes* has the capability of both adhering to and forming biofilms on hydrophobic and hydrophilic surfaces. After seven days of growth at 21°C, *L. monocytogenes* formed significantly more biofilm on stainless steel and floor sealant than it did on nylon. However, attachment and growth on these surfaces was impacted by the growth medium used (Blackman and Frank 1996). This is because available nutrients influence the physicochemical properties of the surface. Nutrients collecting on a surface form what is called a conditioning layer. Conditioning layers form a film on the surface and can facilitate cell attachment. When no conditioning layer was present on stainless steel, it was found that growth medium had minimal impact on *L. monocytogenes* attachment (Hood and Zottola 1997).

2.4 Biofilm Formation and Development

After a cell has irreversibly attached to a surface, a biofilm will begin to develop and mature. This occurs via recruitment of free-swimming bacteria to the biofilm as well as cellular division of the original colonizers. Extracellular polymeric substances (EPS) will also begin to be produced (Lappin- Scott and Costerton 1989). It has been observed in *Pseudomonas aeruginosa* (*P. aeruginosa*) that attachment to a surface can trigger EPS production (Davies and Geesey 1995).

2.4.1 Extracellular Polymeric Substances in Listeria monocytogenes biofilms

Listeria monocytogenes biofilms, as with other bacterial biofilms, are characterized by the production of extracellular polymeric substances. EPS makes up the majority of the biomass in a bacterial biofilm (Flemming and others 2000). Extracellular polymeric substances produced by *L. monocytogenes* consist of proteins, polysaccharides, and extracellular DNA (eDNA). A study by Combrouse and others found that proteins make up a majority of the mass in the EPS of *L. monocytogenes*. Polysaccharides and eDNA were found to be present in similar amounts in the EPS produced by *L. monocytogenes* (Combrouse and others 2013).

EPS production is highly dependent upon the strain of *L. monocytogenes*, as some strains produce significantly more than others. EPS production ability can be grouped based on phylogenetic divisions. Those in Phylogenetic Division II produce significantly higher amounts of extracellular polymeric substances than do those in Division I (Borucki and others 2003). EPS production is also significantly impacted by

the medium the biofilm was grown in, as well as incubation temperature (Combrouse and others 2013).

2.4.2 Biofilm Structure in Listeria monocytogenes

As *L. monocytogenes* biofilms develop, they are capable of creating three-dimensional structures. Scanning electron microscopy images depict cells stacked on top of one another. The cells organize themselves in a net-like structure, with channels separating cell stacks (Marsh and others 2003). Another study observed that *L. monocytogenes* biofilms form different structures under static and flowing conditions. Under static conditions, biofilms formed uniformly. Stacks of cells developed, yet there was no apparent organization to the structure. Under flowing conditions, an organized biofilm developed. Similar to what was observed by Marsh and others (2003), a three-dimensional web-like structure was generated when fluid flow was present (Rieu and others 2008).

2.5 Bacterial Biofilm Dispersal

Dispersion is the final stage in the life cycle of a bacterial biofilm. In this stage, cells break out from the biofilm and change from a stationary, sessile state to a free-swimming, planktonic state (Monroe 2007). The planktonic cells are then free to move to a new location, attach to a surface, and form a biofilm of their own (Kaplan 2010).

Two forms of biofilm dispersion can occur. The first form of dispersion is passive dispersion. External forces that disrupt the biofilm and cause it to break apart result in passive dispersion. The other form of biofilm dispersal is active dispersal. The cells

themselves mediate this type of dispersal (Choi YC 2003; Kaplan 2010). Two chemical compounds known to initiate active dispersion are *cis*-2-decenoic acid and nitric oxide. It is important to note however, that these two compounds themselves do not free the cell from the biofilm matrix (Barraud and others 2009; Davies and Marques 2009). In order for a cell to actually be released from the matrix, it must produce enzymes, such as, proteases, glycosidases, and deoxyribonucleases that will degrade the components of the EPS (Allison 1998; Gjermansen and others 2005; Mann and others 2009).

2.5.1 cis-2-Decenoic Acid

Cis-2-Decenoic acid (CDA) is a monounsaturated fatty acid messenger produced by *Pseudomonas aeruginosa.* CDA allows for cell-to-cell communication and is classified as a diffusible signaling factor (Davies and Marques 2009). When *P. aeruginosa* biofilms were exposed to a 10μM concentration of CDA for 1 hour, significantly more cells were released from the biofilm as compared to the control. CDA exposure to *P. aeruginosa* biofilms at a native concentration of 2.5nM using spent medium also resulted in biofilm dispersion. *Cis*-2-Decenoic acid does not only induce biofilm dispersion in *P.aeruginosa*. It was also found to be effective against other gram-negative and gram-positive bacteria (Davies and Marques 2009).

2.5.2 Nitric Oxide

The gas nitric oxide has also been shown to induce bacterial biofilm dispersion. Gram-negative and gram-positive single species and multispecies were exposed to several molecules that donate nitric oxide at concentrations ranging from 10nM to 10μM. Biofilms were exposed to the nitric oxide donor molecules for 24 hours. It was found that

in all biofilms tested, nitric oxide significantly reduced the surface coverage of the biofilm (Barraud and others 2009).

CHAPTER 3

OBJECTIVES

The objectives of this thesis are:

1. To determine if nitric oxide induces *Listeria monocytogenes* biofilm dispersal.
2. To determine if *cis*-2-Decenoic acid induces *Listeria monocytogenes* biofilm dispersal.
3. To determine if *Listeria monocytogenes* undergoes natural active biofilm dispersal.

CHAPTER 4

MATERIALS AND METHODS

4.1 Media Preparation

4.1.1 Basic Media

Tryptic soy broth (TSB) (BD Bacto, Sparks, MD) was prepared according to the manufacturer's instructions. TSBYE was prepared by adding 6g/L of yeast extract (BD Bacto, Sparks, MD) to tryptic soy broth. Tryptic soy agar (TSA) (BD Bacto, Sparks, MD) was prepared according to manufacturer's instructions. Physiological saline (PS) was prepared by adding 8.5mg/L NaCl (Fischer Scientific, Waltham, MA) in distilled deionized water.

4.1.2 Modified Welsheimer's Broth

The minimal media, Modified Welsheimer's Broth (MWB) was made in 11 different variations. Original MWB composition is shown in Table 1. The other 10 different variations differed only in the carbohydrate source used. Carbohydrates as well as amounts used in each variation are shown in Table 2.

Table 1: Modified Welsheimer's Broth chemical composition

Compound	Concentration	Manufacturer Information
KH_2PO_4	48.20 mM	Fischer Chemical, Fair Lawn, NJ
Na_2HPO_4	115.50 mM	Sigma-Aldrich, St. Louis, MO
$MgSO_4$	1.70 mM	Fischer Chemical, Fair Lawn, NJ
Ferric Citrate	360 µM	Sigma-Aldrich, St. Louis, MO
Glucose	55.50 mM	Aldrich Chemistry, St. Louis, MO
Thiamine	2.96 µM	Fischer Biotech, Fair Lawn, NJ
Riboflavin	1.33 µM	Fischer Biotech, Fair Lawn, NJ
Biotin	2.05 µM	Fischer Scientific, Fair Lawn, NJ
Lipoic acid	24.00 pM	Sigma-Aldrich, St. Louis, MO
Leucine	762 µM	Eastman Kodak Co., Rochester, NY
Isoleucine	762 µM	P-L Biochemicals Inc., Milwaukee, WI
Valine	854 µM	Fischer Biotech, Fair Lawn, NJ
Arginine	574 µM	Fischer Bioreagents, Fair Lawn, NJ
Cysteine	825 µM	Fischer Bioreagents, Fair Lawn, NJ
Methionine	670 µM	Sigma Chemicals Co., St. Louis, MO
Glutamine	411 mM	Fischer Bioreagents, Fair Lawn, NJ

Table 2: Modified Welsheimer's Broth carbohydrate variations

Carbohydrate	Molecular Weight (g/mol)	g/L	Concentration	Manufacturer Information
Glucose	180.16	10	55.50 mM	Aldrich Chemistry, St. Louis, MO
Maltose	342.3	10	29.21 mM	Sigma Chemical Co., St. Louis, MO
Lactose	342.3	10	29.21 mM	Sigma Chemical Co., St. Louis, MO
Fructose	180.16	10	55.51 mM	Sigma Cell Culture Reagents, St. Louis, MO
Cellobiose	342.3	10	29.21 mM	Sigma Chemical Co., St. Louis, MO
Arabinose	150.13	10	66.6 mM	Fischer Scientific, Fair Lawn, NJ
Trehalose	342.3	10	29.21 mM	Acros Organics, Geel, Belgium
Melibiose	342.3	10	29.21 mM	Sigma Chemical Co., St. Louis, MO
Raffinose	504.44	10	19.82mM	Sigma Chemical Co., St. Louis, MO
Sorbose	180.16	10	55.51 mM	Sigma Chemical Co., St. Louis, MO

4.1.3 Phosphate Buffered Saline

A 0.01M solution of phosphate buffered saline (PBS) was used for all indicated experiments. To prepare PBS, the following chemicals were added in specified concentrations: 8 g/L NaCl, 0.2 g/L KCl (Fischer Scientific, Waltham, MA), 1.42 g/L Na_2HPO_4 (Sigma-Aldrich, St. Louis, MO), and 0.24 g/L KH_2PO_4 (EMD Millipore,

Darmstadt, Germany). Final pH was adjusted to 7.4 with HCl. PBS was autoclaved and stored at room temperature (Association of Official Analytical Chemists 1998).

4.2 Preparation of Frozen Stocks and Overnight cultures

Frozen stock cultures of *Listeria monocytogenes* (LM23 Lineage III) (Djordjevic and others 2002). Working cultures were prepared by diluting 100µL of frozen stock into 9.9mL of TSBYE. The inoculated TSBYE was incubated overnight for 18 hours at 32°C. Following overnight incubation, one loop (~10 µL) was taken and streak plated onto TSA. TSA streak plates were wrapped in Parafilm (Pechiney Plastic Packaging, Menasha, WI) and stored at 4°C for up to one month. Before each experiment was conducted, one isolated colony was removed and inoculated in 10mL of TSBYE and incubated at 32°C for 18 hours. This will be referred to as an overnight culture.

4.3 Biofilm Growth

To begin biofilm growth preparation, 100µL of an overnight culture was taken and inoculated in 10mL of glucose MWB. The inoculated tube was then vortexed using a Vortex Genie 2 vortexer (Fischer Scientific, Waltham, MA) for 3 seconds. 100µL of the inoculated MWB was pipetted into the wells of a sterile vinyl Serocluster 96 well "U" bottom plate (microtiter plate) (Corning Incorporated, Corning, NY). Microtiter plates and their lids were sterilized by rinsing in 70% ethanol and were allowed to air dry. Plated were exposed to UV light using a Philips TUV T8 ultraviolet light (Philips Lighting Co., Somerset, NJ) in a NuAire Class II Type A/B3 Biological Safety Cabinet (NuAire, Plymouth, MN) for 15 minutes during the air-drying process (Djordjevic and

others 2002). Wells were filled every other row in the microtiter plate. A six well row of sterile MWB was also filled for use as a blank for total biomass quantification. Wells around the perimeter of the plate were filled with 150µL of sterile distilled water to prevent evaporation during incubation. Once the lids were placed on the plates, the plates were wrapped in Parafilm, leaving 1.5 inches on either of the plate exposed for gas exchange. Plates were wrapped in Parafilm to help prevent contamination and moisture loss during incubation. The inoculated plates were then transferred to a metal box containing a 500mL beaker filled with 200mL water and incubated at 32°C under static conditions for 48 hours.

4.4 Biofilm Biomass Quantification

Total biomass was quantified using the microtiter plate biofilm production assay (Djordjevic and others 2002). To begin the assay, wells in the microtiter plate were rinsed five times with times with 150µL sterile water to remove all planktonic cells and unbound material. Following rinsing, the plates were allowed to air dry completely. Next, wells were stained for 45 minutes with 150µL of 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO). After staining, the crystal violet solution was removed and wells were rinsed five times with 150µL of sterile water. The plates were again allowed to air dry completely. To remove the crystal violet stain, 200µL of 95% ethanol was added to the wells. After one hour, 100µL of the ethanol and crystal violet solution was transferred to a new, sterile microtiter plate. Absorbance was measured at 570nm using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT) and its companion software, Gen5 version 2.05 (BioTek Instruments Inc., Winooski, VT). All of

the steps, with the exception of the absorbance reading were done in a biological safety cabinet.

4.5 Drop Plating

Bacterial cell enumeration was done by drop plating on TSA plates. Five 10µL drops of a single sample dilution were evenly spaced on one quadrant of the TSA plate. Four dilutions were performed on each plate. Inoculated plates were allowed to dry and were then inverted and incubated at 32°C for 24 hours. Bacterial colonies were counted manually following incubation.

4.6 Biofilm Exposure to Nitric Oxide Donors

4.6.1 Biofilm Growth

Biofilms were grown as specified in section 4.3 with the following exception- microtiter plate wells were filled with 150µL of inoculated MWB.

4.6.2 Nitric Oxide Donor Stock Solutions

The nitric oxide (NO) donors used were: molsidomine (N- (ethoxycarbonyl)-3-(4-morpholinyl)-sydnone imine) (Sigma-Aldrich, St. Louis, MO), MAHMA NONOate (6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine) (Sigma-Aldrich, St. Louis, MO), and diethylamine NONOate diethylammonium salt (Sigma-Aldrich, St. Louis, MO). NO donors were stored at -80°C. Stock solutions of the NO donors at a concentration of 10 mM were prepared in PBS and were stored at -80°C as well (Marvasi and others 2014). NO donors used in all experiments were taken from the stock solutions.

4.6.3 Biofilm Treatment of Nitric Oxide Donors

Prior to treatment, spent media in the wells of the microtiter plate was pipetted out and discarded. The wells were then rinsed five separate times with 150μL of PBS to remove any planktonic cells. Immediately before use, NO donor stock solutions were diluted in ice cold PBS to a concentration of 10μM. 200μL of the diluted NO donor solution was added to each well (Marvasi and others 2014). PBS without an added NO donor was used in control wells. The plates were then sealed with Parafilm, leaving 1.5 inches of space uncovered on either side of the plate. Plates were then stored in a metal box containing a 500mL beaker filled with 200mL water at room temperature for 24 hours.

4.6.4 Biofilm Biomass Quantification

Biofilm biomass quantification performed as specified in section 4.4.

4.6.5 Planktonic cell Enumeration

Following 24 hours of treatment with the NO donors, 200μL of the NO donor solution was pipetted out of the each well and put into separate 2.0 mL graduated, natural microcentrifuge tubes (Fischer Scientific, Waltham, MA) containing fresh PBS. The microcentrifuge tubes were then vortexed for 3 seconds and serially diluted. Drop plating for planktonic cell enumeration was performed as specified in section 4.5.

4.7 Biofilm Exposure to *cis*-2-Decenoic Acid

4.7.1 Biofilm Growth

Biofilms were grown as specified in section 4.3.

