

**RAPID DETECTION OF VIABLE BACTERIA IN AGRICULTURAL  
WASH WATERS – A DROPLET-BASED APPROACH**

by  
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A dissertation submitted to Johns Hopkins University in conformity with the  
requirements for the degree of Doctor of Philosophy

Baltimore, Maryland  
September, 2017

## **Abstract**

Foodborne illness is a significant public health concern worldwide, with a global burden of disease comparable to HIV/AIDS, malaria and tuberculosis. The World Health Organization estimated there were approximately 2 billion cases and over 1 million deaths associated with foodborne illness in 2010. In the United States (U.S.) alone, foodborne contamination and associated illness is responsible for an estimated 48 million cases per year, with total health-related costs estimated between \$51 and \$76.1 billion annually. From 1998 to 2008, nearly half of all foodborne illnesses in U.S. were attributable to produce and over 20% were attributable to leafy greens. The food industry must continually evaluate critical control measures for its most vulnerable crops, improve upon detection methods, and maintain collaborative relationships with surveillance networks to lessen prevalence and severity of foodborne outbreaks. The food industry utilizes hazards analysis and critical control point (HACCP) programs to identify and mitigate vulnerabilities in the farm-to-consumer route. Wash waters are essential for removing debris and sanitizing produce before rapidly shipping to the end consumer. If sanitization efficacy is compromised, wash waters can cross-contaminate large batches of previously uncontaminated produce. For this reason, fresh-cut produce wash waters are a critical control point in industrial produce processing facilities. This dissertation assesses inhibition challenges wash waters present to qPCR and a means to overcome these challenges by using common chlorine quenchers. Droplet-based microfluidics, paired with activated fluorescence, is evaluated as a rapid

alternative to detect viable bacterial contamination. The droplet-based method, paired with a FITC-conjugated antibody, achieved excellent sensitivity and specificity for the target bacteria – artificially spiked *Salmonella* – in a produce wash water acquired from a major Mid-Atlantic produce processing facility. Most importantly, viable bacterial detection was achieved in less than five hours – dramatically reducing time needed for traditional culturing that can take days. In-droplet microfluidics shows great promise for preventing produce-associated foodborne outbreaks by potentially providing food industry HACCP program managers a same-day (or same-shift) detection capability.

## Acknowledgments

This research was funded by The Johns Hopkins Education and Research Center for Occupational Safety and Health, sponsored by the National Institute for Occupational Safety and Health, and The Osprey Foundation of Maryland. This dissertation would not have been possible without their funding.

I'd like to thank the Applied Biological Sciences Group, Johns Hopkins University Applied Physics Laboratory, for facilitating and supporting my research activities. Also, Shih-Chi Weng's assistance was critical to this study and much appreciated.

My advisor, Kellogg Schwab, and co-advisor, Charles Young, were invaluable and thoughtful throughout the entire development process. Their guidance cultivated my investigative capabilities, and their patience and understanding for unavoidable life events, such as military mobilizations, is much appreciated.

Finally, I'd like to thank my mother for always being there to help when needed. Her assistance was critical in caring for my son on days when I had to be in the lab well past his bedtime. I'd also like to thank my son, Torin, for being the most remarkable and inspiring three-year-old I know.

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## Chapter 1: Introduction

This dissertation brings together the capabilities and laboratories at the Johns Hopkins Bloomberg School of Public Health and the Johns Hopkins University Applied Physics Laboratory to optimize a proof-of-concept for a rapid isolation, detection, and culturing platform for *Salmonella*, a leading cause of bacterial gastroenteritis from foodborne outbreaks and a pathogen of significant public health concern. Developing robust, same-day platforms and emerging technologies for food-processing facilities and on-site field investigations are a needed capability for regulatory teams and food industry scientists in order to control and prevent *Salmonella* outbreaks (Tauxe et al., 2010). It is critical to develop reliable methodologies for near real-time, same-day (or same-shift) isolation, detection, and live culturing that are highly sensitive and specific in challenging environmental settings. Moreover, there is a clear need for novel rapid, same-day isolation, and detection methods that can enable hazards analysis and critical control point (HACCP) programs to viably culture bacterial organisms present in complex sample matrices, such as wash waters utilized by the produce processing industry (Havelaar et al., 2010).

According to the United States Department of Agriculture's National Agricultural Statistics Service, produce, including head lettuce, leaf lettuce, romaine lettuce, spinach and cabbage, narrowly edged out tomatoes for the most valuable vegetable crop produced in the United States – valued at slightly over \$2 billion dollars in 2016 (USDA Vegetables 2016 Summary, 2017). Given the quantity and wide distribution of produce across the U.S., microbial contamination of produce and associated foodborne illness is a major threat to public health. Across 17 different agricultural commodities, produce alone are responsible

for 2.2 million illnesses per year, or 22% of all foodborne outbreaks – the most among all U.S. agricultural commodities (Painter et al., 2013). Clearly, despite efforts of many researchers and food safety regulators, there still remain significant challenges in insuring produce is safe to consume (Havelaar et al., 2010). Accurate near real-time, same-day (or same-shift) detection and viable culture confirmation of bacterial organisms is currently a capability gap in the produce processing industry. Consequently, countermeasures and mitigation efforts to control foodborne outbreaks can be delayed with subsequent loss of control with respect to the transmission of the microorganism(s) of concern. By leveraging detection platforms for novel culture confirmation methodologies, and optimizing these emerging technologies, industry regulators and public health investigators would have the capability to rapidly, i.e. same-day, diagnose the presence of select bacterial organisms of public health concern and, subsequently, promulgate appropriate countermeasures to mitigate foodborne outbreaks before they occur.

There are many bacterial organisms of public health relevance that can be addressed. This dissertation focuses on *Salmonella enterica* serovar Typhimurium (*Salmonella*), a leading cause of foodborne bacterial enteritis outbreaks from the produce processing industry (HERMAN et al., 2015). This dissertation utilized both a Biosafety Level 1 and Biosafety Level 2 strain of *Salmonella*, ATCC 53647 and ATCC 14028, respectfully. Ten negative bacterial controls were also used, and will be addressed in Chapters 3 and 4 of this dissertation. Utilization of a Biosafety Level 1 *Salmonella* was critical to this project as much of the research was performed outside of a biosafety containment hood. Current isolation, detection, and culturing confirmation for viable bacterial pathogens, such as *Salmonella*, employed by the produce processing industry are time consuming, taking upwards of 3-4

days, and do not provide public health officials or industry regulators sufficient indications or warning to appropriately mitigate recall events (Gil et al., 2009; Hyde et al., 2016).

Hazard analysis and critical control point (HACCP) programs serve as the pathogen control structure at most, if not all, produce processing facilities (Mortimore, 2001). The HACCP structure has eight principles for plan implementation: 1) Describe the product and intended use, 2) Construct and validate a process flow diagram, 3) Identify hazards and control measures, 4) Identify critical control points (CCPs), 5) Establish critical limits, 6) Identify monitoring procedures, 7) Establish corrective action procedures, and 8) Validate the HACCP plan. There are many CCPs that can still be improved by analysis to better address public health threats along the farm-to-table chain, e.g. irrigation water, food handling, storage, etc. This dissertation addresses HACCP capability gaps in detecting bacterial pathogens from waters used in the produce processing and washing sector and focuses on optimizing an emerging technology for same-day culture confirmation. The specific aims of this dissertation were as follows:

- Aim 1) analyze current challenges posed by real-world produce wash waters when using quantitative polymerase chain reaction (qPCR) for direct detection of *Salmonella* deoxyribose nucleic acid (DNA) in complex, real-world produce wash matrices.
- Aim 2) evaluate efficacy and applicability for in-droplet microfluidic use of pre-enrichment and selective medias identified by the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) for *Salmonella* to limit or suppress non-*Salmonella* bacterial species (Zhang et al., 2011).

- Aim 3) optimize and show a proof-of-concept of a fluorescein- (FITC)-labeled antibody specific to the common surface antigen (CSA) of *Salmonella* for culture confirmation of *Salmonella* directly from complex produce wash water matrices using droplet microfluidics. Successful culture results would have the capacity to be microscopically confirmed within 4-8 hours.

Currently, utilizing FDA BAM enrichment and culturing techniques, isolating and confirming a viable culture of *Salmonella* from food samples takes at least 72 hours, when following standardized techniques. This dissertation focuses on optimizing and pairing current methods with microfluidic technologies, and has the potential to drastically reduce viable culturing time by eliminating traditional pre-enrichment and enrichment time of 24-48 hours, and replacing the plating confirmation time of 48 hours with an in-droplet activated fluorescent step only needing a 4-8 hour incubation period. Rapid isolation and detection, with a live culture confirmation capability of approximately 4-8 hours, has the potential to significantly reduce bacterial outbreak occurrence in the U.S. and decrease the overall number of recall events tracing back to the ready-to-eat vegetable industry (Painter et al., 2013; Sivapalasingam et al., 2004). The potential for reduction exists due to the reduced time to confirm a viable pathogen present, thus allowing the target bacterial pathogen(s) could be detected before contaminated produce is shipped from the facility to the consumer.

Rapid identification assays and culture confirmation are essential to provide the needed indications and warning to mitigate, or even prevent, foodborne outbreaks that cause bacterial gastroenteritis – an ongoing threat to public health world-wide (Havelaar et al.,

2015). Examples of current non-culture, rapid methods used for bacterial detection in food are immunoassays, such as immune-analytical systems paired with optics (Jeon et al., 2014), PCR-based assays (will be covered in this chapter), and biosensors (Priyanka et al., 2016). Biosensor technology is an exciting field for food safety professionals, and continues to show great promise moving forward (Arora et al., 2011). For example, the work of Li et al. developing a phage-based biosensor for the direct detection of *Salmonella* on fresh produce is a platform that can be used at multiple stages of a HACCP program (Li et al., 2010).

This dissertation optimized and developed new on-chip microfluidic methods to achieve same-day microbial detection and culturing in a produce wash water acquired from a major Mid-Atlantic fresh produce processing facility. Droplet microfluidics utilizes an on-chip droplet formation device that is potentially capable of direct separation, isolation, enumeration and/or sorting of viable microbial pathogens directly from complex environmental water matrices, such as ready-to-eat vegetable wash waters (Bridle et al., 2014; Sakamoto et al., 2007; Yoon and Kim, 2012). The novel microfluidic culturing method presented in this dissertation could significantly advance sorting, enumeration, isolation and culturing identification of bacterial pathogens in produce wash waters, and other complex environmental water matrices. Moreover, this novel microfluidic culturing technique, coupled with specific fluorescein isothiocyanate (FITC)-labeled antibody signatures for timely and accurate identification, has the potential to confirm viable bacteria from contaminated waters in as little as 4-8 hours – potentially decreasing viable culturing confirmation times more than 10-fold.

Rapid and accurate identification of microorganisms, such as *Salmonella*, in produce wash waters at processing facilities enables public health officials and regulators the capability to promulgate timely employment of appropriately scaled countermeasures to mitigate potential recalls and outbreaks (Hoorfar, 2011). These capabilities also have the potential to develop methods for rapidly identifying non-viable bacterial organisms not requiring an elevated level of response, allowing the promulgation of scaled implementation of countermeasures corresponding to a lower readiness state. Produce processing facilities inherently have a high risk of cross-contamination due to the wash water processing used, particularly the reuse of the water and the potential deactivation of chlorine by organic loads (Weng et al., 2016). Produce wash waters are a CCP where rapid methods of bacterial detections can and should be implemented, as there can be lost chlorine residual, improper cleaning of equipment, poor produce to water ratios, and water quality issues of input water (Holvoet et al., 2012).

Methodologies utilized in this dissertation were evaluated for efficiency, independently and in tandem, for near real-time identification of nucleic acid and viable culture confirmation of *Salmonella* in real-world produce wash water matrices. By using the proof-of-concept research carried out in this project, methods for rapid enumeration/sorting, isolation, detection, and viable culturing of microorganisms can be vastly improved by engineering automated systems. This research focused on assessing the utility of these isolation, detection, and culturing methodologies for rapid, automated, and in-line produce processing facilities. Moreover, these novel capabilities have utility outside the produce processing industry, such as with deploying military units, public health investigation teams, and field-based environmental research worldwide. The three aims described in this dissertation are applicable not only to produce wash waters, but also have the potential to

advance the field of environmental biodetection. This dissertation identified short-comings of qPCR direct detection. This research also optimized separation and culture methods for rapid, same-day viable culturing confirmation of bacteria using in-droplet microfluidics paired with fluorescently-labeled antibody identification.

Molecular methods, such as qPCR, are a highly effective tool in detecting pathogen targets, such as *Salmonella*, with high specificity and relatively high sensitivity in food and water (Ibrahim et al., 2014). PCR methods are well-established, and utilize primers that amplify specific, complimentary genetic sequences on the bacterial pathogen genome. Recently, Murphy et al. evaluated an improved FDA method using qPCR for detecting *Cyclospora cayatanensis* in produce (Murphy et al., 2017). Their findings confirmed the addition of 0.1% Alconox produce wash solution can improve detection, and thus streamline surveillance, response and detection in produce foods. Earlier this year Abakpa et al. usedd PCR to better understand the distribution and routes of transmission of *E. coli* O157:H7 using DNA fingerprinting analysis in the produce growing region of Kano and Plateau States, Nigeria (Abakpa et al., 2017).