4.7.2 Biofilm Treatment of cis-2-Decenoic Acid

Cis-2-Decenoic acid (CDA) (Sigma-Aldrich, St. Louis, MO) was dissolved in 10mL of 100% ethanol (Pharmaco Products, Inc., Shelbyville, KY) to create a 6.2mM stock solution. The stock solution was dispensed in to 1mL aliquots in 2mL microcentrifuge tubes and stored at -20°C for up to one week. Before exposure to biofilms, the stock solution was serially diluted in MWB containing 10% ethanol. CDA was diluted to a concentration of 620nM and 310nM in the MWB with 10% ethanol solution. MWB containing only 10% ethanol was used as a control. Before adding the control or CDA solution, wells were rinsed five times with PS. 0.1mL of the control or two treatment solutions was added to respective wells. The microtiter plates were then covered using Parafilm, leaving 1.5 inches exposed on either side of the plate, and placed in a metal box containing a 500mL beaker filled with 200mL of water. The metal box was then incubated at 32°C for 1 hour.

4.7.3 Biofilm Biomass Quantification

Biofilm biomass quantification performed as specified in section 4.4.

4.7.4 Planktonic Cell Enumeration

Following the 1 hour CDA treatment, 100μL of the control and CDA solutions were pipetted out of each well and put in separate 2mL microcentrifuge tubes containing PS. The microcentrifuge tubes were then vortexed for 3 seconds on high and serially diluted. Drop plating for planktonic cell enumeration was performed as specified in section 4.5.

4.8 Effect of Carbohydrate Source on Biofilm Biomass

4.8.1 Biofilm Growth

Biofilms were grown as specified in section 4.3.

4.8.2 Biofilm Biomass Quantification

Biofilm biomass quantification performed as specified in section 4.4.

4.9 Monitoring Biofilm Biomass, Planktonic, and Sessile cells over Time

4.9.1 Biofilm Growth

Biofilms were grown as specified in section 4.3 with a few minor modifications. Microtiter plates were incubated at 32°C under static conditions for 2, 4, 6, or 8 days.

4.9.2 Biofilm Biomass Quantification

Biofilm biomass quantification performed as specified in section 4.4.

4.9.3 Sessile cell Enumeration

Wells were first rinsed five times with 100µL of PS to remove planktonic cells. After rinsing, the wells were refilled with 100µL of PS. The wells were then swabbed using sterile small cotton tipped applicators (Fischer Scientific, Waltham, MA). The cotton applicator was inserted into the well and allowed to soak up the saline solution, and then it was rotated around the well in a clockwise motion five times to remove the biofilms. The handle of the cotton applicator was removed and the saline soaked tip was placed in a 2mL microcentrifuge tube containing 900µL of PS and 250µL of 700-1,180µm washed, sterile glass beads (Sigma-Aldrich, St. Louis, MO). Microcentrifuge tubes containing the applicator tips were vortexed for a total of 15 seconds in five 3-second pulses. Sessile cell enumeration was done by drop plating as specified in section 4.5.

4.9.4 Planktonic cell Enumeration

To begin planktonic cell enumeration, media in the wells was completely removed and transferred to a 2mL microcentrifuge tube containing 900µL of PS. The microcentrifuge tubes were then vortexed for 3 seconds and serially diluted. Planktonic cell enumeration was performed as specified in section 4.5.

4.10 Effect of Spent Media on Developed Biofilms

4.10.1 Spent Media Collection and Preparation

Spent media was generated in the same conditions biofilms were grown at in section 4.3 with a few modifications. Microtiter plates were incubated at 32°C under static conditions for either 144 or 192 hours (6 or 8 days).

After incubating for 8 days, the spent media from each well was transferred to a 2mL microcentrifuge tube. The microcentrifugetube was placed in an accuSpin Micro microcentrifuge (Fisher Scientific, Waltham, MA) and spun at 10,000 rpm for 3 minutes. The supernatant was removed and filtered through a 25mm 0.22μm syringe filter (Fisher Scientific, Waltham, MA) into a 2mL microcentrifuge tube. Spent media was stored at -20°C for up to one month.

4.10.2 Concentrating Spent Media

After incubating for 6 days, cellobiose MWB spent media from each well was transferred to a 2mL micro centrifuge tube. The tube was placed in a microcentrifuge and spun at 10,000 rpm for 3 minutes. The supernatant was removed and filtered through a 0.22μm syringe filter, using a syringe, into a 2mL microcentrifuge tube. The lids of the microcentrifuge tubes were opened and the tubed were place in a SpeedVac Concentrator Model SVC-100H (Savant Instruments, Inc., Farmingdale, NY) for 3.5 hours. Fresh cellobiose MWB was also placed in the concentrator during the same run as the spent media for use as a control. The initial volume in each tube was 1.8mL. The final volume after being placed in the concentrator was 0.75 mL, indicating the spent media and fresh

media had been concentrated 2.4 times. Following concentration, the concentrated spent media (CSM) and concentrated fresh media (CFM) were filtered again through 0.22μm syringe filter into another 2mL microcentrifuge tube. Both concentrated medias were stored at -20°C for up to one week.

4.10.3 Biofilm Growth

Biofilms were grown as specified in section 4.3

4.10.4 Treatment of Biofilms with Spent Media

Wells containing biofilms were rinsed 5 times with PS to remove planktonic cells. Biofilms were exposed to spent media collected at 8 days, using fresh media as a control. Biofilms were also treated with CSM, using CFM as a control. Treatment times were 2 and 24 hours at room temperature. During treatment with spent media, the microtiter plates were covered using Parafilm, leaving 1.5 inches exposed on either side of the plate. The plates were stored at room temperature in a metal box containing a 500mL beaker filled with 200mL of water.

4.10.5 Biofilm Biomass Quantification

Biofilm biomass quantification was performed as specified in section 4.4.

4.10.6 Planktonic cell Enumeration

Media in the wells was completely removed and transferred to a 2mL microcentrifuge tube containing 900μL of PS. The microcentrifuge tubes were then

vortexed for 3 seconds and serially diluted. Planktonic cell enumeration was done by drop plating, as specified in section 4.5.

4.10.7 Sessile cell Enumeration

Following the removal of the media, wells were first rinsed five times with 100µL of PS to remove any remaining planktonic cells. After rinsing, the wells were refilled with 100µL of PS. The wells were then swabbed using a sterile cotton tipped applicator. The cotton applicator was inserted into the well and allowed to soak up the saline solution, and then it was rotated around the well in a clockwise motion five times to remove the biofilms. The handle of the cotton applicator was removed and the saline soaked tip was placed in a 2mL microcentrifuge tube containing 900µL of PS and 250µL of 700-1,180µm washed, sterile glass beads. The microcentrifuge tubes containing the applicator tips were vortexed for a total of 15 seconds in five 3-second pulses.

Sessile cell enumeration was done by drop plating, as specified in section 4.5.

4.11 Scanning Electron Microscopy

4.11.1 Biofilm Growth

Sterile test tubes (16x150mm) (Fischer Scientific, Waltham, MA) were filled with 10mL of either lactose MWB or cellobiose MWB. 100µL of an overnight culture was added to the test tubes. The tubes were then vortexed for 3 seconds on high. Using flame-sterilized forceps, a clean, sterile stainless steel cylinder (diameter=1.2cm, height=

0.35cm) was dropped into the test tube and adjusted until it laid flat on the bottom of the tube. The test tubes were then incubated at 32°C for 2, 4,6 or 8 days.

4.11.2 Planktonic cell Enumeration

Planktonic cell numbers were enumerated on days 2, 4, 6 and 8. Media was pipetted out of the test tube containing the stainless steel chip and transferred to a sterile test tube. The test tube containing just the media was then vortexed for 3 seconds and serially diluted in PBS. Planktonic cell enumeration was done by drop plating, as specified in section 4.5.

4.11.3 Sessile cell Enumeration

Sessile cell numbers were enumerated on days 2, 4, 6 and 8. After the media had been removed from the inoculated test tube, the stainless steel cylinder was removed using flame-sterilized forceps. The chip was rinsed with 1mL of PBS on each side to remove any planktonic cells and placed in a sterile Falcon 50mL conical centrifuge tube (Fischer Scientific, Waltham, MA) containing 10mL PBS and 1 gram of 700-1,180μm glass beads. The chip was vortexed in the conical tube for 30 seconds. 0.1mL was pipetted out of the conical tube and serially diluted. Sessile cell enumeration was done by drop plating, as specified in section 4.5.

4.11.4 Scanning Electron Microscopy Sample Preparation

Media was pipetted from the test tubes containing the stainless steel chip and discarded. Stainless steel chips were removed from the test tube using flame-sterilized

forceps. Each side of the chip was rinsed with 1mL of PBS. The stainless steel chip containing the biofilm was then dried using a graded ethanol series of 35%, 50%, 70%, 95%, and 100% ethanol. To dry, chips were dipped in the ethanol solution using flame-sterilized forceps, starting with the 35% ethanol solution. The chip was then transferred to a sterile petri dish and allowed to air dry completely in a NuAire biological safety cabinet. Once dry, the chip was dipped in the following solution and steps were repeated until the chip had been dipped in each ethanol solution. The stainless steel chip was fixed to a mount using double sided adhesive carbon tape (Structure Probe, Inc., West Chester, PA) and placed in a Cressington 108auto sputter coater (Cressington Scientific Instruments, Watford, UK) where it was sputter coated with gold for 30 seconds.

4.11.5 Scanning Electron Microscopy

The biofilm samples for both cellobiose MWB grown biofilms and lactose MWB grown biofilms were observed in a JCM-6000 bench top SEM (JEOL USA, Inc., Peabody, MA) using the JCM-6000 software (JEOL USA, Inc., Peabody, MA). Images were taken at 650x magnification.

4.12 Statistical Analysis

Biofilm biomass quantifications were performed with six replications for each of two individual measures. Planktonic cell and sessile cell enumerations were performed with three replications for each of two individual measures. The exception to this was for scanning electron microscopy and concentrated spent media dispersion experiments. For these experiment, planktonic and sessile cell enumerations were replicated three times in

one measure. Microscopy images were taken from one individual sample per day. Statistical analysis was performed using Prism 5 Version 5.0a (GraphPad Software, Inc., La Jolla, CA) for Mac OS X. Two-tailed unpaired t tests were performed for indicated experiments with a p-value of 0.05. One-way analysis of variance tests were performed with Tukey's post test with a p-value of 0.05.

CHAPTER 5

RESULTS

5.1 Determination of Ability of Nitric Oxide to Induce Active Biofilm Dispersion

In other bacteria, nitric oxide has been shown to induce dispersion from biofilms (Barraud and others 2009). Therefore, three NO donors (molsidomine, MAHMA and DNDS) were tested for their ability to induce active biofilm dispersion of biofilms of *Listeria monocytogenes* strain LM23. Dispersion inducing ability was investigated using crystal violet staining to indicate biofilm biomass (Figure 1) or planktonic cell enumeration (Figure 2). After exposure to the NO donors for 24h, two of the NO donors, MAHMA and DNDS were not significantly different than the control (Figure 1). Molsidomine, however, had significantly higher biofilm biomass than the control. If the NO donor caused dispersion of the biofilm, it was expected that the level of biomass stained by crystal violet would be decreased due to loss of bacteria from the biofilm and a breakdown of EPS. However, if a significant portion of EPS remained on the plate, there may not be measurable differences using crystal violet staining, therefore levels of planktonic cells were also analyzed.

The number of planktonic cells was determined after exposure to NO donors (Figure 2). An increase number of number of planktonic cells (log CFU/mL) present in the media of microtiter plate after exposure to NO donors would be indicative of induction of biofilm dispersion. The addition of MAHMA and molsidomine to the biofilms was found to have no significant difference in the number of planktonic cells as

compared to the control. DNDS however, had a significantly lower number of planktonic cells than the control. Based upon the biomass staining and planktonic cell counts, it does not appear that NO induces dispersal of *L. monocytogenes* biofilms.

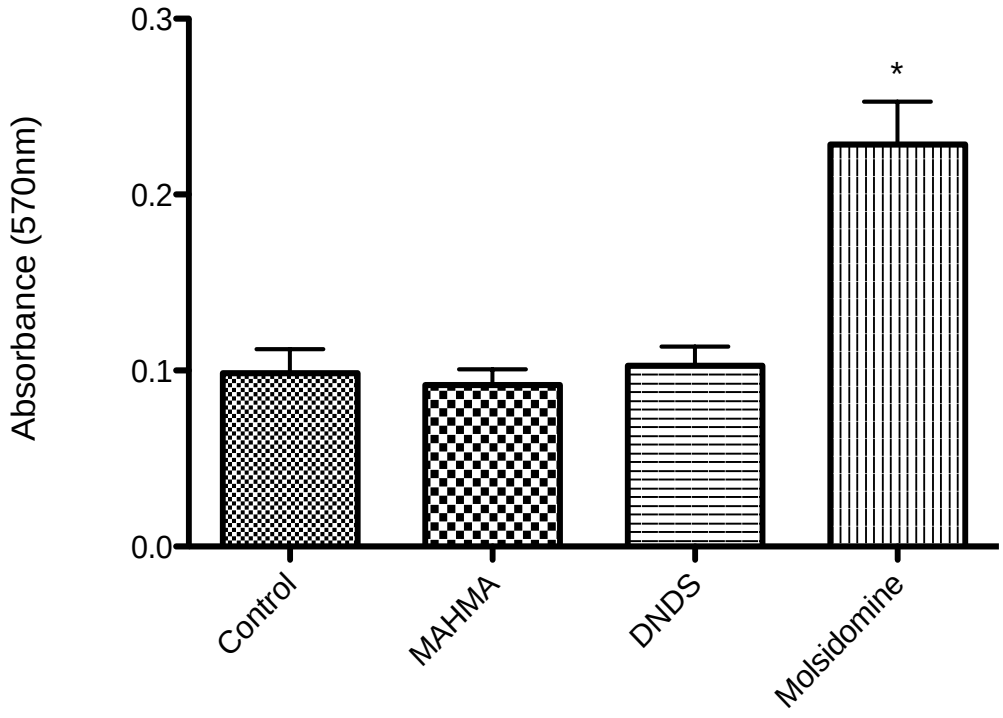


Figure 1: *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet after treatment with a NO donor. Results reported in absorbance at 570nm. MAHMA NONOate: (6-(2-Hydroxy-1-methyl-2 nitrosohydrazino)-N-methyl-1-hexanamine). DNDS: diethylamine NONOate diethylammonium salt. Molsidomine: (N- (ethoxycarbonyl)-3-(4-morpholinyl)-sydnone imine). Nitric oxide donors used at a concentration of 10μM. A (*) indicates a significant difference from the control. Unpaired t test performed, $p<0.05$.

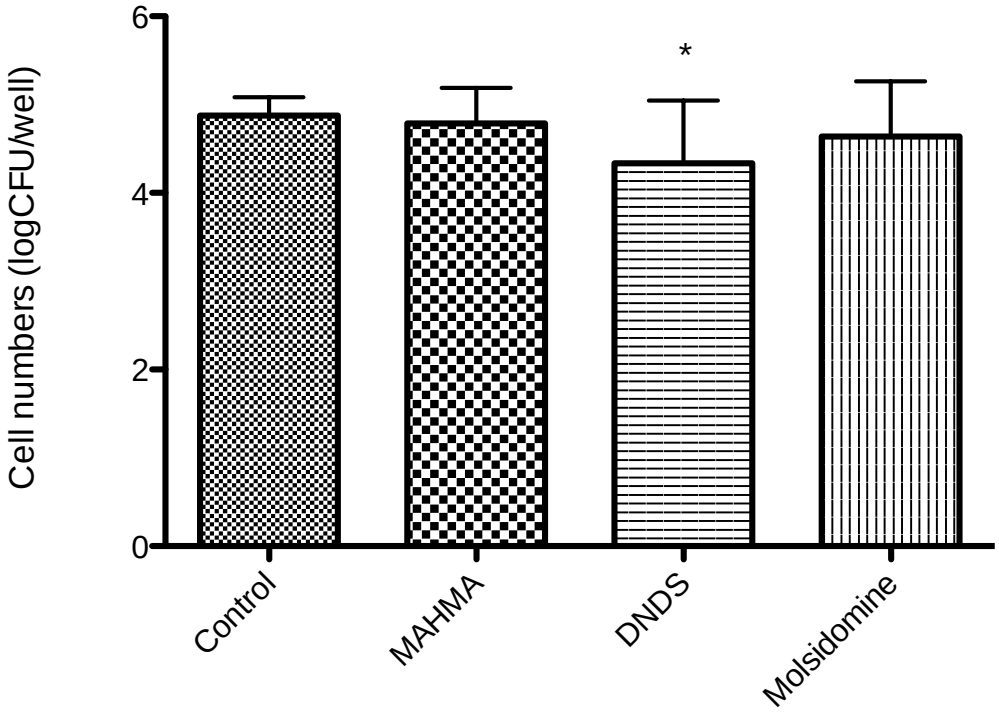


Figure 2: *Listeria monocytogenes* (strain LM23) planktonic cell numbers after treatment with a NO donor. Results reported in logCFU/well. MAHMA NONOate: (6-(2-Hydroxy-1-methyl-2 nitrosohydrazino)-N-methyl-1-hexanamine). DNDS: diethylamine NONOate diethylammonium salt. Molsidomine: (N- (ethoxycarbonyl)-3-(4-morpholinyl)-sydnone imine). Nitric oxide donors used at a concentration of 10μM. A (*) indicates a significant difference from the control. Unpaired t test performed, $p<0.05$

5.2 Determination of Ability of *cis*-2-Decenoic Acid to Induce Active Biofilm Dispersion

Cis-2-Decenoic acid is a fatty acid that has been observed to induce biofilm dispersion in gram-positive bacteria (Davies and Marques 2009). In this experiment, the chemical messenger, CDA, was investigated for its ability to induce dispersion of *L. monocytogenes* biofilms. Similar to the previous experiment, two different analyses were used to determine if biofilms were being dispersed. These two analyses were: biofilm biomass quantification and planktonic cell enumeration (Figure 3 and 4, respectively).

Two different concentrations of CDA, 310nM and 620nM, were screened and compared to a control. It was found that neither concentration had significantly different biomass as compared to the control following the treatment time (Figure 3). No significant difference in planktonic cells was found between the control and 620nM treatment (Figure 3). However, for biofilms treated with a 310nM concentration of CDA, it was found that planktonic cell numbers were significantly ($p<0.05$) lower than the control.

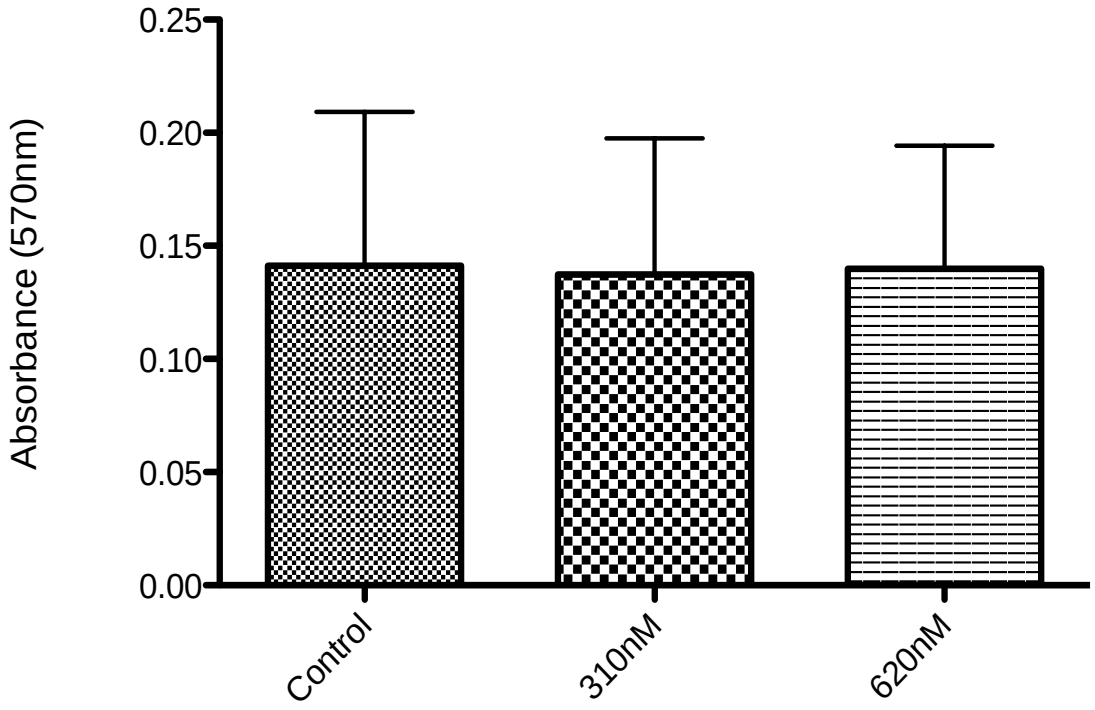


Figure 3: *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet after treatment with CDA. Results reported in absorbance at 570nm. A (*) indicates a significant difference from the control. Unpaired t test performed, $p<0.05$.

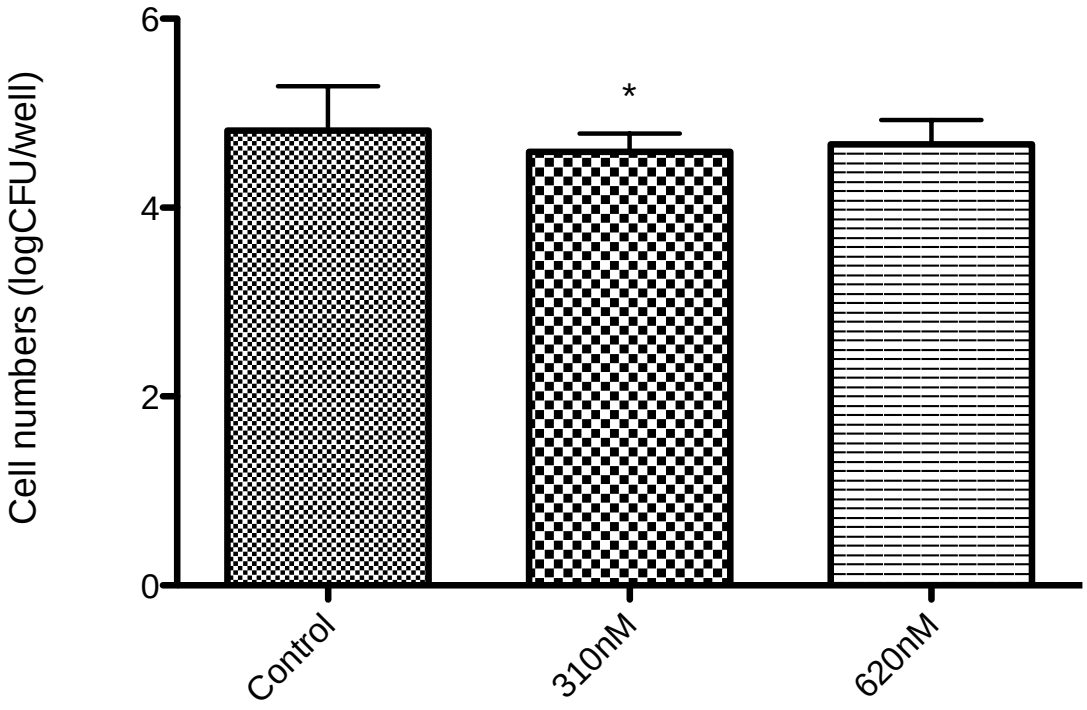


Figure 4: *Listeria monocytogenes* (strain LM23) planktonic cell numbers after biofilm treatment with CDA. Results reported in logCFU/well. A (*) indicates a significant difference from the control. Unpaired t test performed, $p<0.05$.

5.3 Evaluation of Effect of Carbohydrate Source on Biofilm Biomass

A variety of carbohydrates were evaluated to determine their influence on biofilm biomass. MWB was used as the base growth medium and glucose was exchanged for the other carbohydrates (Table 2). The results of this experiment are found in Figure 5.

Biofilm biomass varied significantly depending on the carbohydrate utilized during growth. Maltose exhibited the greatest amount of biomass, with a mean absorbance of 0.326. While the mean absorbance for biomass of biofilms grown in trehalose was lower than maltose, no statistical difference was found between the two. These two carbohydrates produced significantly more biofilm than the other 8 carbohydrates analyzed. Conversely, *L. monocytogenes* grown in raffinose generated the least amount of biomass, with a mean absorbance of 0.022. Biofilm biomass produced for sorbose, melibiose, and arabinose were not statistically different than raffinose and all fell of the low end of the spectrum for absorbance readings. When grown in lactose MWB, significantly more biofilm was generated than when grown in raffinose MWB. However, biofilm production in lactose MWB was not statistically different than biofilms grown in arabinose, melibiose, sorbose, or cellobiose MWB. Cellobiose fell in the middle in terms of biofilm production and was only found to be statistically different than maltose, trehalose, and raffinose. Biofilms grown in glucose and fructose MWB produced similar amounts of biomass and were not statistically different than cellobiose MWB. When compared to glucose as a control, the carbohydrate source utilized can significantly increase, decrease, or produce no significant effect on biofilm biomass.

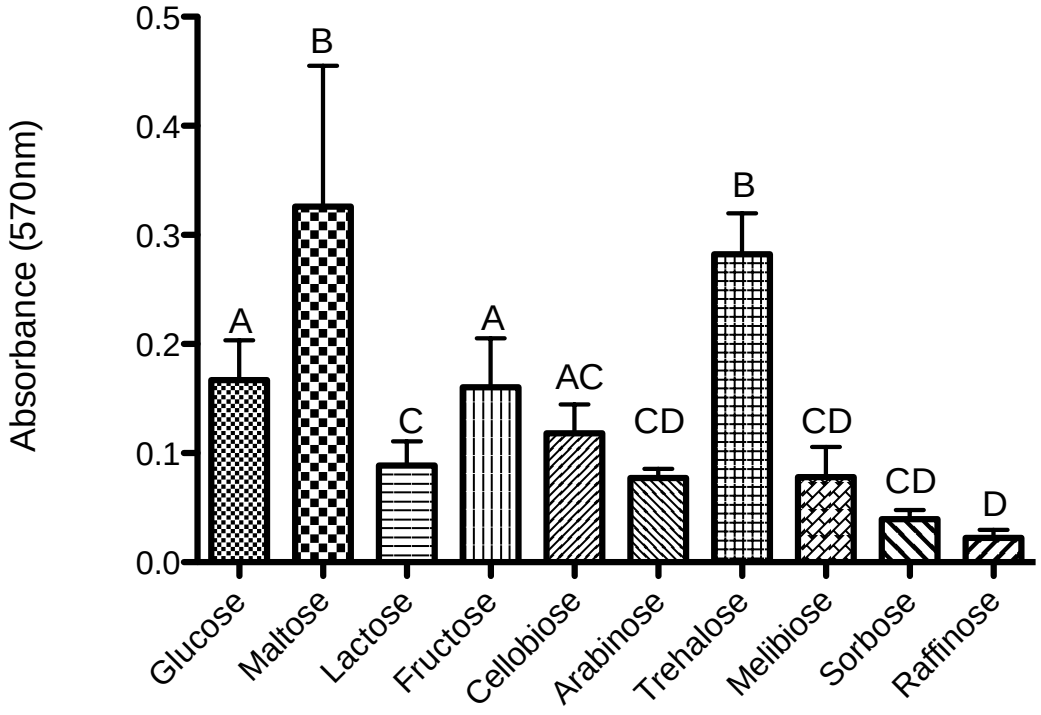


Figure 5: *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet produced in different carbohydrate variations of MWB. Results reported in absorbance at 570nm. Different letters indicate a significant difference. One-way analysis of variance performed with Tukey's post test, $p<0.05$

5.4 Tracking Changes in Biofilm Biomass, Planktonic cells, and Sessile cells over Time

In order to determine if there is any natural dispersion induction in *L. monocytogenes,* seven carbohydrates were selected based on varying levels of biofilm production at 48h (Figure 5). The level of biofilm biomass (crystal violet staining), planktonic cell numbers (CFU/well) and sessile cells (from swabbed biofilms) were tracked over the course of eight days. The carbohydrates tested were glucose (Figure 6), maltose (Figure 7), lactose (Figure 8), fructose (Figure 9), cellobiose (Figure 10), arabinose (Figure 11) and trehalose (Figure 12). In these experiments, dispersion of biofilms was defined as a statistically significant decrease in sessile cell numbers and biofilm biomass, and a statistically significant increase in planktonic cells occurring over the same time interval.

For biofilms grown in glucose MWB (Figure 6), significant decreases in biofilm biomass, sessile cell numbers were observed over the course of the experiment. Sessile cell numbers decrease initially, then had no significant change between days 4 and 8; however a significant decrease was measured between days 2 and 8. Planktonic cell numbers decreased significantly between each time interval**,** indicating that dispersion of cells from the biofilm was not occurring when glucose was the carbohydrate source (Figure 6B).

When maltose was added to MWB (Figure 7), planktonic cell numbers remained unchanged through the entirety of the 6 tracked days when maltose MWB was the growth medium. Sessile cell numbers significantly decreased between days 4 and 6, and also

significantly decreased over the 2 to 8 day period. Biofilm biomass significantly decreased between days 4 and 6 and also was significantly less at day 8 as compared to after 2 days of development (Figure 7B)

When grown in lactose MWB (Figure 8), a significant increase in planktonic cell numbers and a significant decrease in sessile cell numbers were quantified between measurements taken at days 2 and 4. For days 4 through 8, no significant change in sessile or planktonic cell numbers was observed. Between days 2 and 8, sessile cell numbers significantly decreased, while planktonic cell numbers significantly increased. No significant change was seen in biofilm biomass during any of the time intervals where measurements were taken (Figure 8B).

Biofilm biomass, sessile, and planktonic cell numbers all significantly decreased between days 2 and 4 when grown in fructose MWB (Figure 9). During days 4 through 6, sessile cell numbers continued to significantly decrease, while planktonic cell numbers began to significantly increase. No significant change in biofilm biomass occurred from days 4 through 8. Between days 6 and 8, sessile cell numbers remained constant, while planktonic cell numbers significantly decreased. From days 2 through 8, all three variables measured significantly decreased (Figure 9B).