There are clear advantages to utilizing PCR methods of detection over traditional culturing detection methods. The primary advantages relatable to this capability are specificity and time. If reliable primers are used and there is enough DNA template available to amply, a researcher or regulatory scientist can confirm or deny the presence of a select bacterial pathogen of interest in as few as 1-3 hours. However, there are a number of hurdles and disadvantages to utilizing PCR methods for rapid, same-day detection of bacterial pathogens. Chief among them is isolating enough template DNA of the bacterial



pathogen of interest in a highly dilute sample. If contaminated, agricultural wash waters and other environmental waters are likely to contain highly dilute amounts of the bacterial pathogen of interest. Therefore, some type of concentration method up front, such as filtration, will be necessary. Also, detecting the DNA of a target pathogen using PCR does not necessarily equate to the presence of a viable, pathogenic bacterial organism (Bonilauri et al., 2016). It simply means there is DNA from the pathogen of interest in the sample.

For purposes of uniformity and variable isolation in this dissertation, a known amount of *Salmonella* DNA was spiked into real-world wash waters. It is important to note the methods used here are translatable to direct detection of a highly contaminated sample only, and extensive filtration, purification, and/or culturing would be necessary in a highly dilute sample. This dissertation utilized seven real-world wash waters, stratified by four physiochemical variables, which were evaluated for the propensity to cause qPCR inhibition. In a series of experiments, DNA amplification and qPCR efficiency was the metric used to assess inhibition. The findings of these experiments showed the challenges researchers and regulatory scientists face even when working with a highly contaminated sample where direct detection can be employed. Also, based on a review of peer-reviewed research and publications, this is the first known study to quantify qPCR inhibition from real-world agricultural wash waters and correlate the degree of qPCR inhibition to specific physiochemical characteristics.

This dissertation culminates with the use of activated fluorescence in-droplet with the use of microfluidics, and reports the encapsulation of a single *Salmonella* cell for isolation, detection, and confirmation. While this dissertation pairs novel applications of in-droplet

microfluidics with already established methods, there are many diverse applications of microfluidics. From a waterborne pathogen standpoint, microfluidics is used for sample processing and detection (Bridle et al., 2014). Balasubramanian et al. show great utility in using electrophoretic transport and electrostatic trapping in a microfluidic chip for sample processing for MS2 in potable bottled water (Balasubramanian et al., 2007). Agrawal et al. used quantum dots in an immunosensor microfluidic chip to detect both *E. coli* and *Salmonella* from large water volumes (Agrawal et al., 2012). Lay et al. devised a raindrop bypass filter to process *Cryptosporidium* and *Giardia*, and despite clogging issues, were able to achieve successful capture by leveraging a novel raindrop bypass architecture (Lay et al., 2008). The previous studies show the utility of using microfluidic technology for sample processing uses, but microfluidics have also shown great capabilities for detection of viral, bacterial, and protozoal waterborne pathogens. Connelly et al. performed microfluidic pre-concentration of feline calicivirus in water samples leveraging a liposome-based microfluidic detector (Connelly et al., 2012). You et al. developed a handheld lab-on-chip device to detect *E. coli* O157:H7 using optical particle immunoagglutination from a laboratory simulated iceberg lettuce wash (You et al., 2011). Angus et al. developed a field-deployable microfluidic biosensor for detection of *Cryptosporidium* in field water samples (Angus et al., 2012). The previously listed microfluidic detection research, particularly You et al., is much-like the research undertaken in this dissertation. However, this dissertation isolates a single *Salmonella* bacterial cell and subsequently performs growth incubation with a FITC-conjugated antibody specific to *Salmonella*'s common surface antigen. This allows for bacterial separation from the sample and rapid culture confirmation.

As mentioned earlier, the FDA BAM identifies specific steps to take when identifying *Salmonella* in the food industry, and an essential step in traditional culture confirmation of *Salmonella* is selective enrichment. Samples taken from food or water consist of a mixed culture, resulting in a mixture of both benign and pathogenic bacteria – not all of which are *Salmonella*. By performing selective enrichment, with specific nutritional components, pH, and appropriate temperature to encourage only *Salmonella* growth, the investigator is able to discourage the growth of non-*Salmonella* bacterial species (Busse, 1995). Optimizing the media used to support growth of *Salmonella*, and suppress the growth of unwanted bacteria, in microfluidic droplets was essential to ensure maximum, detectable growth of *Salmonella*. As covered briefly before, generation time and the corresponding fluorescent signatures of the encapsulated *Salmonella* was essential in specifically determining the presence of *Salmonella*. Per the FDA BAM, there are a number of growth mediums for *Salmonella* commercially available.

In this dissertation, a pre-enrichment, selective, and universal media for *Salmonella* was evaluated for in-droplet use. Bacterial counts from droplets formed on-chip were performed in TSB, a universal growth media, at pre-designated time intervals following standard laboratory techniques and the FDA BAM. This was initially carried out, before exploring a selective media, to ensure growth in-droplet can be achieved. While this dissertation examines droplet microreactors for highly sensitive enumeration microscopy, this work is a proof-of-concept for a rapid viability confirmation capability that can eventually be used for rapid fluorescent quantitation and potentially sorting in future studies. Specific in-droplet growth, by leveraging a selective broth, is only the first step in developing a methodology for *Salmonella* isolation and detection. This dissertation explored the effects of temperature and

length of incubation to maximize *Salmonella* droplet growth, and the resulting fluorescent signature creating a reliable and detectable signal. This dissertation also demonstrated the auto fluorescence capacity of TSB, a universal growth media for a number of bacterial species, and the undesirable signal this auto fluorescence created in-droplet at low fluorescently-labeled antibody concentrations. Moreover, this dissertation presents data supporting a fluorescence-quenching property of the Rappaport-Vassiliadis (RV) broth that is likely due to low pH effects on the FITC fluorophore. The suspected pH-quenching property of RV broth was overcome by using a 10-fold increase in available FITC-labeled antibody. Both the auto-fluorescence of TSB and fluorescence-quenching properties of RV broth were unexpected experimental hurdles, and should prove useful to researchers and industrial scientists moving forward with fluorescent-based detection methods utilizing culturing methods in microfluidic droplets.

It is necessary to mention the use of a proprietary carrier oil that is capable of maintaining an aerobic environment in the droplets. Of note, this dissertation utilizes Dr. Mazutis', of the Experimental Soft Condensed Matter Group at Harvard University, proprietary fluorinated carrier oil that maximizes oxygen transfer into the on-chip droplets (Mazutis et al., 2013). This dissertation utilizes this proprietary fluorinated carrier oil to maximize bacterial growth in droplets formed on-chip. With all bacterial growth, lag and exponential growth phases play an integral role in culture confirmation and can be affected by many factors, and in this study there needed to be an appropriate count of *Salmonella* per droplet to achieve successful detection (Broughall et al., 1983). *Salmonella* was expected to perform well in-droplet for this dissertation as *Salmonella* has proven to thrive in austere conditions and retain virulence and, more importantly, grow in a range of environments

(Runkel et al., 2013). For our purposes, maximum growth at approximately 4-8 hours is acceptable for rapid fluorescence detection from produce wash waters; therefore, the slight lag times before exponential phase growth is not of great concern.

This dissertation evaluated samples with varying levels of *Salmonella* concentrations to best assess the culturing sensitivity of the on-chip microfluidic droplets, and ensure the encapsulation of a single *Salmonella* bacterial cell in-droplet for incubation and specific detection. This dissertation evaluated similar bacterial species and common agricultural contaminants, such as *E. coli* and other coliforms, in order to assess specificity in media evaluation trials and in-droplet incubation. In order to gain specificity for *Salmonella* viable culture confirmation in the microfluidic system, we capitalized on the selectivity of RV broth paired with fluorescently-labeled antibody specific to the common surface antigen of *Salmonella*. Statistical analysis was carried out using Microsoft Excel 2016 and STATA 11 statistical software.

Developing rapid, same-day capabilities and viable culturing methods for on-site platforms, food-processing facilities laboratories, and field investigations is a needed capability for public health regulatory teams and food industry scientists in order to prevent microbial recalls and outbreaks. It is critical to advance the field of microbial detection by developing reliable methodologies for near real-time, same-day (or same-shift) isolation, detection, and viable culturing that are highly sensitive and specific in challenging agricultural and environmental water settings. There is a clear need for technical capabilities that can be applied to bacterial organisms present in complex sample matrices, such as wash waters utilized by the produce processing industry. The dissertation presented here capitalizes on

capabilities from multiple research sectors of Johns Hopkins University, and aims to advance the field of biodetection for the exposure sciences and environmental epidemiology.

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## **Chapter 2: qPCR Inhibition and Chloramine Quencher Effectiveness in Produce Wash Waters Collected at a Major Mid-Atlantic Produce Processing Facility**

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### **2.1: ABSTRACT**

There are approximately 48 million cases of foodborne illness per year in the United States (U.S.), and these illnesses are assessed to be responsible for about 128,000 hospitalizations and 3,000 deaths per year, according to the U.S. Centers for Disease Control and Prevention (CDC). Bacterial pathogens, such as *Salmonella typhimurium* (*Salmonella*) and Shiga toxin-producing *Escherichia coli* (*E. coli*) are the leading bacterial causes of foodborne outbreaks stemming from leafy greens in the U.S., and accounted for 18% and 11%, respectively, of all foodborne outbreaks attributed to leafy green vegetables in the U.S. from 1973 to 2012. Quantitative polymerase chain reaction (qPCR) is routinely used as a tool to monitor and detect pathogens in food processing plants. This study investigated the inhibitive potential for qPCR from seven wash waters acquired from a major Mid-Atlantic ready-to-eat produce processing facility, including leafy green, onion, and tomato wash waters. Each wash water

underwent physiochemical analysis for total chloramine, turbidity, pH, and conductivity. Physiochemical properties were then plotted against qPCR total threshold cycle (Ct) inhibition. Logarithmic trend lines of plotted data were fit for *rpoD* ( $r^2 = 0.88$ ) and *siuA* ( $r^2 = 0.9$ ) gene targets, and these data suggest chloramines are responsible for the majority of qPCR inhibition in the produce wash waters tested. Chlorine quenchers, such as sodium thiosulfate (ST) and/or sodium ascorbate (SA), are effective in reducing qPCR inhibition by 79% and 51%, respectively. Findings suggest chlorine quenchers, such as ST and SA, can be utilized to reduce qPCR inhibition in direct detection methods. Moreover, this rapid quenching method can be carried out with few resources on-site for effective screening of highly contaminated samples.

## 2.2: INTRODUCTION

Each year 1 in 6 U.S. citizens become ill from consuming a contaminated food or beverage (Cody and Stretch, 2014). Bacterial pathogens, such as *E. coli*, *Campylobacter jejuni* (*Campylobacter*), *Listeria monocytogens* (*Listeria*), and *Salmonella*, are leading causes of food and water contamination in the U.S. (Cooley et al., 2014; Waage et al., 1999). *E. coli* is a commensal bacteria of the intestinal tract of humans, but the six pathotypes that cause disease are typically spread via food or water, or contact with people or animals (Gomes et al., 2016). According to the CDC, *E. coli* causes an estimated 203,000 illnesses per year, and is responsible for approximately 21 deaths. *Campylobacter* is the cause of Campylobacteriosis, generally considered to be vomiting, cramping and diarrhea, and the CDC estimates Campylobacteriosis infects between 1.3 million and 850,000 people in the U.S. annually, and is responsible for approximately 76 deaths. Listeriosis, caused by *Listeria*, is only responsible

for an estimated 1,600 cases per year, but the CDC reports a mortality rate near 85% because Listeriosis largely affects newborns, expecting mothers, the elderly, and those that are immune suppressed. While the cases of Listeriosis are relatively rare, the dangers to the U.S.'s most vulnerable populations make *Listeria* a significant foodborne pathogen of concern. *Salmonella* is estimated to be the major U.S. bacterial foodborne pathogen with 1.2 million illnesses and 450 deaths annually (Scallan et al., 2011). *Salmonella* has a high annual incidence of 15.2 illnesses per 100,000 people (Wilken et al., 2014). A rapidly increasing concern with respect to many bacteria, is the growing threat of antibiotic resistant strains (van den Bogaard and Stobberingh, 2000). The CDC estimates 2 million people become infected with antibiotic resistant bacteria, and 23,000 of those infected die annually. Bacterial contamination in the fresh-cut produce industry is of primary concern to regulators and consumers, as contaminated produce wash waters are capable of contaminating previously uncontaminated produce during the farm-to-fork process (Doyle, 2015). Antibiotic resistant strains of both *Salmonella* and *E. coli* are increasingly being detected in fresh and ready-to-eat produce worldwide (Vital et al., 2017). Sensitive and rapid detection of antibiotic resistant strains is a public health priority, and developers should focus on novel capabilities to address detection.