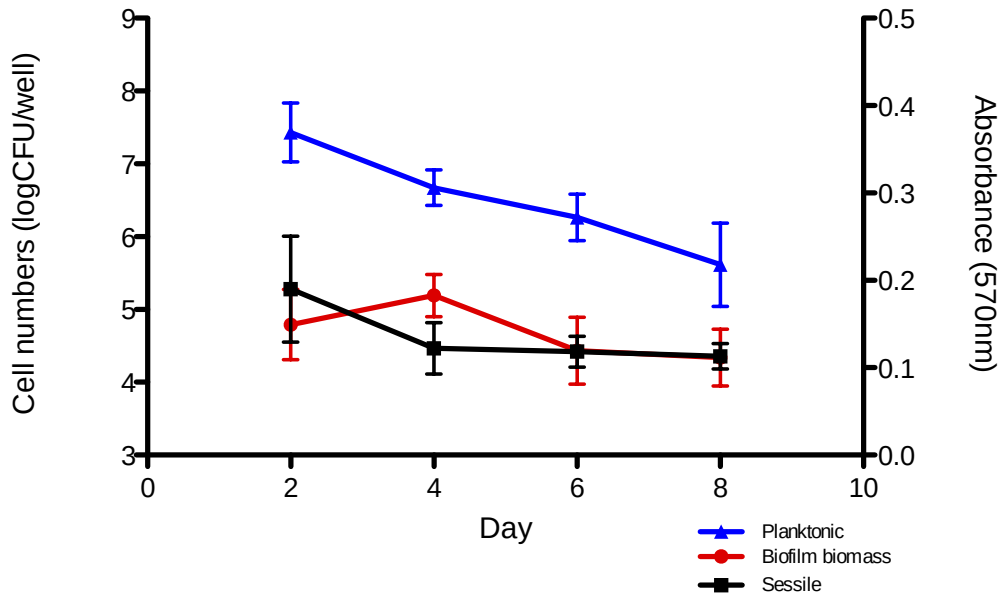
For biofilms grown in cellobiose MWB (Figure 10), planktonic cell numbers decrease during days 2 through 4, while sessile cell numbers and biofilm biomass did not change. During days 4 through 6, biofilm biomass again exhibited no significant change. Sessile cell numbers significantly decreased, while the number of planktonic cells significantly increased. Biofilm biomass and sessile cell numbers remained stable from days 6 to 8, while the number of planktonic cells significantly decreased. From the initial

measurement at day 2 to the final measurement at day 8, biofilm biomass, sessile cells, and planktonic cells all significantly decreased (Figure 10B).

Development in arabinose MWB (Figure 11) from days 2 through 4 resulted in a significant decrease in biofilm biomass, while sessile and planktonic cell numbers did not significantly change. During days 4 through 6, the number of planktonic cells significantly increased. Biofilm biomass and the number of sessile cells did not change significantly. A similar trend was seen for days 6 to 8 except that planktonic cell numbers decreased significantly. Over days 2 through 8, the number of planktonic cells significantly increased, biofilm biomass significantly decreased, and sessile cell numbers were not significantly different (Figure 11B).

In trehalose MWB (Figure 12), there was an initial decrease in sessile and planktonic cell numbers during days 2 through 4. Biofilm biomass did not significantly change during this time period. For days 4 through 6, however, biofilm biomass significantly increased, while sessile and planktonic cells numbers remain statistically unchanged. During days 6 through 8, all three variables measured significantly decreased. Biofilm biomass and the number of planktonic cells did not change between the initial and final measurements, however, sessile cell numbers significantly decreased during this time (Figure 12B).

A

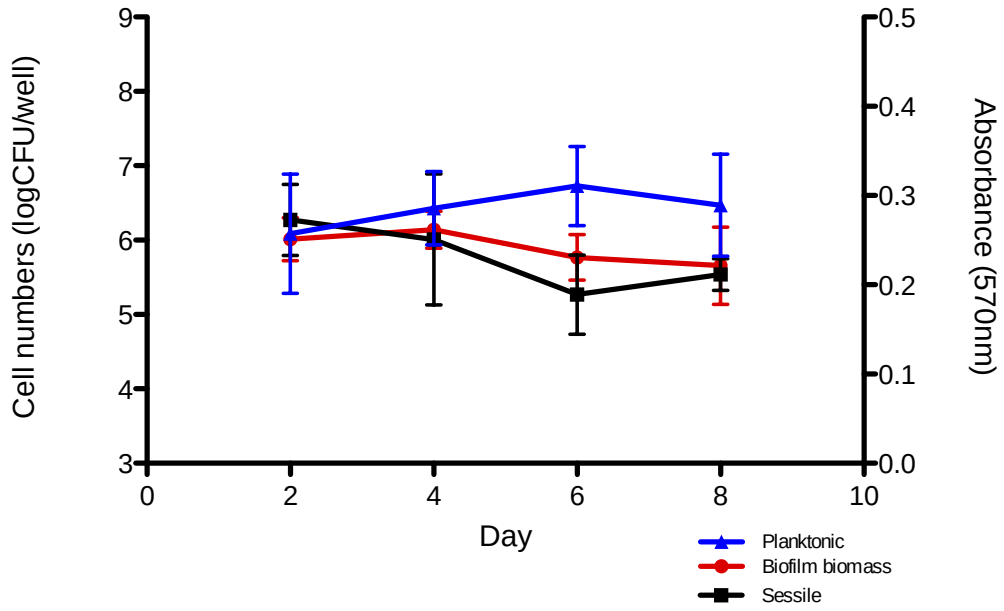


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 6: Changes in biofilms grown in Glucose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in glucose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported as logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A

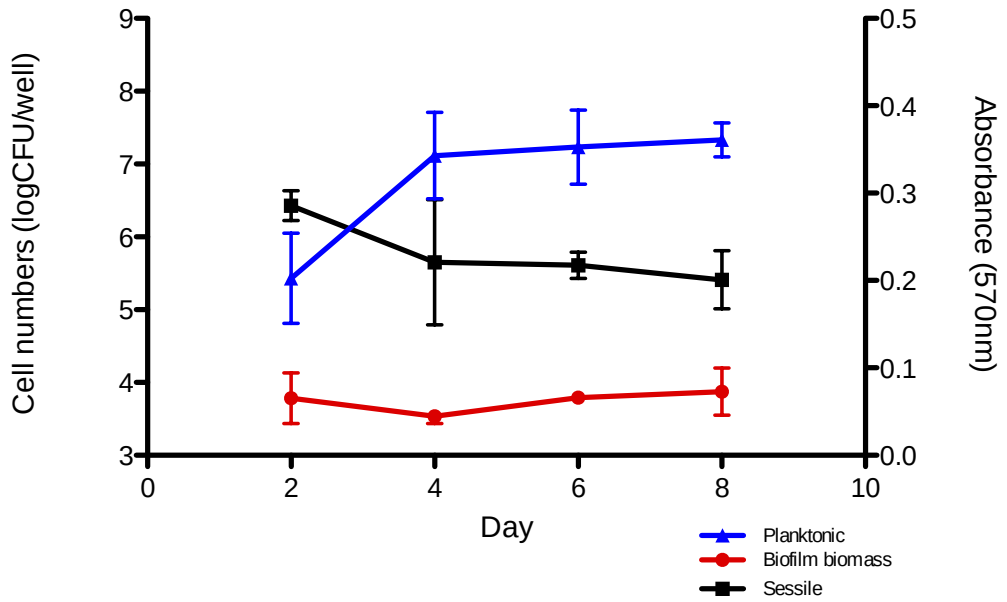


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 7: Changes in biofilms grown in Maltose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in maltose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported in logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A

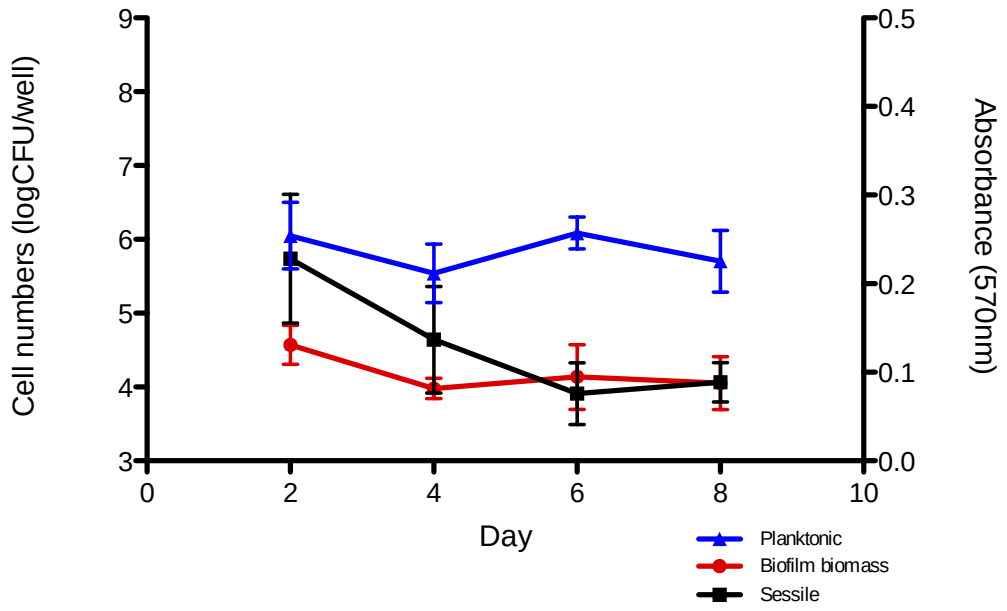


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 8: Changes in biofilms grown in Lactose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in lactose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported as logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A

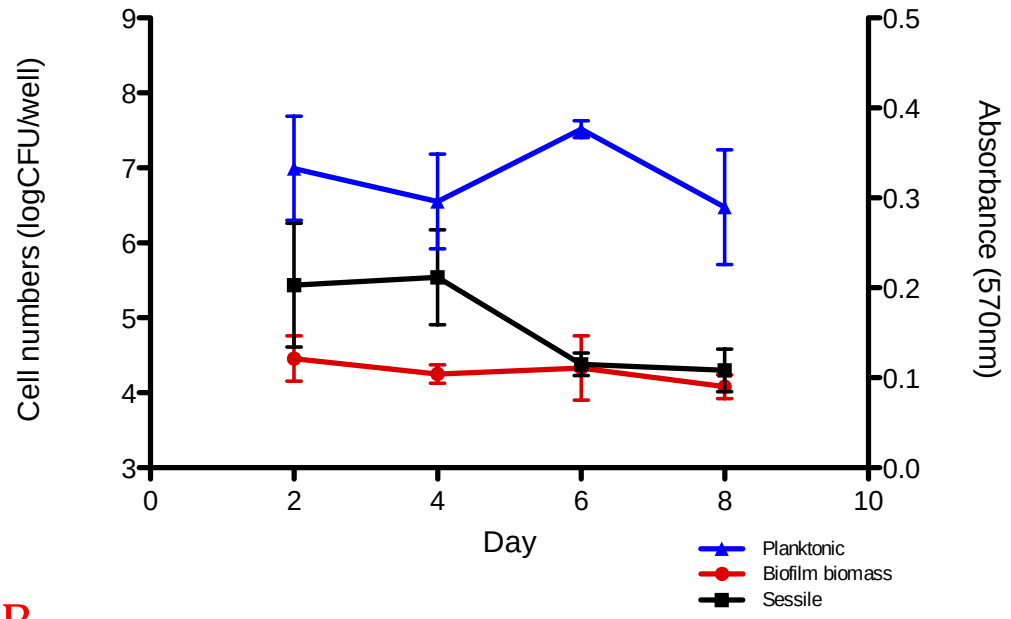


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 9: Changes in biofilms grown in Fructose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in fructose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported in logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A

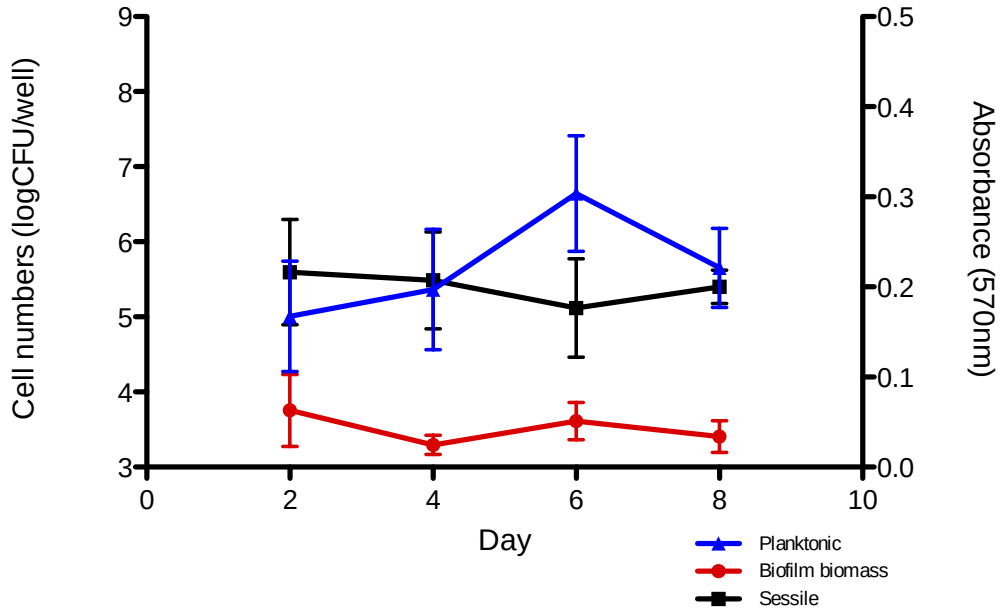


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 10: Changes in biofilms grown in Cellobiose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in cellobiose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported in logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A

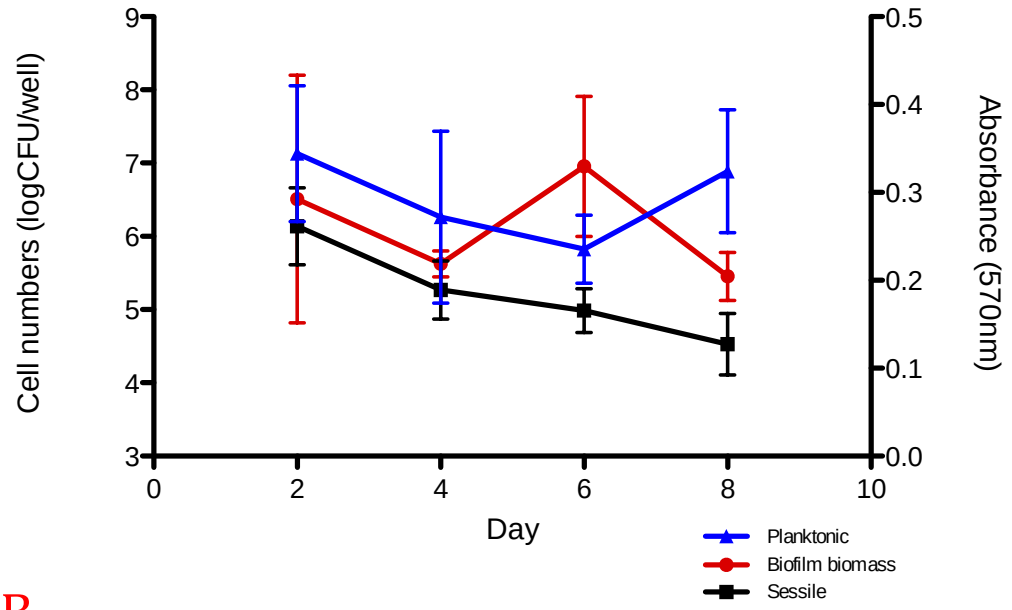


B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 11: Changes in biofilms grown in Arabinose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in arabinose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported in logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

A



B

Days	Biofilm biomass	Sessile	Planktonic
2-4			
4-6			
6-8			
2-8			

Figure 12: Changes in biofilms grown in Trehalose MWB over time. A) *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet, sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in trehalose MWB. Biofilm biomass is measured in absorbance at 570nm. Plate counts done for planktonic and sessile reported in logCFU/well. **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

5.5 Evaluation of Effect of Spent Media on Developed Biofilms

In this experiment, spent media was collected from biofilms after 8 days of development. Planktonic cell numbers as well as biofilm biomass were quantified after 2 hours (Figure 13) and 24 hours (Figure 14) of biofilm exposure to the spent media.

After 2 hours (Figure 13), no significant difference was found in planktonic cell numbers between the control and spent media treated samples. For biofilm biomass, microtiter plate wells treated with spent media had significantly more biofilm biomass than that of the control wells. After 24 hours (Figure 15), planktonic cells numbers in control wells were significantly higher than wells treated with 8 day spent media. Yet, no significant difference in absorbance between the control and spent media samples was detected.

Additionally, spent media was collected from biofilms after 6 days of development and was concentrated by removing water in the media. Planktonic cells and sessile cells were enumerated after 2 hours (Figure 15) and 24 hours (Figure 16) of exposure to the CSM. At 2 hours (Figure 15), the number of planktonic cells was significantly higher in microtiter plate wells treated with CSM at compared to the control. No significant difference between control and CSM samples for sessile cell numbers was found after 2 hours. After 24 hours (Figure 16), sessile cell numbers were significantly higher in the control. The number of planktonic cells was not significantly different between control and CSM treated wells.

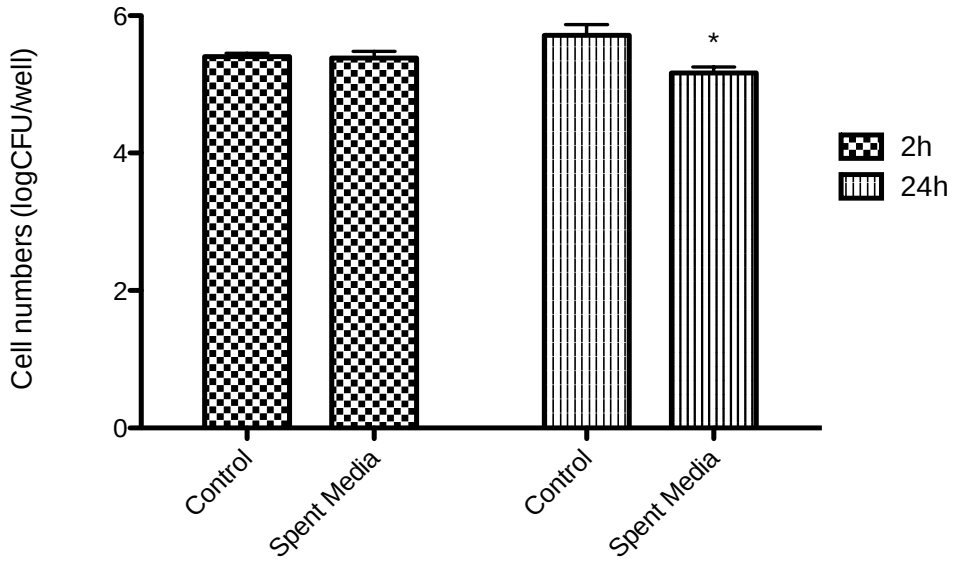


Figure 13: *Listeria monocytogenes* (strain LM23) planktonic cell numbers after treatment for 2 hours and 24 hours with cellobiose MWB spent media collected after 8 days. Results reported in logCFU/well. A (*) indicates a significant difference. Unpaired t test performed, $p<0.05$.

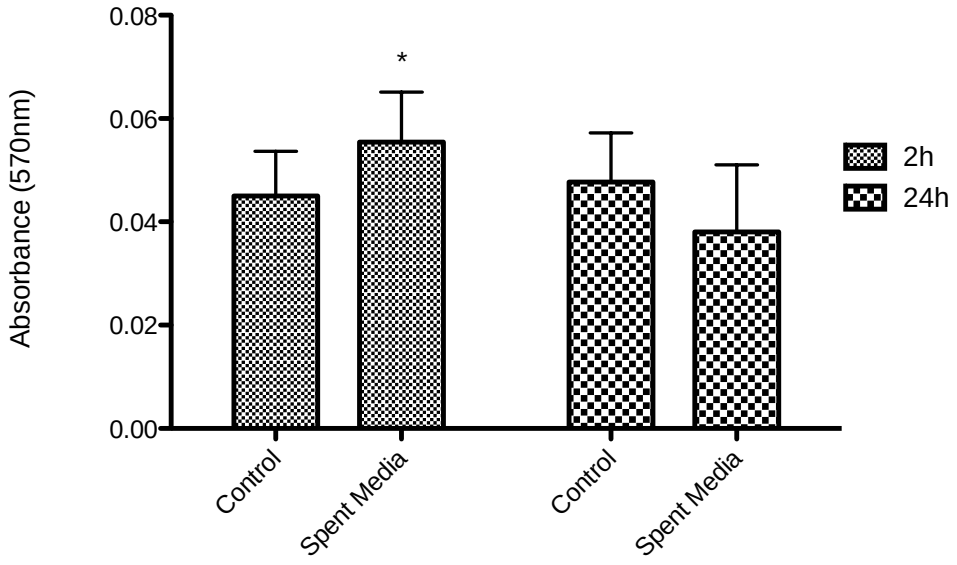


Figure 14: *Listeria monocytogenes* (strain LM23) biofilm biomass stained with crystal violet after treatment for 2 hours and 24 hours with cellobiose MWB spent media collected after 8 days. Results reported in absorbance at 570nm. A (*) indicates a significant difference. Unpaired t test performed, $p<0.05$.

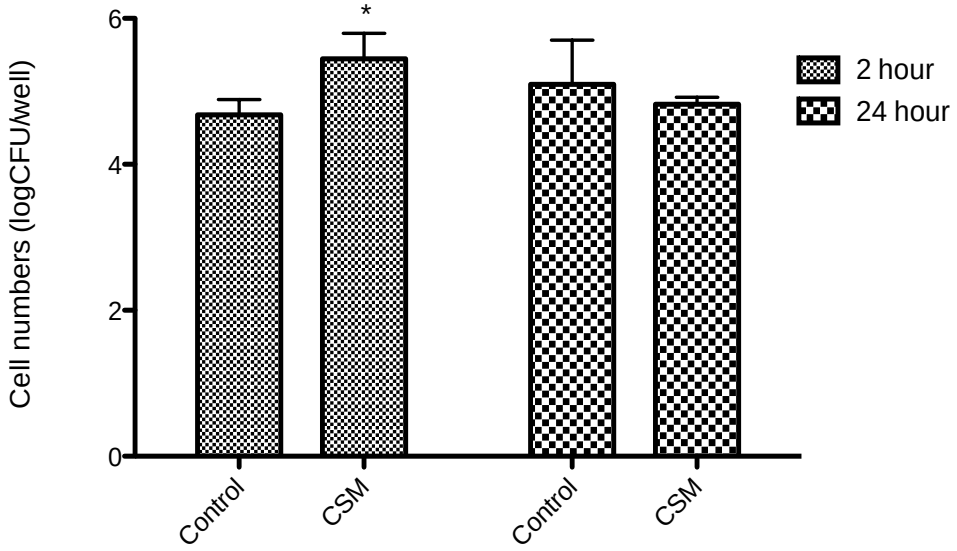


Figure 15: *Listeria monocytogenes* (strain LM23) planktonic cell numbers after treatment for 2 hours and 24 hours with cellobiose MWB concentrated spent media (CSM) collected after 6 days. Cellobiose MWB concentrated fresh media was used as a control. Results reported in logCFU/well. A (*) indicates a significant difference. Unpaired t test performed, $p<0.05$.

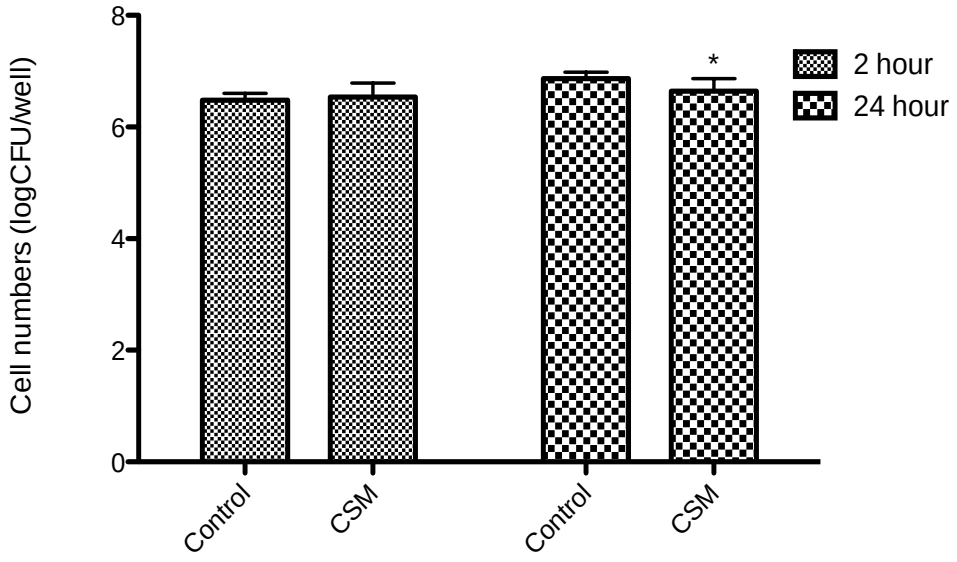


Figure 16: *Listeria monocytogenes* (strain LM23) sessile cell numbers after treatment for 2 hours and 24 hours with cellobiose MWB concentrated spent media (CSM) collected after 6 days. Cellobiose MWB concentrated fresh media was used as a control. Results reported in logCFU/well. A (*) indicates a significant difference. Unpaired t test performed, $p<0.05$.

5.6 Observation of Biofilms using Scanning Electron Microscopy in tandem with Planktonic and Sessile cell Enumeration

In this experiment, biofilms were grown statically on stainless steel chips. Images were taken using a SEM at 650x magnification. Biofilm samples were observed for qualitative differences after 4 and 6 days of growth. Biofilms were grown in either cellobiose MWB (Image 1-6) or lactose MWB (Image 7-12). Quantitative data of planktonic and sessile cell numbers were also collected for cellobiose MWB (Figure 17) and lactose MWB (Figure 18).

A clear qualitative difference in SEM images for biofilms grown in cellobiose MWB for 4 (Image 1-3) and 6 (Image 4-6) days can be seen. After 4 days, large three-dimensional structures can be seen rising off of the surface of the stainless steel cylinder. Very few cells not encased in EPS are visible. Larger biofilms have wrinkled, or bubble-like appearance. After 6 days however, the landscape of the stainless steel cylinder is much different in appearance. Much smaller biofilm structures are present and are more densely packed than the larger structures seen after 4 days. Also, a large amount of cells that do not appear to be encased in any EPS can be seen.

Quantitative data of sessile and planktonic cell numbers (Figure 17) for this experiment are used to give more insight into the qualitative data obtained from the SEM images. Whereas images taken after 6 days depict a more densely packed stainless steel cylinder, quantitative data reveal that sessile cell numbers significantly decreased over this time period. Sessile cell numbers also decrease between days 2 and 4, yet remained

statistically unchanged between days 6 and 8. The number of sessile cells was significantly less at day 8 than they were at day 2. Planktonic cell numbers also significantly decreased between days 2 and 4. From days 2 through 8, planktonic cell numbers significantly decreased just as the number of sessile cells did.

Visible differences can be seen at 4 (Image 7-9) and 6 (Image 10-12) days of growth on stainless steel cylinders in lactose MWB. At 4 days, surface attached cells are randomly scattered on the cylinder. Biofilms do not appear to be forming, as no EPS production is apparent. The same holds true for the sample imaged at 6 days. However, the cells on the cylinder have become organized into one-dimensional net-like structures. The structures vary in their degree of order, with Image 10 exhibiting the most structure. However, some degree of structure is seen in each image.

Quantitative data were also collected for biofilms grown in lactose MWB (Figure 18) to supplement the qualitative microscopy images. A significant increase in the number of planktonic and sessile cells was measured between days 2 and 4. Then, between days 4 and 6, both significantly decreased and remained statistically unchanged from days 6 through 8. Between the first enumeration on day 2 and the final enumeration on day 8, sessile and planktonic cells were statistically unchanged.