The modern food system in the U.S. hardly resembles the food system of 60 years ago. For instance, the U.S. Department of Agriculture (USDA) estimates that in 2016 vegetable crop yield was valued at nearly \$13.4 billion and occupied 2.6 million acres (USDA, 2017). This “modernization” of the U.S. food industry is a direct result of agricultural industrialization over the past 60 years.(Wallinga, 2009) Agricultural industrialization, also

referred to as food industrialization, has precipitated large-scale production of single-crop (plant and animal) monocultures, increased technology in food production, and globalized the food system (Floros et al., 2010). Unfortunately, food industrialization has also negatively impacted public health and welfare, such as increasing the potential spread of contaminated foods to consumers worldwide (Stuckler and Nestle, 2012). Given the size, reach, and potential vulnerabilities (to both producer and consumer) of the food industrial complex, it was necessary to put in-place a robust apparatus that could help safe-guard food safety and protect consumers from dangerous foods.

The U.S. Food and Drug (FDA) Bacteriological Analytical Manual (BAM) is used by food industry scientists and regulators to guide decision making and planning for suspected contaminated food events. The FDA BAM is a collection of approved and preferred methods by FDA analytical laboratory scientists charged with detection of pathogens in food or cosmetics. The FDA BAM has four general guidelines and principles: 1) Food Sampling and Preparation of Sample Homogenate, 2) Microscopic Examination of Foods and Care and Use of the Microscope, 3) Aerobic Plate Count, and 4) Investigation of Food Implicated in Illness. The FDA BAM has 25 total chapters with a chapter dedicated to each of the general guidelines and principles. The remaining 21 chapters are dedicated to FDA-validated methods used by regulatory, academic, and governmental scientists to guide isolation and detection techniques for common food contaminants.

The Hazard Analysis and Critical Control Point (HACCP) structural concept was born in 1971 by Dr. Howard Bauman at Pillsbury Co., and now serves as the most widely

employed pathogen control structure in the U.S. industrial food complex (Bernard, 1998). The HACCP structure has eight principles for plan implementation: 1) Describe the product and intended use, 2) Construct and validate process flow diagrams, 3) Identify hazards and control measures, 4) Identify critical control points, 5) Establish critical limits, 6) Identify monitoring procedures, 7) Establish corrective action procedures, and 8) Validate the HACCP plan (Mortimore, 2001). A significant principle in HACCP programs for fresh-cut produce safety in the farm-to-consumer route is the pre-washing/washing of ready-to-eat vegetables at mechanized, industrial vegetable wash facilities (Holvoet et al., 2012; Unnevehr, 2000). This “wash” step occurs shortly after the vegetables arrive to a facility (López-Gálvez et al., 2009). After going through the wash protocol, these vegetables are rapidly shipped to consumers or retail destinations in their region, marketed as ready-to-eat, and assumed to be safe for consumption. However, evidenced by the Chipotle *E. coli* outbreak of 2015, these ready-to-eat vegetables can be unknowingly contaminated with bacterial pathogens. Since these vegetables are consumed raw, unlike meats that are usually cooked, the wash critical control point (CCP) is essential to protect the population from foodborne outbreaks in the farm-to-consumer chain.

Properly chlorinated produce wash water, with a lasting residual, is critical to achieve food safety (Van Haute et al., 2013). These produce wash waters must be able to inactivate pathogens commonly found transiting the farm-to-fork route, such *E. coli*, *Salmonella*, and *Listeria* (Deng et al., 2014; Ruiz-Cruz et al., 2007). Many dynamics are ongoing during the processing at produce processing facilities. Organic load and free chlorine availability are key aspects of on-line produce wash procedures (Shen et al., 2013a). As organic loads build,

more free chlorine, often in the form of sodium hypochlorite, needs to be added in order to achieve effective disinfection of vegetables being processed (Gil et al., 2009; Weng et al., 2016). For this reason, researchers have performed studies validating the need for sanitization in produce wash waters. Munther et al. carried out research studying the overall effectiveness by generating a mathematical model to better understand efficacy of chlorine, chlorine levels, cross-contamination and pathogen survival during washing procedures (Munther et al., 2015). Also, a pilot scale study evaluating chlorine efficacy for pathogen inactivation and cross-contamination was carried out by Lou et al. with the U.S. Department of Agriculture in Beltsville, MD (Luo et al., 2012).

Guidance from the U.S. Food Standards and Modernization Act (FSMA) explicitly states if an agricultural water comes in contact with food that is to be consumed, this water must be free of all detectable *E. coli*, as this organism is used as an indicator organism for fecal contamination. This research aim addresses bacteria, specifically *Salmonella*, that would be in direct contact with ready-to-eat vegetables via processing wash water. More importantly to note, FSMA directs the detectable limit for the indicator bacteria in these waters should be as low as possible. Filtration is often used to concentrate dilute bacterial samples, and reach the lowest possible detection limit per unit volume. Filtration for detection of bacteria has been used for quite some time, and membrane filtration systems will likely remain a stable technique in the practice (Grant, 1997). There are many filtration capabilities available for the detection of bacteria in waters, some examples are filtration enrichment paired with microfluidics, filtration paired with bioluminescence, and culture-

based chromogenic membrane filtration (Maheux et al., 2014; Shinozaki et al., 2016; Wu et al., 2017).

Relevant detection methods typical to wash water and agriculture are either culture- or molecular-based. Culturing techniques for isolation and detection are often paired with molecular techniques, such as qPCR. Molecular techniques, such as qPCR, are well-established and utilize specific primers, specific thermocycling conditions, and a master mix to generate logarithmic replication of the target strand of DNA. PCR product is generated through a process of DNA strand separation, primer annealing, and DNA extension. DNA concentration is then calculated by determining the threshold cycle, or Ct, of amplified DNA product compared to baseline fluorescent signal. There are some distinct advantages and disadvantages to both conventional and molecular detection. Molecular approaches, usually a PCR-based system, are fast, i.e. 1 to 3 hours depending on preparation time and cycling conditions. The primary advantage to PCR-based systems is time. However, conventional PCR-based systems cannot confirm viability of the pathogen, since only presence of the DNA is being detected. Culture methods can confirm viable and infective bacteria. Confirming the presence of a viable and infective bacterial target is the advantage of classic cultural methods. The chief draw-back to culture methods is the time involved. Traditional culturing techniques are the most time-consuming techniques used for isolation and detection of pathogens in the environment, and are the cornerstone of the FDA BAM. These processes can take up to 4-5 days, depending on the concentration of the bacteria and the physical state, i.e. injured cells (Rizzo et al., 2004). According to FDA BAM and AOAC methods for detection, culture techniques generally use a pre-enrichment step utilizing a



food source easily metabolized by the bacteria, followed by an enrichment step. This final step of enrichment utilizes broth media with a specific formulation that encourages growth of the target bacteria, but discourages the growth of unwanted bacteria that are likely present. Once enrichment is complete, selective agar plating techniques are utilized to isolate individual colonies of the target bacteria. The key aspect of selective plating involves the physical characteristics of the bacteria growth on the plates. This distinguishes target bacteria from non-target bacteria. Generally, each step of this process takes 18-24 hours, with the exception of pre-enrichment which can take 36-48 hours.

To have the potential to detect low concentrations of pathogenic bacteria in hundreds of gallons of wash water there is a need for an extended period of time to isolate the organism, likely by some type of advanced filtering technique with subsequent culturing of the live organism, and is often paired with DNA purification for the use of molecular approaches for detection of target nucleic acid. Following FDA BAM guidance, this would involve multiple enrichment steps, and finally selective plating for confirmation. This all assumes the low level of concentration of the pathogen of interest, bacteria in this example, isn't lost during filtering or simply not sampled from the hundreds of gallons of water. Overall, this live culture confirmation process would take 3-4 days minimum. Another option for scientists is to use molecular techniques. Molecular techniques, such as qPCR, are relatively rapid techniques to positively identify the presence of bacterial target DNA. However, standard qPCR techniques will not provide insight into the viability of the bacteria of concern – only providing confirmation bacterial DNA is present in the sample (Law et al., 2015). As with live culture confirmation, concentrating the sample is an important

requirement. Additionally, there is the need for more than one bacterial cell to perform molecular analysis, using qPCR. Thus, under current practices, the FSMA guidance regarding the detection of the lowest detectable limit possible is largely an aspirational goal, and there is a clear need for industry, government, and academic researchers to develop novel methods capable of detecting a single bacteria in a relatively large volume of water.

In order to address microbial contamination in water present in fresh produce processing tanks, it is necessary to understand the real-world chemical and physical obstacles presented by the wash waters. There has been many studies undertaken investigating pathogenic detection methods in simulated or synthetic wash waters (Banach et al., 2015; Jung et al., 2014; Lee et al., 2014; Petri et al., 2015). In general, these approaches utilized a known amount of plant material, ground up the material, and spiked this ground plant material to the water. This laboratory-borne synthetic wash water was then used as the water matrix for study for bacterial growth and/or detection studies. In contrast, this research used seven real-world wash waters, i.e. wash waters from a major Mid-Atlantic fresh produce processing facility (not laboratory-borne synthetic), to study molecular detection challenges using qPCR. This facility provided wash waters from their baby spinach, cabbage, celery, shredded lettuce (two separate lines), onion, and tomato processing lines. Since the physiochemical properties of these real-world wash waters varied by line, and these properties are suspected to influence bacterial deactivation and detection, the wash waters were then analyzed for certain chemical and physical properties, including chloramine concentration, turbidity, pH, and conductivity (Van Haute et al., 2013; Weng et al., 2016). Following analysis of these chemical and physical properties, dilutions of the wash water

were generated and a known concentration of purified DNA from *Salmonella* was spiked into each wash water dilution. The qPCR threshold cycle (Ct) was used as the measurement of interest in this study. The goal of this molecular detection research was not to assess if one bacterial cell of *Salmonella spp.* could be detected, but was to take the first step in investigating practical challenges and assess the amount of qPCR inhibition caused by real-world vegetable wash waters. Inhibition was calculated and statistical analysis was carried out to identify the effect of pure wash waters, and to elucidate any association with chemical and physical properties present in the representative wash waters under test.

## **2.3: MATERIALS AND METHODS**

Strain of *Salmonella spp.* and DNA Extraction:

An overnight culture of *Salmonella enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 53647) was grown from an isolated colony on a tryptic soy agar plate (bioMerieux). QIAamp DNA Mini Kit (Qaigan) was then used to extract and purify *Salmonella*. Protocols and procedures provided by Qaigan were followed without modification. A 260nm/280nm ratio measurement using the NanoDrop 2000-C (ThermoFisher Scientific) was determined to quantify DNA yield from the Qaigan spin-column extraction kit. Final DNA yield was 75 ng/uL. Extracted DNA was aliquoted and stored at -20C until use.

Primers Used:

Two separate primer sets were used in this study to target *Salmonella* sigma factor and virulence-associated gene, ropD and siiA, respectively. The forward, reverse, and product base pair size are: ropD forward 5-‘ACATGGGTATTCAGGTAATGGAAGA’-3, reverse 5-‘CAGTGCTGGTGGTATTTTCA’-3, with a 75 base pair product (Barbau-Piednoir et al., 2013); siiA forward 5-‘ACGACTGGGATATGAACGGGGAA’-3, reverse 5-‘TCGTTGTACTTGATGCTGCGGAG’-3, and has a 107 base pair product (Ben Hassena et al., 2015).

#### PCR Equipment, Reagents, and Cycling Parameters:

Applied Biosystems 7500 Fast Real-Time PCR System was used for all qPCR amplification experiments. PowerUp SYBR Green Master Mix was used according to manufacturer protocols (Applied Biosystems). Reaction volumes per well were 20 uL consisting of 10 uL PowerUp SYBR 2x master mix, 1 uL of 16 uM forward primer (800 nM/20 uL reaction volume), 1 uL of 16 uM reverse primer (800 nM/20 uL reaction volume), and 8 uL of wash water dilution under evaluation or the control PCR-grade water. The DNA concentration used was 30 pg/uL in the final 20 uL solution volume of SYBR Green master mix, primers, and wash water or PCR-grade water control. Real-time cycling conditions were run on the fast setting and cycling conditions were as follows: 2 minute hold for UDG activation at 50C, 2 minute hold for Dual-Lock DNA polymerase at 95C, 40-45x cycles of 3 seconds denature at 95C and 30 seconds anneal/extend at 60C.

#### Wash Water Collection and Characterization:

Seven real-world, industrial agricultural wash waters were acquired at a major Mid-Atlantic fresh produce processing wash facility. Wash water samples were collected for baby spinach, shredded lettuce (two separate lines), onion, cabbage, tomato, and celery lines. Negative controls were performed in parallel with each experiment to ensure collected wash water samples were not contaminated with the target organism DNA used for assessment of inhibition. No contamination was detected (data not shown).

#### Wash Water Characterization:

Collected wash waters for all vegetable samples were analyzed for free chlorine and organic chloramines by the N,N-diethyl-*p*-phenylene diamine colorimetric method (APHA-AWWA-WEF, 2012). Turbidity, conductivity, and pH were also measured by Hach 2100N Turbidimeter (Hach Company, CO), Hach Sension5 portable conductivity meter (Hach Company, CO) and accutupH<sup>+</sup> probe (ThermoFisher Scientific), respectively.