5.6.1 SEM Images of Biofilms Grown in Cellobiose MWB for 4 days

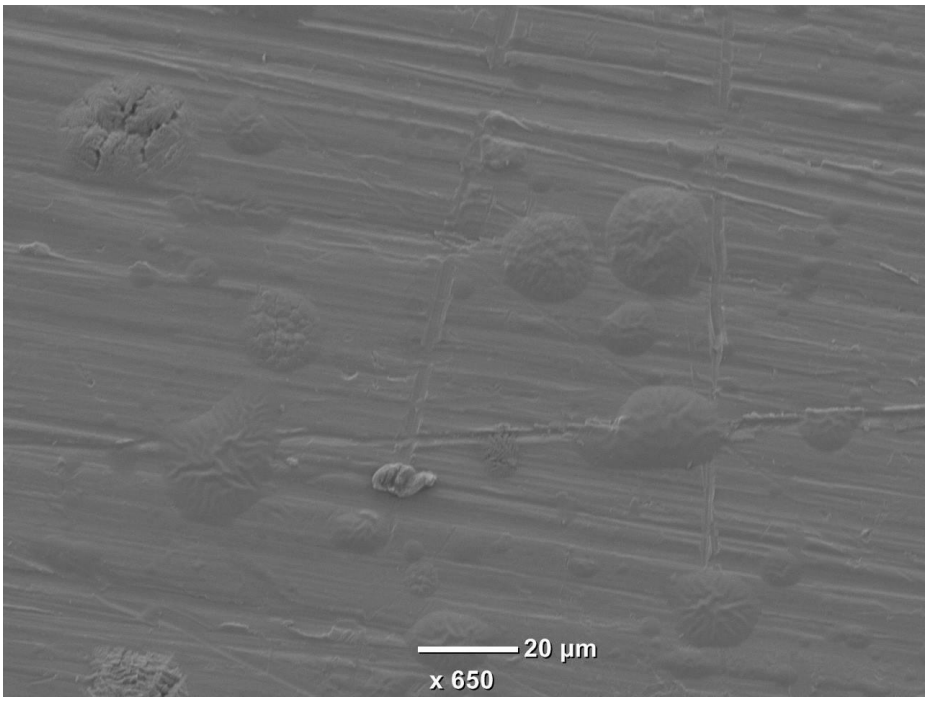


Image 1: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

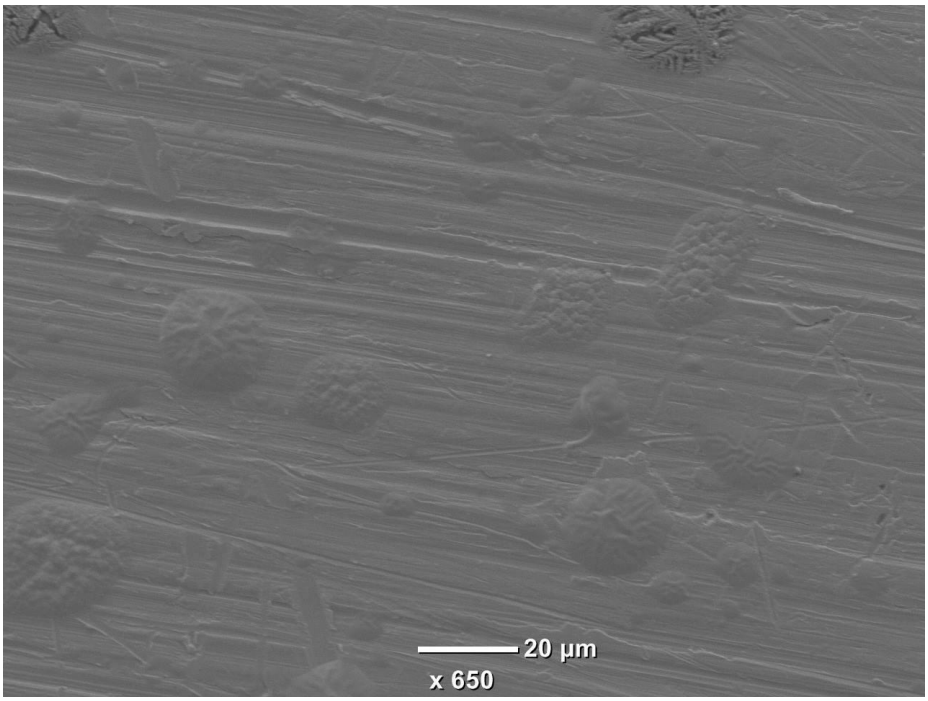


Image 2: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

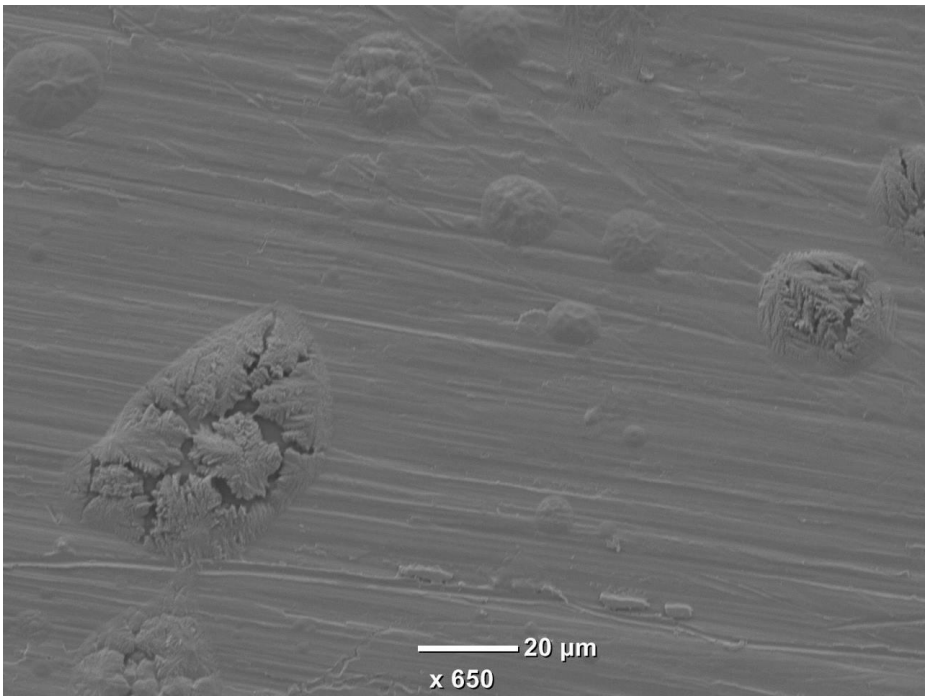


Image 3: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

5.6.2 SEM Images of Biofilms Grown in Cellobiose MWB for 6 days

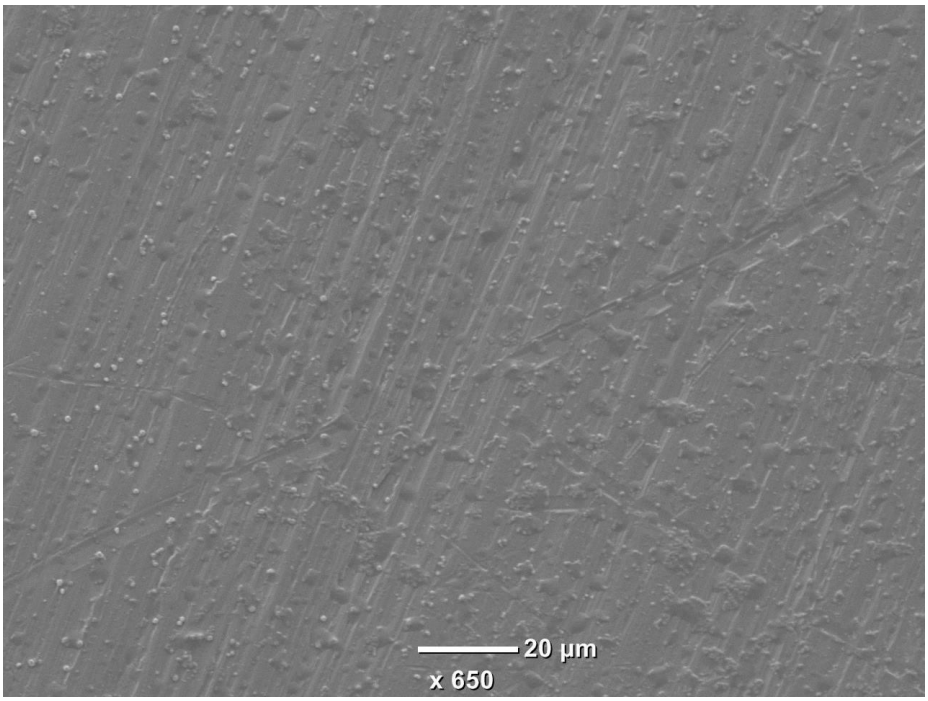


Image 4: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

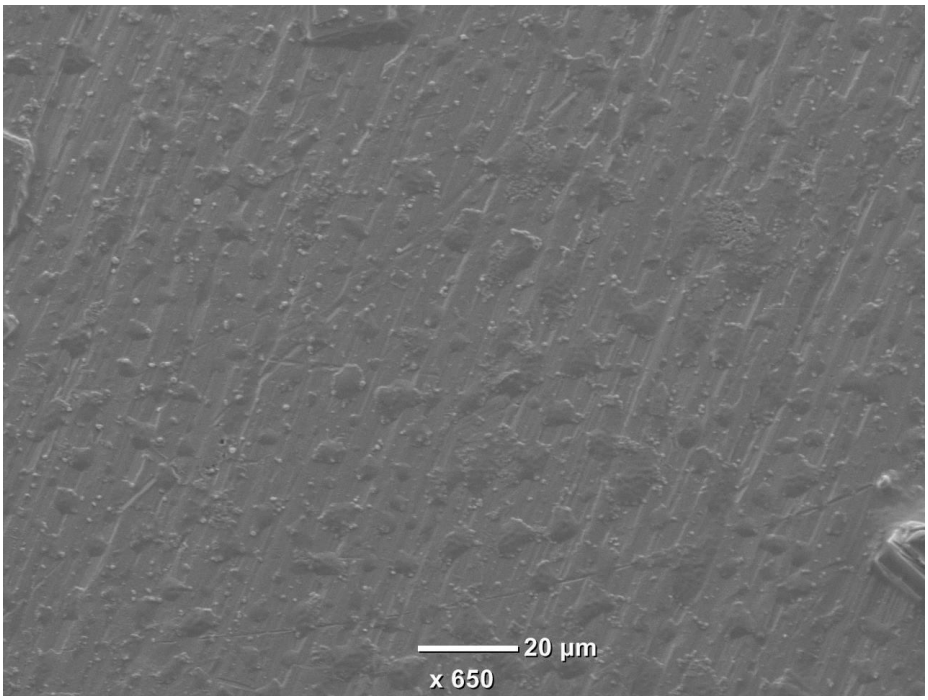


Image 5: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

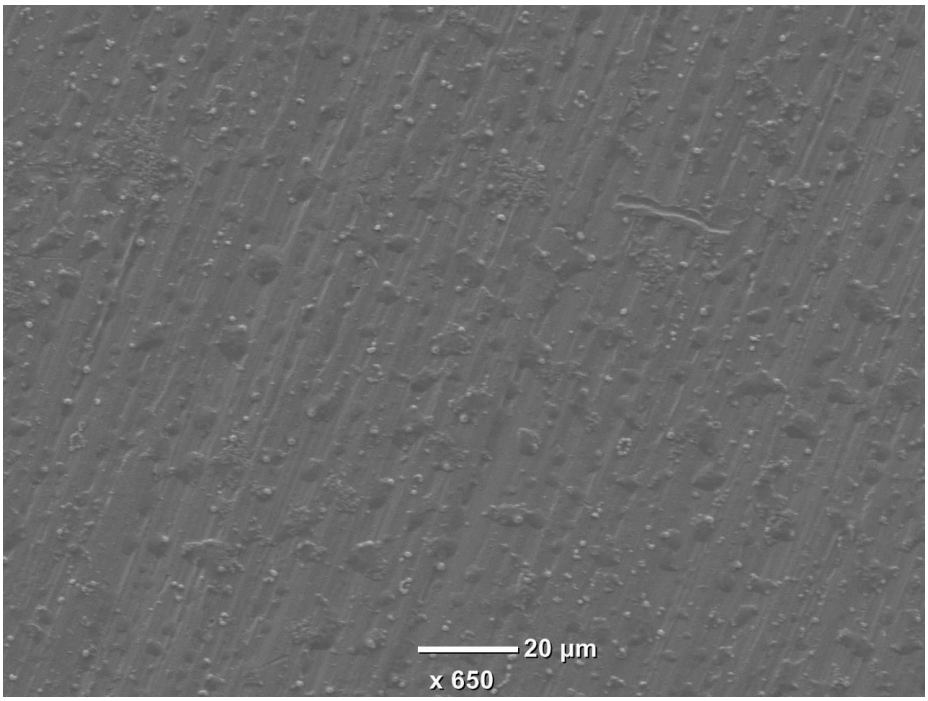
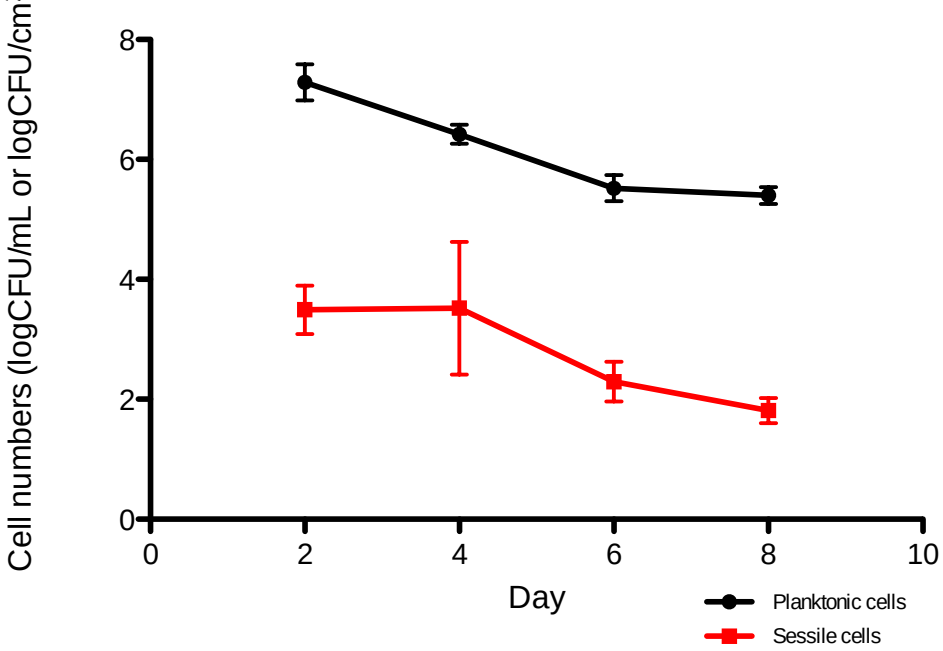


Image 6: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in cellobiose MWB on a stainless steel cylinder. Image taken at 650x magnification.