#### Artificially Spiked Wash Waters and Wash Water Dilutions:

Wash water dilutions were spiked with 0.075 ng/uL *Salmonella* DNA. DNA dilutions were made from stocks at a concentration of 75 ng/uL, and these dilutions were made in either wash water or PCR-grade water control. The final DNA concentration was 600 pg per 20 ul reaction volume. Wash water dilutions were prepared to assess the inhibitory effect of these wash waters on qPCR efficiency. The following wash water dilutions spiked with purified DNA were prepared: 1:1 (pure wash water), 1:2 (1 part wash water – 1 part PCR-grade nuclease-free water), 1:4 (1 part wash water – 3 parts PCR-grade nuclease-free water), and

1:8 (1 part wash water – 7 parts PCR-grade nuclease-free water). PCR-grade nuclease-free water was used to provide a baseline control without inhibition.

Chlorine Quenchers Used:

Sodium thiosulfate (ST), sodium ascorbate (SA), and an equal mixture of SA and ST (SA/ST) were utilized. The molar concentration of both quenchers and the equal mixture were 1 mM. These quenchers were utilized to assess their applicability in quenching the inhibitory effect from pure wash water. These quenchers were also assessed in PCR-grade water to assess if they alone had an inhibitory effect on qPCR.

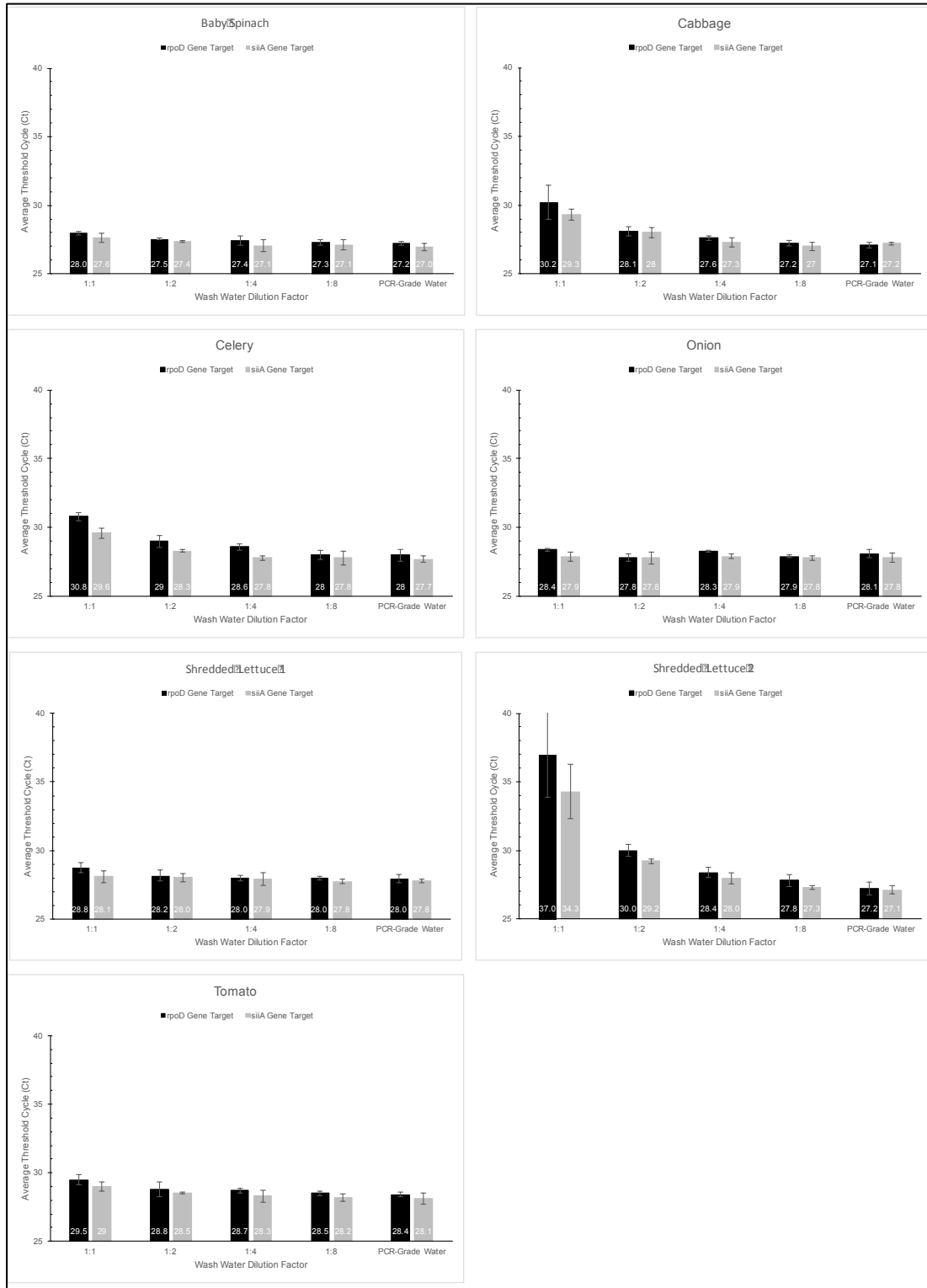
## **2.4: RESULTS**

Inhibition of Collected Wash Waters:

All seven agricultural wash waters exhibited some level of inhibition. The degree of inhibition ranged from approximately 1 to 10 Ct cycles, with one of the shredded lettuce lines being the most inhibitory to qPCR – shredded lettuce line 2 (Figure 2.1; Table 2.1). Using a constant target DNA concentration of 0.075 ng/ml (600 pg per 20 uL qPCR reaction volume) in each sample allowed for the accurate assessment of the contribution a respective wash water had to qPCR inhibition. Moreover, the dilution methodology systematically diluted out the inhibitors present in the respective wash waters. These data indicate organic chloramines present in the wash waters contributed the most to overall inhibition of qPCR (Figure 2.2). Once the wash waters were diluted with PCR-grade water, at gradually increasing concentrations up to pure PCR-grade water, the inhibition was

effectively ablated (Figure 2.1; Table 2.1). These data also indicate non-chloramine-related physical and chemical properties, such as pH, turbidity, and conductivity, do not substantially contribute to qPCR inhibition (Figure 2.2).

Figure 2.1 displays qPCR inhibition data for each of the seven vegetable wash waters, and stratifies by dilution factor. The dilutions range from extracted DNA spiked into pure wash water to the extracted DNA spiked in PCR grade water. DNA concentrations were kept constant across all dilutions at 600 pg per 20 uL reaction volume. The dilution factor listed along each x-axis are as follows, and identified in the Figure 1 title: 1:1 (pure wash water), 1:2 (50% wash water/50% PCR grade water), 1:4 (25% wash water/75% PCR grade water), 1:8 (12.5% wash water/87.5% PCR grade water), and PCR-grade water.



**Figure 2.1:** Levels of qPCR inhibition from the produce wash waters. Produce wash water dilutions with pure wash water (1:1), 1:2, 1:4, and 1:8 ratios were evaluated. PCR-grade water served as the control for comparison. Two molecular targets for *Salmonella*, rpoD and siiA, were used with a consistent DNA concentration of 600 pg per 20ul reaction volume. Each ran in triplicate and error bars represent 95% confidence intervals.



Statistical analysis of the qPCR data was carried out by generating 95% confidence intervals (CIs), stratified by dilution factor. Each wash water dilution CI was evaluated against the standard, which was DNA spiked into PCR-Grade water (Table 2.1). It is important to note qPCR Ct values will have slight inherent variation. When analyzing the CIs, under a normal distribution, non-overlapping CIs indicate statistical significance approximating a p-value  $\leq 0.05$ . Moreover, in this analysis, CIs that overlap by less than 1/100<sup>th</sup> of a decimal point are highlighted as nearly significant (*italics only*), but not listed as statistically significant. Statistically significant CIs are shown in bold and italics, and are annotated in the Table 2.1 legend. Regarding overlapping and non-overlapping 95% CIs, all CI dilutions are compared to the CI standard of PCR-grade water.

**Table 2.1:** Summary of Means and 95% Confidence Intervals by Dilution Factor for Wash Waters and Gene Target Under Test. For statistical analysis, all dilutions were compared to the standard of PCR-grade water.<sup>a,b</sup>

Wash Water Type	Dilution Factor	ropD Mean (n=3)	ropD 95% Conf. Interval	siiA Mean (n=3)	siiA 95% Conf. Interval		
<b>Baby Spinach</b>	1:1	<b>27.97</b>	<b>27.68</b>	<b>28.25</b>	27.63	26.87	28.39
	1:2	27.33	26.39	28.27	27.37	27.22	27.51
	1:4	27.43	26.63	28.23	27.07	26.06	28.07
	1:8	27.30	26.87	27.73	27.13	26.33	27.93
	Standard	27.23	26.95	27.52	26.97	26.34	27.59
<b>Cabbage</b>	1:1	<b>30.23</b>	<b>27.50</b>	<b>32.97</b>	<b>29.27</b>	<b>28.39</b>	<b>30.14</b>
	1:2	28.07	27.31	28.83	28.00	27.25	28.75
	1:4	27.57	27.19	27.95	27.27	26.55	27.98
	1:8	27.20	25.93	28.47	26.97	26.25	27.68
	Standard	27.10	26.67	27.53	27.20	26.95	27.45
<b>Celery</b>	1:1	<b>30.83</b>	<b>30.21</b>	<b>31.46</b>	<b>29.63</b>	<b>28.83</b>	<b>30.43</b>
	1:2	29.03	28.09	29.97	28.30	28.05	28.55
	1:4	28.63	28.12	29.15	28.07	27.69	28.45
	1:8	28.03	27.32	28.75	27.77	26.65	28.89
	Standard	27.97	27.03	28.91	27.70	27.20	28.20
<b>Shredded Lettuce 1</b>	1:1	28.77	27.97	29.57	28.10	27.20	29.00
	1:2	28.17	27.29	29.04	28.03	27.41	28.66
	1:4	28.00	27.57	28.43	27.93	26.93	28.94
	1:8	28.00	27.75	28.25	27.77	27.39	28.15
	Standard	27.97	27.25	28.68	27.80	27.55	28.05
<b>Shredded Lettuce 2</b>	1:1	<b>37.00</b>	<b>30.14</b>	<b>43.86</b>	<b>34.30</b>	<b>29.95</b>	<b>38.65</b>
	1:2	<b>30.00</b>	<b>29.01</b>	<b>30.99</b>	<b>29.23</b>	<b>28.85</b>	<b>29.61</b>
	1:4	28.40	27.65	29.15	27.97	27.03	28.91
	1:8	27.80	27.55	28.05	27.30	27.05	27.55
	Standard	27.23	26.23	28.24	27.10	26.44	27.76
<b>Onion</b>	1:1	28.37	27.99	28.75	27.93	27.17	28.69
	1:2	27.83	27.26	28.41	27.80	26.81	28.79
	1:4	28.33	28.19	28.48	27.83	27.21	28.46
	1:8	27.90	27.65	28.15	27.77	27.39	28.15
	Standard	27.93	27.79	28.08	27.77	27.05	28.48
<b>Tomato</b>	1:1	29.53	28.73	30.33	29.00	28.25	29.75
	1:2	28.80	27.66	29.94	28.47	28.32	28.61
	1:4	28.70	28.27	29.13	28.27	27.26	29.27
	1:8	28.53	28.15	28.91	28.23	27.61	28.86
	Standard	28.43	28.05	28.81	28.07	27.19	28.94

a: Bold and Italicized Values Indicate Statistical Significance

b: Italicized Values Indicate Nearly Statistically Significant

Following CI analysis, an analysis of variance (ANOVA) was performed on the dilution dataset in order to assess if the means across the five dilutions groups and two primers, stratified by wash water type (10 total), were statistically significantly different. ANOVA analysis, without further correction or adjustment, indicates each wash water type had statistically significant differences in the means for 7 of the 14 groups (Table 2.2).

ANOVA alone can be misleading when addressing the entire group and lacks the capability to fill information gaps regarding which dilution group mean(s) are contributing to the overall statistical significance in ANOVA. Therefore, a Bonferroni correction was performed in order to isolate mean(s) within groups responsible for the overall statistical significance concluded by performing ANOVA (Table 2.2).

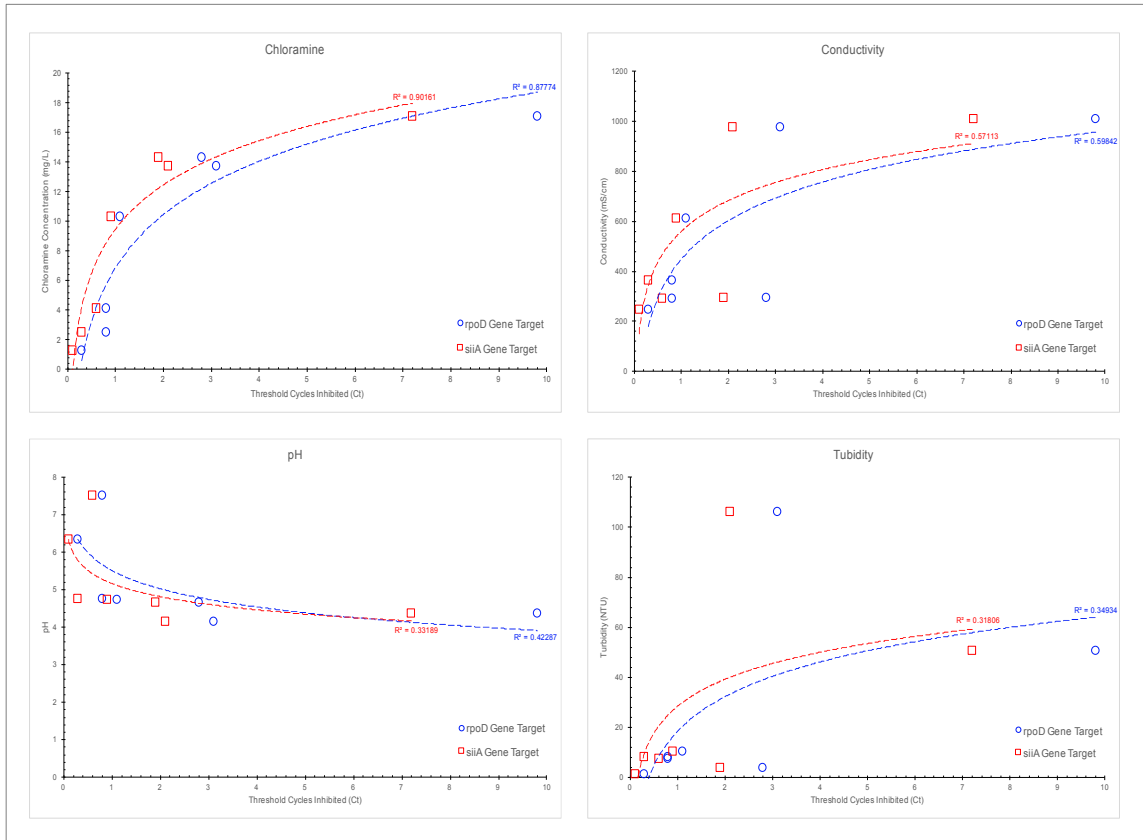
**Table 2.2:** ANOVA and Bonferroni Analysis Identifying Dilution Influencing F-Statistic Significance

<b>Wash Water Sample and Gene Target</b>	<b>ANOVA of Dilution Means (F-Statistic, 0.05)</b>	<b>Dilution Influencing Significance (Bonferroni)</b>
Baby Spinach ropD	0.03	1:1
Baby Spinach siiA	0.10	NA
Cabbage ropD	0.01	1:1
Cabbage siiA	0.01	1:1, 1:2
Celery ropD	0.01	1:1, 1:2
Celery siiA	0.01	1:1
Shredded Lettuce 1 ropD	0.02	1:1
Shredded Lettuce 1 siiA	0.55	NA
Shredded Lettuce 2 ropD	0.01	1:1
Shredded Lettuce 2 siiA	0.01	1:1
Onion ropD	0.01	1:1
Onion siiA	0.95	NA
Tomato ropD	0.01	1:1
Tomato siiA	0.03	1:1

*1:1 Pure Wash Water and 1:2 50% Wash Water*

Wash water samples were analyzed for four physiochemical properties; total chloramines, turbidity by Nephelometric Turbidity Units, pH, and conductivity. Physiochemical analysis results were then plotted against total inhibition (Figure 2.2). For each wash water sample evaluated, total inhibition is described as the difference in Ct needed

to amplify DNA between pure wash water (1:1) and PCR-grade water. Figure 2.2 displays qPCR inhibition Ct for each vegetable wash water by the four physiochemical properties analyzed.



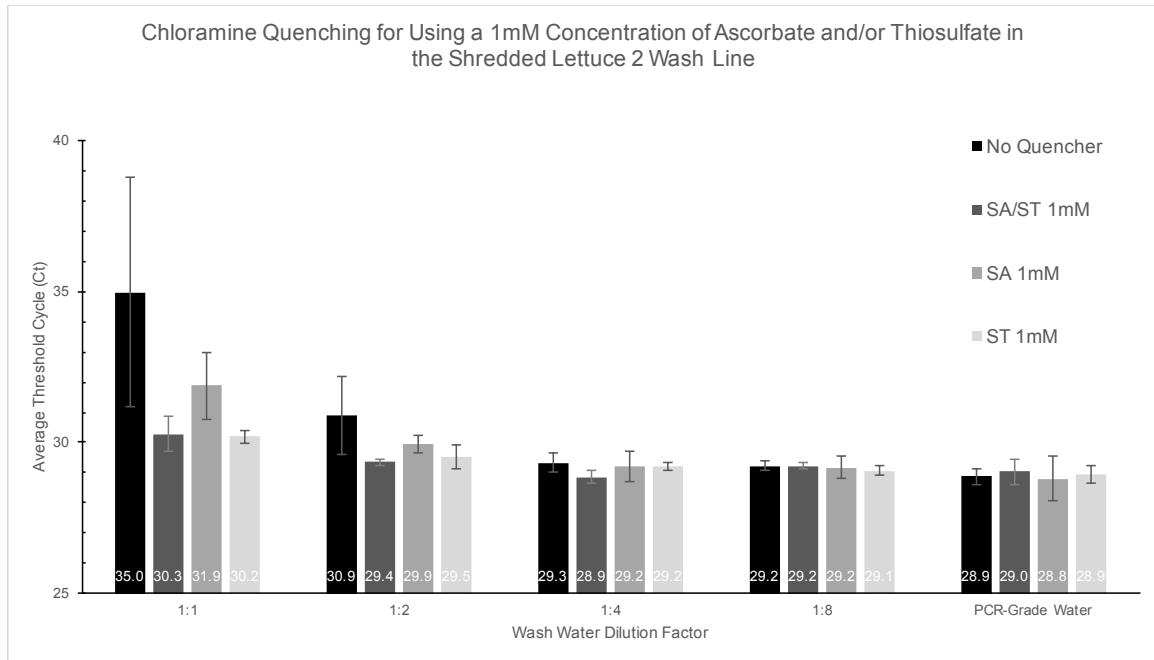
**Figure 2.2:** Scatterplot Summary of the Effect Wash Water-based Physiochemical Constituents had on Overall qPCR Inhibition

Plotted data show a relationship between total organic chloramine concentration and amount of inhibition observed (Figure 2.2). Once organic chloramines were determined to significantly contribute to overall inhibition in the wash waters, a chloramine quenching experiment using the most inhibitory wash water, Shredded Lettuce 2, was carried out. A 1 mM concentration of quencher was used to neutralize the organic chloramine concentration

present in the Shredded Lettuce 2 wash water line. In this experiment, only the *rpoD* gene target was used because this primer set exhibited the most qPCR inhibition and was considered to be the more challenging experimental condition of the two (Figure 2.1; Table 2.1). In addition to assessing qPCR inhibition removal from a real-world wash water, an additional objective of this experiment was to confirm there is not a statistically significant effect on qPCR performance, or reduced sensitivity of the qPCR, by the use of 1 mM concentrations of ST, SA, or the ST/SA mixture (Figure 2.3; Table 2.3).

**Table 2.3:** The Following Table Summarizes the Raw Ct Values Represented in Figure 3

Quencher	PCR-Grade Water	1:8	1:4	1:2	Undiluted Wash Water
None	28.73	29.27	29.57	32.1	38.85
None	28.74	29.34	29.41	30.74	32.82
None	29.14	29.09	29.03	29.85	33.25
SA/ST	28.63	29.16	28.64	29.46	30.84
SA/ST	29.07	29.2	28.96	29.27	29.84
SA/ST	29.38	29.34	28.98	29.32	30.14
SA	28.25	28.84	28.82	30.17	31.45
SA	28.63	29.23	29.69	29.66	33.01
SA	29.54	29.46	29.07	29.97	31.16
ST	28.64	29.12	29.26	29.5	30.24
ST	28.99	28.9	29.46	29.9	30.35
ST	29.16	29.16	29.42	29.21	29.99



**Figure 2.3:** Quenching of Chloramine-related Inhibition Stratified by 1mM SA, ST, and SA/ST Quenchers Analyzed Across Four Dilution Factors (1:1, 1:2, 1:4, and 1:8). PCR-Grade Water Served as the Control. All quenchers under test were ran at 1 mM (SA/ST = 0.5mM SA and 0.5mM ST). All conditions ran in triplicate.

ANOVA was performed on the quenching dataset in order to assess if the means across the dilutions statistically significantly differ, stratified by quencher type. As before, ANOVA alone can be misleading when addressing the entire group and lacks the capability to fill information gaps regarding which dilution group mean(s) are contributing to the overall statistical significance in ANOVA. Therefore, a Bonferroni correction was performed in order to isolate mean(s) within groups responsible for the overall statistical significance concluded by performing ANOVA (Table 2.4). The statistical analysis in Table 2.4 shows a clear trend of mean variation increasing from PCR-grade water to undiluted wash water, and also reinforces the quenchers used, SA/ST, SA, and ST, do not statistically inhibit qPCR sensitivity at 1 mM.

**Table 2.4:** Wash Water Dilutions with Corresponding F-statistic Indicating an Overall Difference in Means. The Bonferroni Correction Identifies the Variables Causing an Overall Statistically Significant, or nearly Statistically significant, Finding

Dilution	F-Statistic	Bonferroni Correction by Quencher
PCR-Grade Water	0.93	None
1:8	0.66	None
1:4	0.19	None
1:2	0.06	No Quench-SA/ST
Undiluted Wash Water	0.03	No Quench-ST

## 2.5: DISCUSSION

This study is the first to evaluate attributable factors to qPCR inhibition from real-world vegetable wash waters, and aids in better understanding the challenges posed by these complicated agricultural water matrices. There was one published finding that attributed its qPCR inhibition to a chlorine compound. Buttner et al.'s work investigating the decontamination efficacy of chloride dioxide gas against residual *Bacillus subtilis* (a surrogate for *Bacillus anthracis*) remaining on furniture after a bioterrorism attack in the workplace (Buttner et al., 2004). A few other peer-reviewed articles listed inhibition, but referred to it as undetermined.

These data suggest organic chloramines are the primary constituent influencing qPCR inhibition when employing direct detection techniques against wash waters (Figure 2.1 and 2.2; Table 2.2). These data also indicate there is potential for other constituent factors, such as pH or conductivity, to explain at least some of the wash water inhibitive potential (Figure 2.2). Chloramine concentration appears to have a logarithmic relationship with overall inhibition, having more of an impact on qPCR inhibition at 1-10 mg/L (Figure 2.2).

Data indicated overall inhibition plateaus at or above 10 mg/L chloramines present in the wash water (Figure 2.2). In both chemical and biological detection contexts, it is common for researchers to quench available chlorine in water samples (Shen et al., 2013b). Quenchers used are generally a sulfate compound, such as sodium thiosulfate, or compounds such as ascorbic acid (or sodium ascorbate) (Zhang et al., 2015). However, the effect these quenchers have on qPCR analysis for direct detection has not been explored to a great extent. In addition to identifying the primary constituent causing qPCR inhibition (Figure 2.2), this study indicates relatively high molar concentrations (1 mM) of either sodium thiosulfate or sodium ascorbate do not inhibit qPCR (Figure 2.3). Application of these quenchers to the most challenging wash water, shredded lettuce 2 line, suggests these quenching methods are effective and should be included in a qPCR direct detection protocol for chlorinated waters, without compromising qPCR sensitivity.

A published finding specifically addressed ST not inhibiting qPCR in developing an internal control and evaluation standard for qPCR assays to detect *Helicobacter pylori* in drinking water (Sen et al., 2007). Sen et al. used a chlorine concentration of 1.5 to 0.86 mg/L and a ST concentration of 0.1% to 1%. Here, chlorine quenchers show promise in drastically reducing qPCR inhibition for direct detection. The trends for inhibition reduction were statistically significant per confidence interval analysis across the quencher variables and stratified by dilution factor (Figure 2.3). ANOVA analysis was carried out on the groups of means across the three quencher variables under test, ST, SA, and a ST/SA mixture, when added to a wash water that had been found to be inhibitory to qPCR (Figure 2.1; Table 2.1). The groups of Ct means amplified from the pure wash water were statistically significantly



different, and statistical significance decreased (F-statistic increase) as the wash water became more dilute (Table 2.4). Regarding pure wash water, addition of 1 mM of ST alone and ST/SA reduced qPCR inhibition by 79%, and addition of SA alone reduced qPCR inhibition by 51% (Figure 2.3; Table 2.3). Moreover, statistical analysis using ANOVA, with an F-statistic of 0.93, strongly suggests there is no statistically significant difference between purified DNA amplification in PCR-grade water or purified DNA in PCR-grade water with a quencher added (Table 2.4). This is an important finding as it suggests using quenchers at a 1 mM concentration will not inhibit or reduce the sensitivity of qPCR analysis, and is the first time this has been reported for SA.