5.6.3 Planktonic and Sessile cell Enumeration for Biofilms Grown in Cellobiose MWB

A



B

Days	Sessile	Planktonic
2-4		
4-6		
6-8		
2-8		

Figure 17: Changes in biofilms grown in Cellobiose MWB over time. A) *Listeria monocytogenes* (strain LM23) sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in cellobiose MWB. Planktonic cells reported in logCFU/well. Sessile cells reported in logCFU/cm^2 **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

5.6.4 SEM Images of Biofilms Grown in Lactose MWB for 4 days

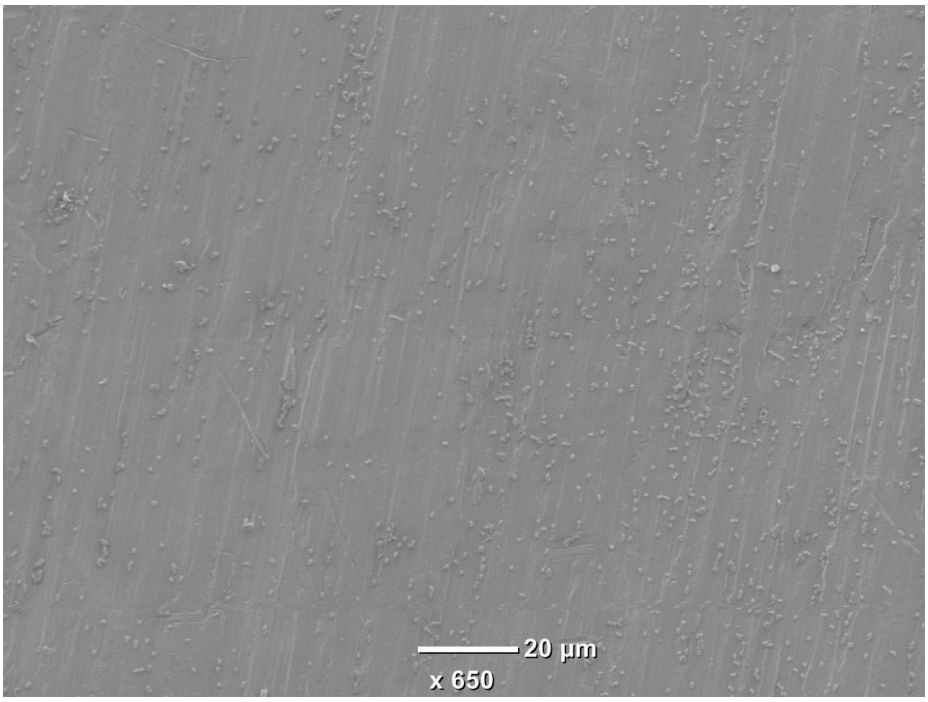


Image 7: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

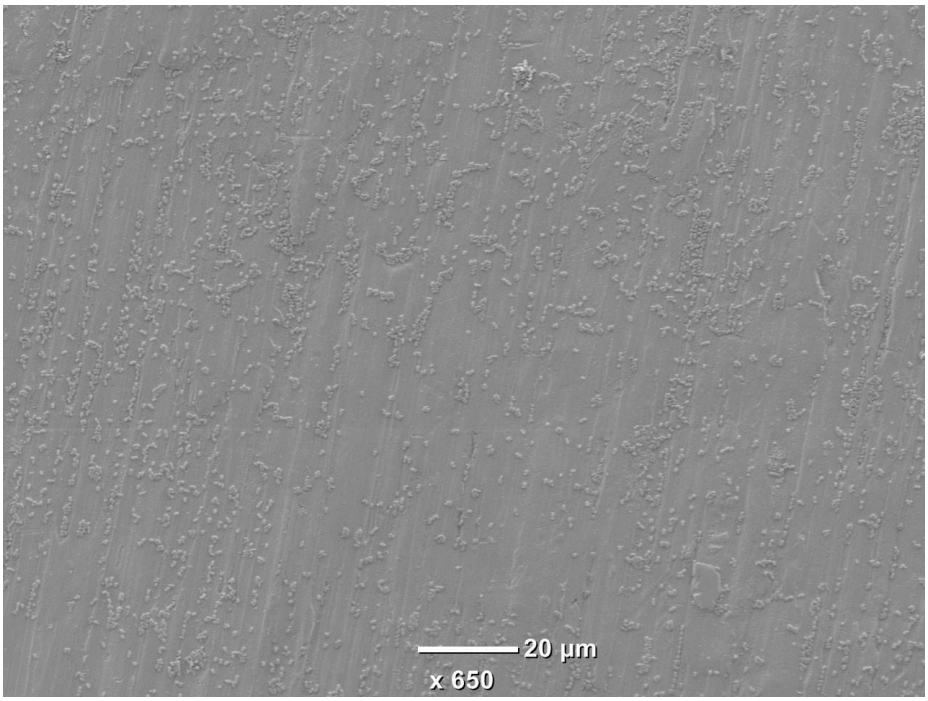


Image 8: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

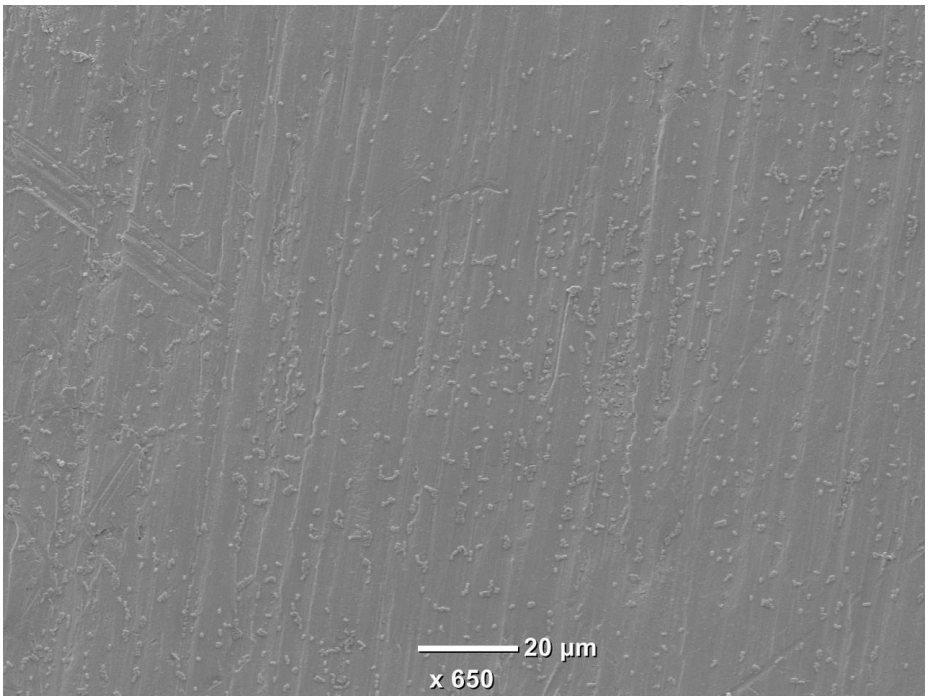


Image 9: *Listeria monocytogenes* (strain LM23) biofilm grown for 4 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

5.6.5 SEM images of biofilms grown in lactose MWB after 6 days

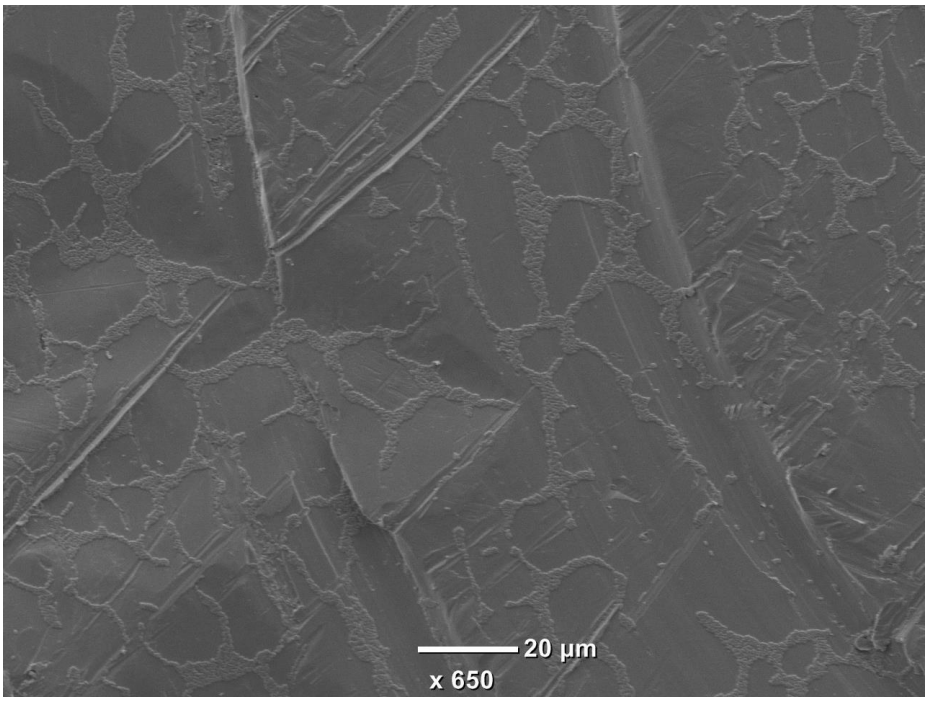


Image 10: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

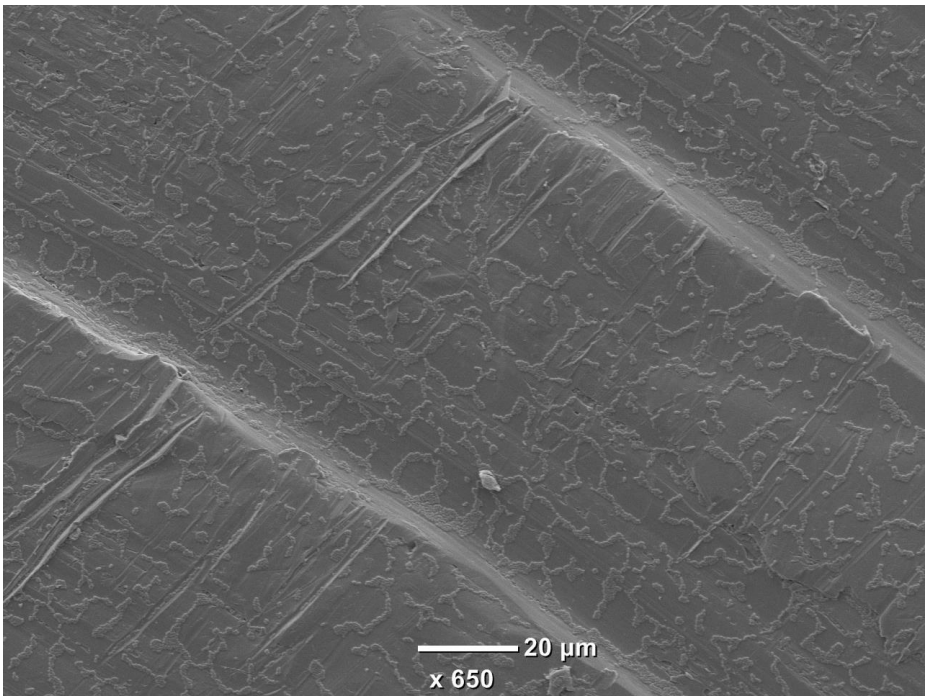


Image 11: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

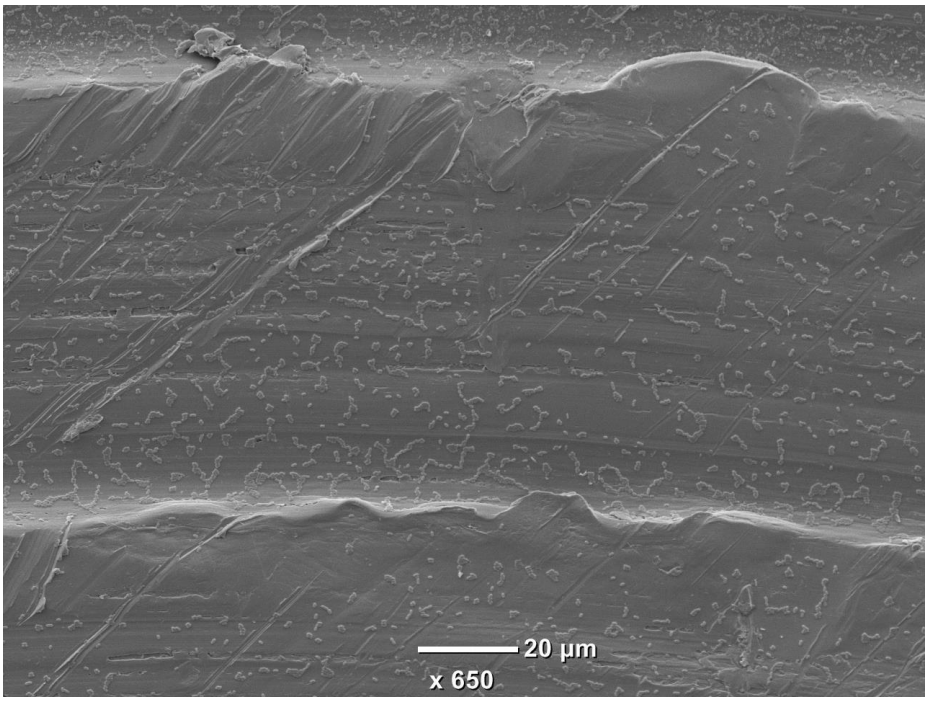
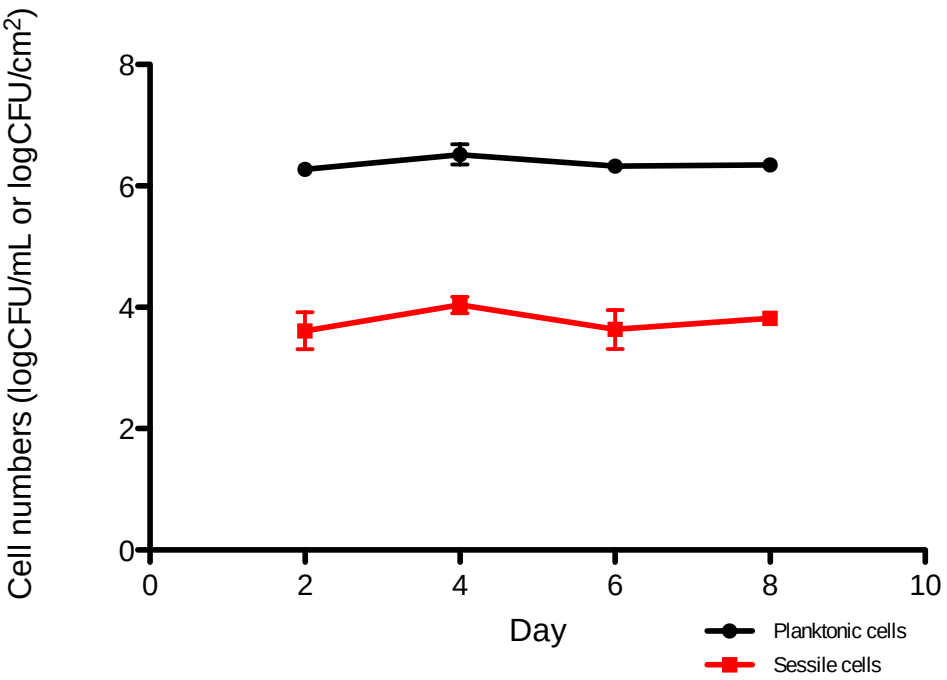


Image 12: *Listeria monocytogenes* (strain LM23) biofilm grown for 6 days in lactose MWB on a stainless steel cylinder. Image taken at 650x magnification.

5.6.6 Planktonic and Sessile cell Enumeration for Biofilms Grown in Lactose MWB

A



B

Days	Sessile	Planktonic
2-4		
4-6		
6-8		
2-8		

Figure 18: Changes in biofilms grown in Lactose MWB over time. A) *Listeria monocytogenes* (strain LM23) sessile cell and planktonic cell numbers over the course of 8 days for biofilms grown in lactose MWB. Planktonic reported in logCFU/well. Sessile reported in logCFU/cm^2 **B)** Statistical analysis of changes of different time intervals (left most column). Cells with a red fill indicate a significant decrease, green a significant increase, and gray fill indicate no significant change. Unpaired t test performed on the two days measurements were taken in a given time interval, $p<0.05$.