These quenchers could be quite useful for both rapid laboratory, lab-on-chip applications, or potentially on-site mobile qPCR platforms where performing direct detection in chlorinated waters would necessitate nearby laboratory resources and/or a mobile platform with ease of use (Chin et al., 2017; Tourlousse et al., 2012). Even molecular scientists not utilizing mobile qPCR technologies could potentially make use of these quencher applications. For instance, Di Cesar et al. recently noted residual chlorine levels prevented certain aspects of their work with bacterial communities and antibiotic resistant genes in urban waste waters (Di Cesare et al., 2016). By quenching the chlorinated wash water, or any applicable chlorinated water, on-site research scientists and standards regulators could rapidly test for the presence of molecular markers originating from their target organism. There are limitations to carrying out qPCR methods on-site when compared to laboratory. For instance, the sample taken would need to be highly contaminated to ensure there's enough genetic material present in order to gather reliable

data and amplification. If the target organism is present at low concentrations, a rapid filtration and concentration step would need to be carried out on-site. It is likely if the target organism is present at very low concentrations, this method of on-site quenching, with or without filtration and concentration, and follow-on qPCR detection is not feasible. Consequently, conventional microbiological culturing methods would be more feasible. These methods would include pre-enrichment and selective enrichment for the target bacteria, followed by either DNA extraction for laboratory-based qPCR for molecular detection and/or culture techniques using selective agar plates for the target organism.

There are three key items with respect to these data: 1) Chloramines present in fresh-cut produce wash waters were found to significantly contribute to qPCR inhibition; 2) There is no significant reduction in qPCR sensitivity by using SA/ST, SA, or ST at a 1 mM concentration; and 3) Bonferroni analysis suggests either SA/ST or ST alone perform well in reducing the inhibitory effects of organic chloramines in qPCR direct detection.

## **2.6: CONCLUSION**

Pathogenic contamination by bacteria, such as *Salmonella*, in agricultural and environmental waters is a significant threat to public health and will remain a chief concern to industrial, local, state, and federal stakeholders (Craun et al., 2010; Doyle et al., 2015; Scallan et al., 2011). There are many CCPs within HACCP programs for the ready-to-eat vegetable industry including harvesting, handling, large batch washing, and distribution to the consumer (Hyde et al., 2016; Panisello et al., 2000). This research highlights obstacles in direct molecular detection using qPCR at the wash water CCP. More significantly, by

measuring qPCR inhibition of real-world wash waters and analyzing these wash waters for physiochemical properties precipitating inhibition, organic chloramines were found to be responsible for the majority of qPCR inhibition and reduction in amplification sensitivity. Common chlorine quenchers, such as ST and SA, were found to be effective in chloramine-related qPCR inhibition reduction and did not impair annealing and extension of the amplicon at concentrations of 1 mM. Currently, the standard for same-day detection, if feasible, is molecular-based qPCR detection of the target organism. Depending on concentration of the pathogen present, these same-day qPCR methods can potentially sample and measure directly, or following some type of concentration or filtering. However, there are many limitations to carrying out qPCR direct detection methods on matrices, such as vegetable wash waters. For instance, the target bacteria would likely be highly dilute in the sample and more traditional culturing methods advocated by the FDA BAM would be necessary in order to achieve high enough cell counts suitable for bacterial DNA extraction and subsequent detection by way of qPCR. There are some promising same-day detection capabilities on the forefront of agricultural industry pathogen regulation and bacterial detection research for agricultural and environmental waters. Emerging technologies for pathogenic bacteria detection in water, such as in-droplet microfluidics, show promise for same-day live culture confirmation. Future research, however, should first focus on optimizing developing and optimizing methods for rapid bacterial culturing from agricultural and environmental waters, and subsequently engineer platforms capable of being installed on-site at ready-to-eat vegetable wash facilities. Eventually, the ideal capability would merge and leverage rapid culture confirmation and qPCR molecular detection.

**Acknowledgements:** Dr. Shih-Chi Weng, Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health; Applied Biological Sciences Group, Johns Hopkins University Applied Physics Laboratory; ERC Grant; The Osprey Foundation of Maryland.

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# **Chapter 3: Evaluation of Rappaport-Vassiliadis Fluorescence and Use as a Selective Broth Media for Specifically Cultivating *Salmonella* in Microfluidic Droplets**

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## **3.1: ABSTRACT**

There are approximately 48 million cases of foodborne illness per year in the U.S., according to the U.S. Centers for Disease Control. *Salmonella typhimurium* (*Salmonella*) is one of the leading causes of bacterial gastroenteritis in the U.S., and has been implicated in the contamination of fresh produce. Developing methods to rapidly detect viable bacterial pathogens, such as *Salmonella*, are a priority for the U.S. and in the best interest of public health. In-droplet microfluidics is an emerging technology that can isolate a single bacterial cell and has the potential to be integrated into detection of bacteria in agricultural and environmental water. This research examined growth specificity and anti-fluorescence propensity of a selective media for *Salmonella*, Rappaport-Vassiliadis (RV), and its utility for in-droplet selection for pure culture growth and detection from a single encapsulated cell.

Tryptic soy broth (TSB) and buffered peptone water (BPW) were also evaluated. A panel of 12 bacterial species was evaluated – 2 *Salmonella* species and 10 non-*Salmonella* species. These data indicate RV is 100% specific for *Salmonella* when grown at 41.5°C, however, the growth rates are substantially reduced and would likely be problematic for rapid in-droplet microfluidic detection. Growth rates and selectivity at 37°C were also evaluated and RV completely suppressed 80% of negative controls, with only the non-specific growth of *Enterobacter* and *Citrobacter* isolates. *Salmonella* growth rates remained optimal at 37°C, and would likely support in-droplet detection capabilities. The addition of a fluorophore-conjugated antibody, in tandem with RV broth, could provide optimal growth rates for detection and achieve near 100% specificity. Fluorescence properties of medias under test was evaluated. RV broth reduced fluorescence intensity of fluorescein isothiocyanate (FITC), a common fluorophore used in laboratory research. At 0.1, 1, and 10 ug/ml concentrations in RV broth, FITC-conjugated *Salmonella* antibody consistently exhibited approximately one log<sub>10</sub>-reduction in fluorescent signal intensity when compared to TSB, BPW, and phosphate buffered saline. This reduction in fluorescence intensity likely is due to the low pH of RV broth. Also, these data indicate TSB has a high level of auto-fluorescence which must be accounted for in all fluorescent assays utilizing low fluorescently-labeled antibody concentrations.

### **3.2: INTRODUCTION**

Bacterial contamination of ready-to-eat vegetables is a significant concern for food safety scientists and regulators worldwide (Mercanoglu Taban and Halkman, 2011). *Salmonella*, and other pathogenic bacteria such as *Escherichia coli* (*E. coli*) and *Shigella* are responsible for

approximately 48 million bacterial outbreak cases in the U.S. per year (Newell et al., 2010; Scharff, 2015). According to the Food Standards and Modernization Act (FSMA) of 2011, the target for a lower limit of detection for these bacteria should be as low as possible. Ideally, detection of one organism per unit volume would be the best limit of detection. It is reasonable to assume this detection volume would be a 1 liter sample from a larger water source. Common methods for bacterial detection are traditional culture and molecular analysis. Traditional culture methods use established protocols developed by the Food and Drug Administration (FDA) and Association of Official Analytical Chemists (AOAC). Steps for both FDA and AOAC protocols include bacterial-dependent pre-enrichment, selective enrichment, and selective plating process. These conventional methods are relatively straightforward and low-cost, however, a time investment between 48-72 hours can be the limiting factor for rapid (i.e. less than one day) detection (Gracias and McKillip, 2004). Molecular analysis tools being utilized for foods include quantitative polymerase chain reaction (qPCR), quantitative reverse transcriptase polymerase chain reaction (RT-qPCR), and loop-mediated isothermal amplification (LAMP). Zheng et al. performed a comparative analysis of conventional culturing, qPCR, RT-qPCR, and LAMP methods on cilantro, lettuce, parsley, spinach, tomato, and jalapeno pepper artificially spiked with *Salmonella* (Zhang et al., 2011). The authors report that all four methods were comparable in all aspects except for time. Molecular analysis and detection was faster and equally as efficient as conventional methods. Given traditional culturing time for viable confirmation or the template DNA requirements for the lower limit of molecular detection, it is nearly impossible to achieve a detection limit of one bacterial cell per liter of sample volume in less than 12 hours (Law et al., 2015). There would need to be time investment up-front for pre-enrichment, enrichment, and/or

selective enrichment to effectively carry out culture or molecular detection at such a low concentration (Andrews and Hammack, 2001).

The U.S. vegetable industry is actively looking for approaches to improve detection limits and reduce the time needed to specifically and sensitively detect the lowest possible number of bacterial pathogens in the food-to-table processing chain (Jung et al., 2014). Limitations revolving around this issue are not trivial, and they include capability of equipment, current methods used, and the life cycle of the bacteria (Havelaar et al., 2010). Current methods used for culturing a bacterial species of interest in foodstuffs or agricultural waters involve some combination of sampling, concentrating, pre-enrichment broth, universal enrichment broth, selective broth enrichment, and plating using selective agar media (Andrews and Hammack, 2001). This process is well-established by the FDA Bacteriological Analytical Manual (BAM) for the bacterial pathogens and if appropriately implemented can reliably identify the presence or absence the viable bacteria of concern. While established and reliable, there are limiting factors with these traditional methods – most notably time. Sampling and concentrating could take as little as a few hours, however, pre-enrichment step(s), selective enrichment and selective plating can take 18 to 24 hours at each step. Pre-enrichment steps are sample dependent and will largely depend on the degree of injury the target bacteria had encountered, i.e. sanitizers, chlorine stress, level of dessication, etc. (Zheng et al., 2015). Thus, in order to accurately confirm a live culture of the bacteria of interest it could take at least 3-4 days. Due to the perishable nature of ready-to-eat vegetables, this timeline is a significant issue because vegetables are generally shipped out from industrial vegetable wash facilities to the consumer the same day they are washed

and packaged (Lynch et al., 2009). There is a clear need for reliable methods utilizing emerging technical applications to drastically shorten the time necessary to provide a live culture confirmation of bacterial contamination (Havelaar et al., 2010).

In-droplet microfluidic detection is an emerging technology with significant promise to potentially reduce the culture confirmation time from days to hours (Hamon and Hong, 2013). With respect to waterborne bacterial pathogens, microfluidics has already been utilized for sample processing and/or detection (Bridle et al., 2014). For example, microfluidics has been utilized for 3D circular lab-on-chip technologies to process water samples and paired with anti-*E. coli* or anti-*Salmonella* quantum dots for fluorescence detection (Agrawal et al., 2012). There has also been the development of a handheld direct detection device using latex microparticles conjugated with an anti-*E. coli* antibody for optical light scattering detection in lettuce samples (You et al., 2011). Microfluidics is well-established in clinical medicine for applications such as screening for antibiotic resistance and clinically relevant biomarkers, and has been described as an ideal platform for point-of-care and remote setting screening for nucleic acid and phenotypic detection of antibiotic resistance (Aroonnuan et al., 2017). In addition to samples processing and screening for antibiotic resistance, microfluidics has shown sound utility in biomarker analysis. Over the past 5-7 years, advances in microfluidic technologies have evolved in the area of sample processing to allow clinicians the capability to more rapidly analyze lipids, small molecules such as glucose, and even nucleic acid (Pagaduan et al., 2015).

In addition to using microfluidic detection, the propensity for medias to selectively culture *Salmonella* and discourage growth of non-*Salmonella* are also an important aspect of successful low titer detection. The pre-enrichment, universal enrichment, and selective enrichment media for *Salmonella* identified by the FDA BAM include buffered peptone water (BPW), tryptic soy broth (TSB), and Rappaport-Vassiliadis broth (RV), respectively (Andrews and Hammack, 2001).

*E. coli* and *Salmonella* are gram-negative *Enterobacteriaceae*, and both can be found in the gastrointestinal tract of an animal host (Winfield and Groisman, 2003). Moreover, both *Salmonella* and *E. coli* are common bacterial contaminants of agricultural and environmental waters of concern to public health (Sivapalasingam et al., 2004). *Citrobacter*, *Enterobacter*, *Proteus*, and *Shigella* are also part of the *Enterobacteriaceae* family, and exhibit some of the same characteristics as *E. coli* and *Salmonella*. For instance, gram-negative members of the *Enterobacteriaceae* family share a common lipopolysaccharide allowing targeted immunotherapy in clinical patients with sepsis (Di Padova et al., 1993).

While members of the *Enterobacteriaceae* family share many similarities, not all members of the family are categorized as coliforms. Coliforms are gram-negative, rod-shaped bacteria, capable of aerobic and facultative anaerobic growth. Coliforms possess  $\beta$ -D-galactosidase capable of cleaving lactose to generate glucose and galactose producing fermentation acid and gas at 37°C (Browne et al., 2010). Though *Citrobacter*, *Enterobacter*, *E. coli* and *Salmonella* are all members of the same phylogenetic family, *Enterobacteriaceae*, only the non-lactose fermenting *Salmonella* is not a coliform. *Salmonella*'s lack of  $\beta$ -D-galactosidase

means chromogenic medias engineered for this enzyme, that are typically used for identification of coliform *Enterobacteriaceae* members, are not effective in identifying *Salmonella*.

*Enterococcus* and *Staphylococcus* are both gram-positive bacteria found in agricultural and environmental waters. *Enterococcus* has shown high prevalence and antibiotic resistance in the farm environment and on produce in Tunisia (Ben Said et al., 2016). The researchers in Tunisia reported *Enterococcus* on over 80% of samples of vegetables from both farms and markets (Ben Said et al., 2016). *Staphylococcus* is best known for the antibiotic resistant strain, methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA has been implicated in farm settings, and found in farm workers on large livestock operations (Smith and Wardyn, 2015). These research studies highlight the importance for developing rapid, viable culturing methods.

Understanding the bacterial growth and life-cycle is critical to developing viable in-droplet detection methods. Bacterial growth is characterized by lag, logarithmic, and stationary phases. Lag phase is a complex and dynamic aspect for the life cycle and growth of bacteria. Lag, or lack of bacterial cell division and biomass growth, is theorized to be medium- and/or temperature-related and observed when bacteria are introduced to a new environment or encounter environmental variations (Dens et al., 2005a). Temperature is a key component of microbial growth, and can induce an intermediate lag phase or initiate exponential biomass growth (Dens et al., 2005b). Environmental parameters, such as temperature, pH, and water activity, have shown observable effects on both lag and

generations times in *Listeria*, particularly an increase in lag time with increased environmental stress (Francois et al., 2006). Lag times and temperature dependence are critical in understanding biomass growth in food, as food will encounter many temperatures from farm to the consumer (Sant'Ana et al., 2012a). Growth rates in the logarithmic phase are also highly dependent on environmental conditions, such as temperature. Sant'Ana et al. modeled the growth rates of *Salmonella* and *Listeria* as a function of temperature, and reported a clear relationship between increasing growth rate with an increase in temperature (Sant'Ana et al., 2012b).

Current research utilizing medias for growth in-droplet has focused on microbial detection and antibiotic resistance, but the field is still developing (Kaminski et al., 2016). Antibiotic resistance of bacterial communities has been a growing area of in-droplet research since antibiotic resistance in the food supply is on the rise (Doyle, 2015). Antibiotic resistant strains of both *Salmonella* and *E. coli* are increasingly being detected in fresh and ready-to-eat produce worldwide (Vital et al., 2017). Investigations have evaluated the presence of antibiotic resistance in *E. coli* isolates at food production systems for leafy green vegetables, including large commercial farms, small-scale farms, and homestead gardens (Jongman and Korsten, 2016). Using phenotypic analysis, researchers were able to find an antimicrobial link between irrigation water and leafy green vegetables. Keays et al. recently focused on using *E. coli* to develop a novel method of rapidly analyzing bacteria for antibiotic resistance (Keays et al., 2016). Their work focused on leveraging optical densities, along with the presence or absence of antibiotics, to increase speed and characterization of antibacterial bacterial strains present in a sample. Moreover, Keays et al. also found bacterial growth in-



droplet to be faster than that of traditional culture methods. Droplet microfluidics promote higher growth rates due to many advantages over traditional culture, such as minimal contamination, rapid mixing within droplets, precise volumes, and small volumes utilized (Churski et al., 2012). Bacterial sorting and enumeration are also an avenue of in-droplet research being pursued. Schaerli and Hollfelder identify the potential utility of using microfluidic droplets for enumeration and sorting purposes, and highlight sorting of a fluorescent compound (or bead) in a droplet as an avenue for experimental biology (Schaerli and Hollfelder, 2009). Dong et al. recently utilized chemotaxis to aid bacterial cell sorting in a two-phase in-line process (Dong et al., 2016). The group achieved sorting by leveraging chemotactic properties of *E. coli*, and directed movement of the bacteria away from a certain region of the microfluidic channel by concentrating a chemoeffector, fluorescein, on the complimentary side of the channel. Following this chemotactic concentration step, *E. coli* was cultivated and enumerated in-droplet. Their work, on both pure and mixed cultures, showed bacterial cells can be encapsulated, cultivated, enumerated, and sorted using propensity of chemotaxis between bacterial species.

Fluorescence is often adapted for use in conjunction with microfluidic technologies for rapid detection of pathogenic bacteria in water samples. Recently, researchers fielded a semi-portable microfluidic device utilizing an Alexa Fluor 488-conjugated anti-*Legionella* antibody to enumerate and detect *Legionella* in cooling tower waters (Yamaguchi et al., 2017). The samples were processed by mixing the water sample directly with the fluorescent dye suspension on-chip and counted optically via a portable high-speed camera. Results were confirmed via fluorescent microscopy. Fluorescent techniques have also been applied to

microfluidics in clinical settings. A microfluidic capillary system capable of detection bacteria in *E. coli*-spiked urine samples was designed to screen for urinary tract infection (Olanrewaju et al., 2017). Their work utilized Alexa Fluor 647-conjugated anti-*E. coli* antibodies, and counts were validated by way of fluorescent microscopy. Long-term growth analysis can also be carried out in-droplet as well. Tanouchi et al. recently observed homeostatic aspects, such as cell size and growth rate control, for *E. coli* in-droplet (Tanouchi et al., 2017). The data gathered were stratified by three temperatures, recorded over 70 generations, and measured by a yellow fluorescent protein.

In-droplet microfluidics utilizing bacterial cells encapsulated with media is an emerging technology offering a number of applications to molecular biologists, cytologists, and microbiologists (Yoon and Kim, 2012). Given its relatively new application, there is much opportunity in optimization research for applications adapting well-established microbiologic methods to miniaturized bioreactor droplets (Bridle et al., 2014). The applicability of growth medias typically used for *Salmonella* in traditional culture methods – a universal broth, pre-enrichment broth, and selective broth for *Salmonella* – should be of interest to inform researchers of the potential for these medias to be used in-droplet as growth medias for *Salmonella*, particularly a *Salmonella*-selective RV broth.

The volume of a microfluidic droplet will depend on the experimental parameters, but a typical microfluidic droplet is in the pico- to nano-liter volume range (Kaminski et al., 2016). Research by Tan et al. indicates droplet size is impacted by the resistance of the microfluidic channel and the mixing of chemical agents used in-droplet (Tan et al., 2004).

Selective media for *Salmonella* could prove valuable when generating a pure culture from a single encapsulated *Salmonella* cell, and should mix well with water samples given both are water soluble.

A pure culture was assessed in these growth experiments because it is expected droplets will encapsulate a single bacterial cell and subsequent growth in-droplet will be needed to confirm presence or absence of *Salmonella* in the sample. The utilization of a selective enrichment broth for the *Salmonella* could potentially inhibit, or greatly suppress, growth of non-*Salmonella* species encapsulated and limit growth in-droplet to only the target bacteria – *Salmonella*. First, the ability of the selective medias to limit growth of non-*Salmonella* must be evaluated.

Turbidity, or optical density, is an established technique in measuring kinetics of bacterial growth. Brewster reported the utility of turbidity measurement of *E. coli* and *Salmonella* in a 96-well microwell plate with 1:10 dilutions from  $10^7$  to 1 CFU/ml (Brewster, 2003). Lingqvist characterized strain variability in *Staphylococcus* when using an automated turbidity measuring system, Bioscreen (Lindqvist, 2006). The results indicate growth measurements among *Staphylococcus* can potentially vary when using turbidity as the measurement of growth kinetics.

In order to optimize the intra-droplet environment, candidate broth medias were evaluated to ensure suitability for *Salmonella*-specific growth. A panel of twelve bacterial

species was evaluated in this study, two *Salmonella* positive controls and ten non-*Salmonella* negative controls, were evaluated. This research used TSB as the universal enrichment media, which also served as the positive control for the bacterial panel. BPW was the pre-enrichment broth media under test, and RV broth was the selective broth media under test. Turbidity measurement was utilized to measure growth and calculate generation times across the twelve bacterial species under test. Medias were also analyzed for fluorescent properties. Data gathered from pure culture growth and fluorescence experiments proved to be informative and translatable for applications involving bacterial growth utilizing in-droplet microfluidic methods.

### **3.3: MATERIALS AND METHODS**

#### Bacterial Species/Strains:

There were twelve bacterial strains used. Two *Salmonella* species were used as positive controls and 10 non-*Salmonella* were used as negative controls (Table 3.1). *Salmonella* ATCC 53647 was acquired from ATCC. Following an overnight culture at 37°C from a loop of lyophilized material, a loop from the overnight culture was streaked onto a tryptic soy agar plate (bioMerieux) to isolate a single *Salmonella* ATCC 53647 colony for stock generation. Following overnight culture incubation of the isolated colony at 37°C, stocks were generated and stored in 10% glycerol. The remaining 11 bacterial species were acquired from Ellen Forsyth, Johns Hopkins University Applied Physics Laboratory.

**Table 3.1:** Bacterial Species/Strains Utilized in Growth Experiments

Bacterial Strain	ATCC ID	Other Designation(s)	Biosafety Level
<i>Citrobacter freundii</i> (Braak) Werkman and Gillen	8090	NCTC 9750	1
<i>Enterobacter aerogenes</i> Hormaeche and Edwards	13048	NCDC 819-56, NCTC 10006	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	11775	NCTC 9001	2
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	13706	CIP 104337, NCIB 12416	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	700609	CN13	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	700891	HS(pFamp)R	1
<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz	19433	NCTC 775, NCDO 581	2
<i>Proteus hauseri</i> O'Hara et al.	13315	NCTC 4175 strain Lehmann, NCIB 4175	2
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> Rosenbach	6538	FDA 209	2
<i>Salmonella enterica</i> subsp. <i>enterica</i> (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium	14028	CDC 6516-60	2
<i>Salmonella enterica</i> subsp. <i>enterica</i> (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium	53647	Chi4062	1
<i>Shigella sonnei</i> (Levine) Weldin	25931	NCDC 1120-66, CIP 104223	2

### Media and pH Measurements:

Three broth medias were utilized, and include TSB (Sigma), BPW (10g peptone, 5g sodium chloride, 3.5g disodium phosphate, 1.5g mono-potassium phosphate, deionized water; autoclaved for 15 minutes at 121°C – all components from Sigma), and RV (Becton Dickinson). Media for Bioscreen experiments was at 1x concentration, and media used in-droplet was at an initial 2x concentration. A 2x media was encapsulated in-droplet because the two contributing sample inputs are merged upon droplet formation. This creates a 50/50 mixture. The pH of all medias and the phosphate buffered saline (PBS) control were measured using a pH meter (VWR Symphony SB70P Digital, Bench-model pH Meter).

### Growth Conditions, Equipment, and Measurement:

Bacteria were grown for 24 hours at either 37°C or 41.5°C (RV only) and turbidity measurements were taken every 15 minutes using an automated absorbance device (BioScreen). Given the objective of this research was to examine the limiting capability of

RV media for non-*Salmonella* bacterial species, all samples were grown at 37°C – the optimum growing temperature for the *Salmonella* utilized. The control panel was also grown at 41.5°C in the RV broth for 24 hours, per media manufacturer instructions. Of note, optimal growing temperature for the *Enterobacter* strain used is 30°C. The starting cell concentration for all bacterial species was 10<sup>3</sup> colony-forming unit (CFU)/ml and all bacterial species/strains studied were performed in triplicate.

Each bacterial species/strain used in this study was cultivated from stocks by an overnight culture. Streak plates for isolation were then performed for each bacterial species and allowed to grow overnight. Isolated bacterial colonies were then added to PBS until an absorbance of 0.05 ± 0.005 at 660nm was reached. An additional absorbance, 0.1 ± 0.05 at 600nm, was measured to ensure equipment was working properly. This absorbance value was determined to provide a 10<sup>9</sup> CFU/ml suspension in a preliminary spread plating experiment for all twelve bacterial species under test (data not shown). Starting with a 10<sup>9</sup> CFU/ml suspension in PBS a 1:10 dilution series was repeated until reaching a 10<sup>5</sup> CFU/ml concentration in PBS. Spread plating for CFU/ml counts were performed on tryptic soy agar plates (bioMerieux) in duplicate during this experiment to ensure CFU/ml concentrations were accurate, and all concentrations were found to have a starting concentration of 10<sup>9</sup> CFU/ml. A 1:10 dilution series was performed in order to achieve 10<sup>5</sup> CFU/mL concentration in PBS. A 1:100 dilution was then performed by transferring 10 microliters of 10<sup>5</sup> CFU/ml into 990 microliters of media. A 1:100 dilution was performed at this stage to minimize the input of PBS, and thus not diluting the media under test. A 200 microliter aliquot of 10<sup>3</sup> CFU/mL bacterial concentration in media were then added per well

of the proprietary Bioscreen Honeycomb 100-well plate. Three replicates were performed per bacterial species/strain in respective media.

#### Determination of Generation time:

Growth curves were generated for each bacterial strain, and stratified by each of the four conditions under test. The slope of the bacterial log-phase growth was utilized for the calculation of generation time. Length and slope of the log-phase varied across all bacteria. To calculate the slope a log-linear trend line was fit to the log-phase, and an  $r^2$  and natural log equation for each condition under test was attained.