CHAPTER 6

DISCUSSION

6.1 Determination of Ability of Nitric Oxide to Induce Active Biofilm Dispersion

Three nitric oxide donors were screened for their ability to induce active biofilm dispersion in *Listeria monocytogenes* strain LM23. Active biofilm dispersion ability was evaluated by measuring biofilm biomass stained with crystal violet and planktonic cells after treatment with a NO donor molecule. Nitric oxide was chosen to be tested due to previous findings that it can induce significant active biofilm dispersal in other gram-positive bacteria. The three donor molecules, MAHMA, DNDS, and molsidomine were used, as they are low in cost and commercially available (Barraud and others 2009). Biofilms were treated with NO donors for a time period of 24 hours at a concentration of 10μM as previous studies have found this time and concentration for these molecules to be effective at inducing dispersion in *Escherichia coli* and *Salmonella enterica* (Marvasi and others 2014). Unfortunately, these three specific compounds have not been shown to induce biofilm dispersion in gram-positive bacteria, yet other NO donor molecules have been found to be effective against gram-positive bacteria at the same concentration and treatment time (Barraud and others 2009).

When investigating the effect of NO donors on biofilm biomass, it is expected that biofilm biomass will significantly decrease as compared to an untreated control. This is because an active dispersion event is characterized by the breakdown of EPS components and the reversion of sessile cells to their planktonic state (Allison 1998; Gjermansen and others 2005; Monroe 2007; Mann and others 2009). However, as seen in Figure 1, no such

effect was measured. MAHMA and DNDS were not significantly different than the control. For molsidomine, an interesting effect was observed, the absorbance measured from the crystal violet stained biofilm biomass was significantly higher than the control. This effect has been observed before in *Pseudomonas aeruginosa*. When treated for 24 hours with millimolar concentrations of the NO donor, sodium nitroprusside, *P. aeruginosa* biofilms increased in biomass and planktonic biomass decreased (Barraud and others 2006)

As a secondary means of evaluating active biofilm dispersal, planktonic cell numbers were measured after treatment with the NO donors (Figure 2). Barraud and others also determined that high concentrations of sodium nitroprusside resulted in a decrease in planktonic biomass (2006). While planktonic biomass was not measured for this experiment, no significant difference was found between molsidomine treated wells and control wells. Additionally, no significant difference in planktonic cell numbers was found for MAHMA. DNDS, on the other hand, had planktonic cell numbers that were significantly lower. This suggests that a concentration of 10μM of DNDS could potentially be killing planktonic cells, which could possibly invalidate the results for planktonic cell numbers for this compound, as true planktonic cell numbers would not have been quantified.

Coupling the results obtained from measuring planktonic cell numbers and biofilm biomass, these data do not suggest that MAHMA or molsidomine cause active biofilm dispersion when exposed to *L. monocytogenes* biofilms at a concentration of 10μM for 24 hours. More investigation into the effect of DNDS on *L. monocytogenes* cells needs to be done to validate the results obtained in this study, as it cannot be ruled out that it was not

detrimental to sessile cells as well, which could have impacted results obtained for biofilm biomass quantification.

6.2 Determination of Ability of *cis*-2-Decenoic Acid to Induce Active Biofilm Dispersion

The compound *cis*-2-Decenoic was chosen to be investigated for its potential to induce active biofilm dispersal in *L. monocytogenes*, as previous research has found it to be effective against gram-positive bacteria (Davies and Marques 2009). Furthermore, a treatment time of 1 hour at concentrations of 310nM and 620nM, as used in this experiment, were shown to be effective at inducing active biofilm dispersion in the gram-positive bacterium, *Bacillus cereus* (Sepehr and others 2014). Previous studies using CDA measured the optical density of released cells to determine if dispersion was occurring (Davies and Marques 2009; Sepehr and others 2014). In this experiment, active biofilm dispersion was again measured by two different means, including the quantification of biofilm biomass and planktonic cell enumeration (Figure 3-4).

Compared against the control, neither a 310nM nor 620nM concentration of CDA produced a significant difference in biofilm biomass stained with crystal violet (Figure 3). For planktonic cell number enumeration, no significant difference was found between the control and 620nM CDA treatment (Figure 4). When CDA was used at a concentration of 310nM, planktonic cell numbers were significantly lower than the control. This is the opposite of what is expected, as planktonic cell numbers should increase in a dispersion event. A significant decrease at a concentration of 310nM is not

likely due to CDA being toxic to *L. monocytogenes* cells, as no significant difference in planktonic cell numbers compared to the control was seen at 620nM.

Data from biofilm biomass quantification and planktonic cell enumeration do not suggest that CDA induces active biofilm dispersion in *L. monocytogenes* at concentrations of 310nM and 620nM when treated for 1 hour.

6.3 Evaluation of Effect of Carbohydrate Source on Biofilm Biomass

After investigating the effect of two known biofilm dispersal agents, nitric oxide and *cis*-2-Decenoic acid, on *L. monocytogenes* biofilms, the next research objective for this thesis was to determine if natural active biofilm dispersion occurs in this organism. The first step in investigating natural active biofilm dispersal was to examine the influence of carbohydrate source on biofilm development. Carbohydrate source has previously been shown to influence biofilm formation. Kim and Frank used *Listeria monocytogenes* Scott A grown in different carbohydrate variations of MWB and found that biofilms covered different amounts of area on stainless steel based on the carbohydrate used (1995). Using this information, it was hypothesized that carbohydrate source could potentially have an impact on biofilm dispersal as well. For this reason, 10 different carbohydrate variations of MWB were screened for use in investigating natural active dispersion in *L. monocytogenes* (Figure 5).

The effect of carbohydrates on biofilm development was measured by quantifying biofilm biomass stained with crystal violet (Djordjevic and others 2002). This method offers a quick and convenient way to determine biofilm formation in a microtiter plate

well. Additionally, since the test is done in microtiter plates, several carbohydrates can be screened at once with a large number of replicates, making this an ideal method for this experiment. One downside to this method, however, is that there is no way to tell differences in EPS production or sessile cell numbers, as only biofilm biomass (EPS + sessile cells) can be measured.

It was determined that the carbohydrate source used for growth can significantly increase or decrease biofilm biomass as compared to glucose (Figure 5). Trehalose was found to significantly increase biofilm biomass as compared to glucose after 2 days of incubation. This result is similar to those found by Kim and Frank (1995). They discovered that trehalose MWB significantly increased biofilm area coverage on stainless steel slides after 1 and 4 days of incubation as compared to glucose MWB. Additionally, the same study found that cellobiose MWB and fructose MWB had significantly more biofilm area coverage after 1 day of incubation as compared to biofilms grown in glucose MWB (Kim and Frank 1995). Looking at Figure 5, this effect was not measured for *L. monocytgenes* strain LM23. After 2 days of incubation in microtiter plates, biofilms grown in fructose MWB and cellobiose MWB were not significantly different in biofilm biomass from those grown in glucose MWB. While the study by Kim and Frank is not a perfect comparison to this experiment due to differences in strain and growth surface, it does agree with the finding that carbohydrates can significantly influence biofilm development (1995).

6.4 Tracking Changes in Biofilm Biomass, Planktonic cells, and Sessile cells over Time

After confirming that the carbohydrate source used for growth can influence biofilm biomass production, seven of the carbohydrates were chosen for investigation into active biofilm dispersion (Figure 6-12). The reasoning behind this was that if carbohydrate source can influence biofilm biomass, it could potentially influence the late stages of biofilm development as well (i.e. dispersion). The carbohydrates chosen represented those that were significantly lower, significantly higher, and not significantly different from glucose in biofilm biomass (Figure 5). This was done to gain a complete picture of any possible effect on natural dispersion that carbohydrate source may have. The carbohydrates screened were, glucose (Figure 6), maltose (Figure 7), lactose (Figure 8), fructose (Figure 9), cellobiose (Figure 10), arabinose (Figure 11), and trehalose (Figure 12). A potential dispersion event was characterized by the following criteria- a significant decrease in biofilm biomass and sessile cell numbers, along with a significant increase in planktonic cell numbers.

Of the seven carbohydrates screened (Figure 6-12), none exhibited trends corresponding with a potential dispersion event as mentioned above. However, three carbohydrates were identified that met two of the three criteria of biofilm dispersion mentioned above. Biofilms grown in fructose MWB (Figure 9) and cellobiose MWB (Figure 10) both displayed a significant decrease in sessile cell numbers synchronized with a significant increase in planktonic cell numbers between measurements taken after 4 and 6 days of growth. Yet, both significantly decreased in planktonic and sessile cells

between days 2 and 8. Biofilms grown in lactose MWB (Figure 8) significantly decreased in sessile cell numbers, while significantly increasing in planktonic cell numbers over days 2 through 4 of growth as well as over days 2 through 8. For biofilms grown in arabinose MWB (Figure 11), a significant decrease in biofilm biomass and a significant increase in planktonic cells numbers were recorded after days 2 through 8.

The results of this experiment were inconclusive as to if dispersion occurred in any of the biofilms analyzed, as not all three criterions for dispersion were met. Being one of the candidates that displayed two of the three criteria for dispersion, cellobiose MWB was chosen to further investigate to confirm or refute whether active biofilm dispersion had occurred.

6.5 Evaluation of Effect of Spent Media on Developed Biofilms

In an attempt to explain the phenomena observed in cellobiose MWB, along with other carbohydrate variations of MWB, spent media was collected from biofilms grown in microtiter plates for 8 days (Figure 13). It was hypothesized that spent media could contain a chemical messenger molecule that could induce biofilm dispersion, and that this compound would accumulate in static growth conditions. If an unknown compound was indeed causing the perceived dispersion event seen in cellobiose MWB (Figure 10), then treating fresh biofilms with spent media should cause the same effect. The idea behind this experiment came from a study conducted by Davies and Marques. In this study, the researchers used spent media from *Pseudomonas aeruginosa* to induce dispersion in fresh *P. aeruginosa* biofilms (Davies and Marques 2009).

Biofilms were treated with 8 day spent media for 2 hours and 24 hours (Figure 13-14). These two treatment times were chosen, as dispersion inducing compounds can be effective on gram-positive bacteria after a shorter or longer exposure time (Barraud and others 2009; Sepehr and others 2014).

The data obtained from this experiment suggested that a dispersion event had not occurred in Figure 10. The significant increase in control planktonic cell numbers after 24 hours (Figure 13) seen was likely due to the fact that fresh media was used as the control and the cells were simply in more optimal conditions for growth. The significant increase in biofilm biomass after 2 hours (Figure 14) cannot be explained. An effect similar to what was observed with the NO donor, molsidomine (Fig. 1), could have potentially occurred, yet this is unlikely as the same effect did not occur after 24 hours of exposure to spent media.

Another experiment was done with the same goal of clarifying what was observed in cellobiose MWB (Figure 10). Spent media was collected after 6 days of growth where the potential dispersion event had occurred (Figure 10). Water was removed from the media to concentrate a possible dispersion-inducing compound. This was done as higher concentrations of the dispersion-inducing compound, CDA, have been shown to result in more pronounced dispersion events (Davies and Marques 2009). Whereas the previous experiment had failed to detect any possible dispersion event, concentrating the spent media could create more significant results that would be detected. Planktonic (Figure 15) and sessile cell numbers (Figure 16) were enumerated in this experiment to correspond with the significant changes observed in biofilms grown in cellobiose MWB

between days 4 and 6 (Figure 10). Significantly higher planktonic cell numbers were found in wells treated with CSM (Figure 15). Additionally, significantly higher sessile cell numbers were found in control wells (Figure 16). Both of these findings correspond with the characteristics of a dispersion event. However, significant increases in planktonic cell numbers did not occur in sync with significant decreases in sessile cell numbers as was seen in Figure 10.

The data obtained from the concentrated spent media experiment (Figure 15-16) partially agreed with the hypothesis that biofilms treated with spent media would exhibit the same changes observed between 4 and 6 days of growth for biofilms grown in cellobiose MWB (Figure 10). However, as the data were not fully supportive, further investigation into this phenomenon needed to be done.

6.6 Observation of Biofilms using Scanning Electron Microscopy in tandem with Planktonic and Sessile cell Enumeration

Visual observations of cellobiose MWB grown biofilms was the next step in determining if active dispersion was occurring. Biofilms samples were observed before and after the perceived dispersion event at 4 and 6 days, respectively (Figure 10). Lactose MWB was used as the control growth medium, as no significant changes in biofilm biomass, sessile cell numbers, or planktonic cells numbers were seen during the same time period (Figure 8).

Images taken at days 4 (Images 1-3) and 6 (Images 4-6) for cellobiose MWB grown biofilms are strikingly different. Between days 4 and 6, biofilms underwent a

change from large microcolonies to small microcolonies with cells not bound in EPS visibly apparent. When examining the quantitative data taken, both sessile cell numbers and planktonic cell numbers significantly decreased between days 4 and 6 (Figure 17). These data do not exhibit a significant change in planktonic cell numbers that would be expected if a dispersion event were occurring, nor do they reproduce the effect seen in Figure 10. A possible reason as to why these data did not reproduce those obtained from the 8 day experiment (Figure 10) could in part be due the fact that biofilms were grown on stainless steel as opposed to vinyl, as growth surface has been shown to impact biofilm development (Kalmokoff and others 2001).

Major differences between images taken at 4 (Images 7-9) and 6 (Images 10-12) days for cells grown in the control media, lactose MWB, can be seen. Cells are randomly scattered at day 4, yet at day 6, cells have taken on organized net-like structures. These structures have been observed in *Listeria monocytogenes* Scott A grown under starvation stress in brain heart infusion broth, which contains glucose as the carbohydrate source (Takhistov and George 2004). This previous research points at the possibility of nutrient stress being the initiator of the observed structural changes. Quantitative data did not match up with previous results (Figure 8) for this growth medium either, as significant decreases in sessile and planktonic cell numbers were measured (Fig. 18).

It is clear that the biofilms observed changed dramatically from days 4 through 6, yet quantitative data do not support that these changes were due to a dispersion event occurring. It is likely that the observed changes were structural changes that can occur in

a nutrient limited environment, and that the event was unrelated to dispersion (Takhistov and George 2004).

CHAPTER 7

CONCLUSIONS

The two known dispersion inducing compounds, NO and CDA do not appear to have any influence on *Listeria monocytogenes* strain LM23. The NO donors, MAHMA and molsidomine can be definitively ruled out. MAHMA produced results for planktonic cell numbers and biofilm biomass that were not significantly different than the control. Molsidomine resulted in an opposite effect on biofilm biomass and was not significantly different from the control in planktonic cell numbers. Further testing needs to be done on DNDS as it is possible it was harmful to *L. monocytogenes*. However, it is unlikely it will induce dispersion, as the other NO donors had no effect. CDA also produced negative results. At the two concentrations tested, no significant change in biofilm biomass was measured. A significant decrease in planktonic cell numbers at 310nM likely did not occur, as the same effect was not measured when a 602nM concentration was used.

Carbohydrate source can significantly increase or decrease biofilm biomass as compared to glucose. When *L. monocytogenes* is grown with different carbohydrates under static conditions, cellobiose, lactose, and fructose all exhibited significant changes in sessile and planktonic cell numbers that suggested biofilm dispersion could be occurring. Treatment with concentrated spent media resulted in effects that agreed with what would be expected if dispersion had occurred, yet the data were not fully supportive of a dispersion event. SEM imaging revealed a changing biofilm landscape, but quantitative data failed to reproduce what was initially observed in cellobiose MWB. Whether natural active dispersion occurs in *L. monocytogenes* is inconclusive. The data

presented in this thesis suggests that this is a complex issue that requires further research to fully explain the phenomena observed.

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