#### Microfluidic Consumables, Equipment, and Procedure:

The co-flow microfluidic device utilized in this research was designed by Dr. Linas Mazutis with the Experimental Soft Condensed Matter Group, Harvard University, and published in Nature Protocols (Mazutis et al., 2013). Microfluidic chips, with 16-20 devices per chip, were fabricated by the Research and Exploratory Development Department (REDD), Johns Hopkins University Applied Physics Laboratory (JHUAPL). Microfluidic equipment, consumables, and technical support were supplied by the Applied Biological Sciences Group, JHUAPL. The microfluidic droplet system utilizes a proprietary surfactant (Phasex Corp.) at a concentration of 646 mg per 40 ml 7500 Novec Oil (3M). This surfactant-oil mixture serves as the carrier medium and aids in droplet formation of the sample input(s) on-chip. Flow rates reported in the Nature Protocol were 180:90:90 ul/hr (2:1:1 flow rate ratio) for the surfactant-oil mixture and the two sample inputs (Mazutis et al., 2013). Flow

rates utilized in this dissertation were modified slightly and were 2:0.5:0.5 (surfactant-oil to sample(s) input flow rate). Slight modifications were made for uniformity of observed droplet diameter upon formation during experiments. The on-chip device had one surfactant-oil input and two sample inputs. If two sample inputs are utilized, the overall input flow rate of the samples must be split between the two inputs. For example, if the flow rate of the surfactant-oil input is 2,000 ul/hr, sample input A will be 500 ul/hr and sample input B will be 500 ul/hr. Flow rates typically were 1,000 ul/hr surfactant oil input, and 250 ul/hr for each sample input. Samples were loaded into a syringe using a blunt fill needle (BD Blunt Fill Needle). The fill needle was then removed and replaced with a precision needle (BD Precision Glide). Tubing was connected between the sample syringe and the microfluidic device on-chip (95 Durometer LDPE, Scientific Commodities Micro Medical Tubing, 0.015" I.D x 0.043" O.D.). Pumps designed for syringe (1 ml to 30 ml volume BD Luer Lock Tip) use were used to regulate flow rates (Harvard Apparatus). Pump flow rate was managed via a software program compatible with Harvard Apparatus (LabVIEW 16, National Instruments). Droplet generation was monitored in real-time using Nikon objectives equipped with a high-speed camera and complimentary software (FASTEC IL5). Droplets were captured in 1.5 ml experimental tubes (Eppendorf).

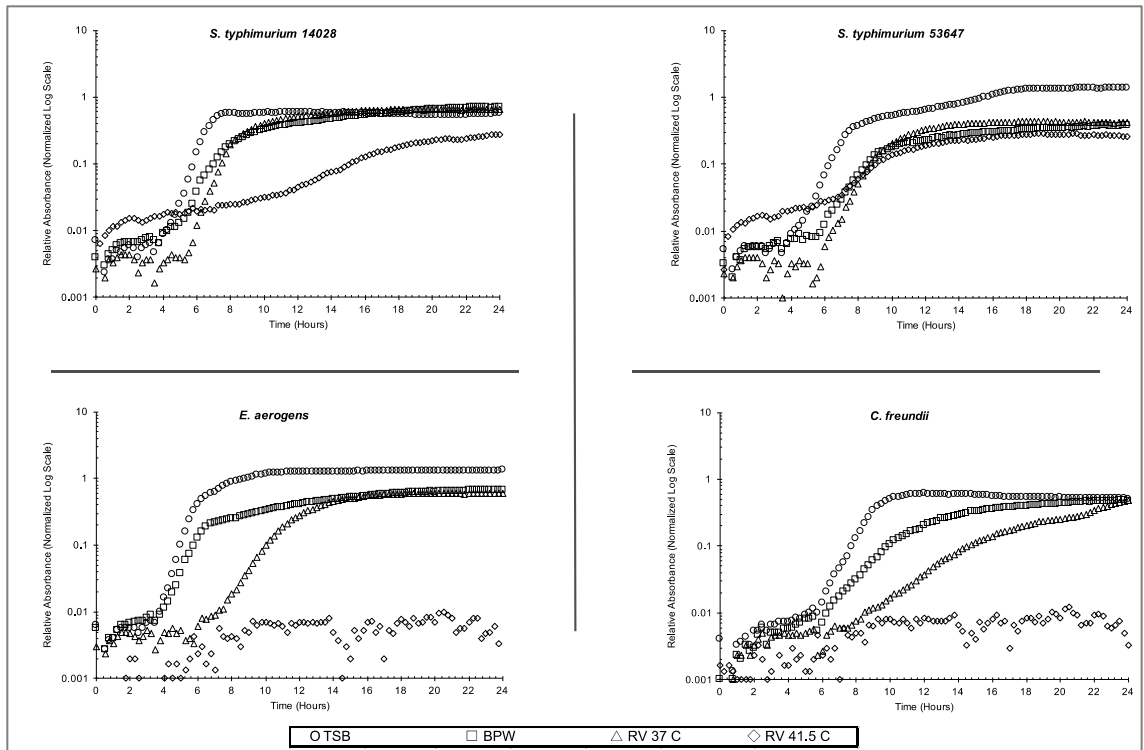
#### Fluorescent Antibody and Fluorescent Measurement Procedures:

A FITC-conjugated antibody (FITC-Ab), anti-*Salmonella*, CSA-1 Antibody (BacTrace), was utilized to measure media (TSB, BPW, and RV) contribution to overall fluorescence at concentrations of 0.1, 1, and 10 ug/ml. Measurements were taken by fluorimeter (Tecan) in a 96-well black bottom plate at 488 nm excitation and 525 nm emission. The FITC-Ab was



also merged into droplets at a 1:300 concentration from FITC-Ab stocks (0.5 mg/ml). Once merged into the droplet, at a 50/50 ratio with the complimentary input sample, the ratio in-droplet was 1:600 FITC-Ab (0.83 ug/ml in-droplet FITC-Ab concentration)

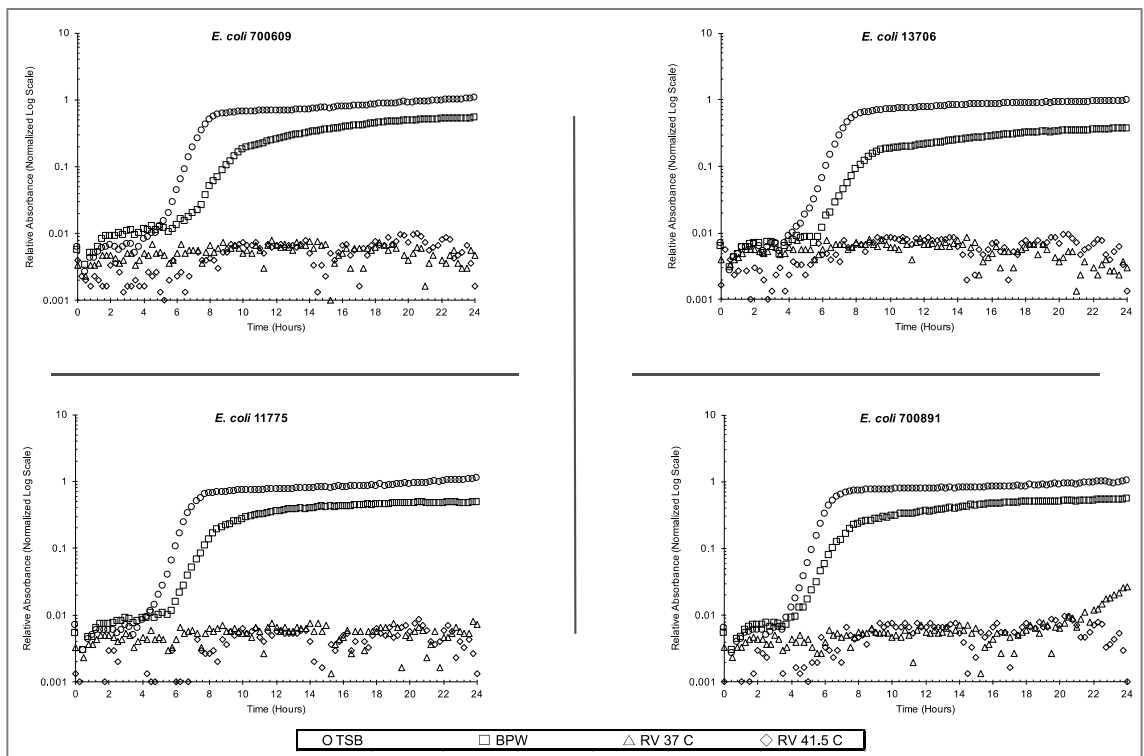
### 3.4: RESULTS



**Figure 3.1:** TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline. Growth kinetics of two *Salmonella* species, *E. aerogens*, and *C. freundii* stratified by media and temperature (RV only). Each value is the mean of three replicates. Values were normalized to background media absorbance. Absorbance measurements were taken at 15 minute intervals for 24 hours.

All twelve bacterial species replicated well in TSB (Figure 3.1, 3.2, and 3.3; Table 3.2). TSB was utilized as a positive control for the media variable under test, and was expected to provide the ideal growth for both *Salmonella* and non-*Salmonella*. TSB growth curves also

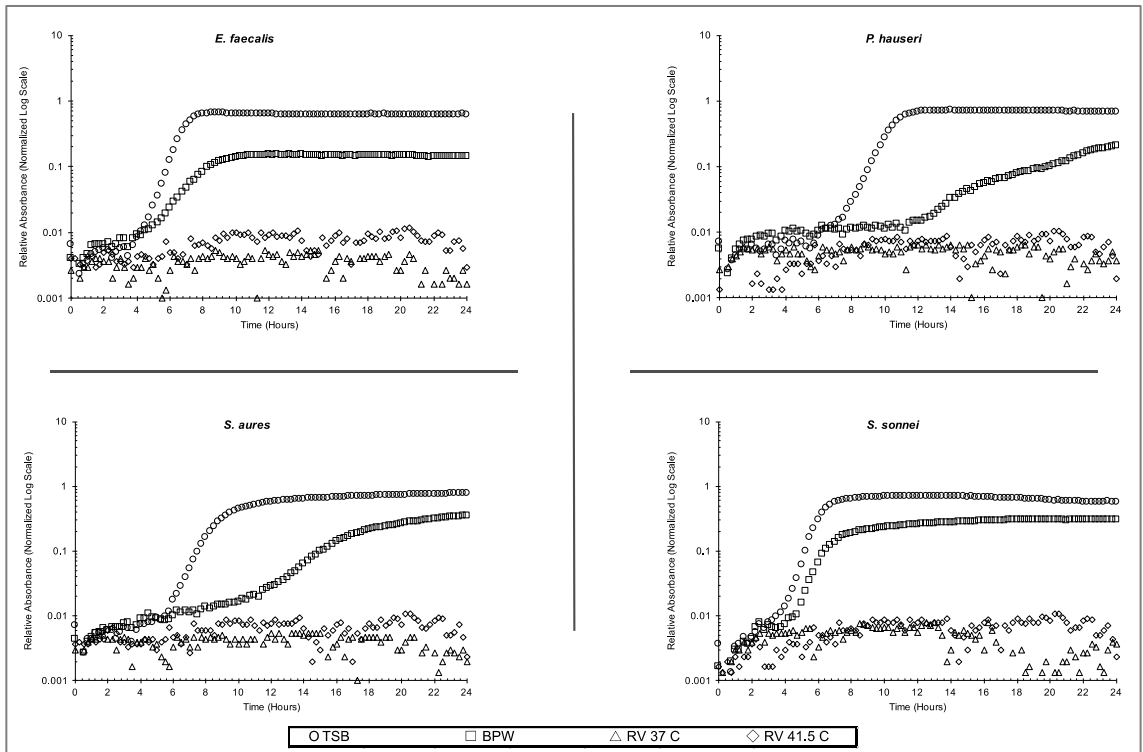
served as the standard for generational growth, or doubling time, across all organisms under the test conditions. Doubling time across all species in TSB ranged from 23.1 to 39.6 minutes (Table 3.2). TSB is a well-known and utilized universal growth media for bacteria in microbiological laboratories. Its use in this study should not be interpreted as useful for suppressing growth of the ten negative control bacterial species.



**Figure 3.2:** TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline. Growth kinetics of four *E. coli* strains stratified by media and temperature (RV only). Each value is the mean of three replicates. Values were normalized to background media absorbance. Absorbance measurements were taken at 15 minute intervals for 24 hours.

All twelve bacterial species under test exhibited some measure of growth in BPW (Figures 3.1, 3.2, and 3.3). Doubling times ranged from 29.3 to 203.9 minutes (Table 3.2). The range of generation times across the twelve bacterial species is an indicator that BPW

either suppresses growth of many non-*Salmonella*, or does not provide the ideal nutrition for some bacterial species under test, i.e. *P. hauseri* and *S. aureus* (Figure 3.3; Table 3.2). While the two *Salmonella* strains performed well in BPW, the fastest generation time in BPW was *Shigella*, not *Salmonella* (Table 3.2).



**Figure 3.3:** TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline. Growth kinetics of four *E. faecalis*, *P. hauseri*, *S. aureus*, and *S. sonnei* stratified by media and temperature (RV only). Each value is the mean of three replicates. Values were normalized to background media absorbance. Absorbance measurements were taken at 15 minute intervals for 24 hours.

RV broth media at 37°C promoted the desired growth of both *Salmonella* strains and inhibited the growth of 8 out of 10 non-*Salmonella* bacteria. However, two of the non-*Salmonella* negative controls, *Citrobacter* and *Enterobacter*, were able to replicate in the RV media (Figure 3.1; Table 2). Doubling times for *Salmonella* 028 and *Salmonella* 647 were 27.5 and 39.8

minutes (Table 3.2), respectively. Doubling times for *Citrobacter* and *Enterobacter* were 103.5 and 49.2 minutes (Table 3.2), respectively. RV broth proved very effective at eliminating or suppressing growth of most non-*Salmonella* and encouraging the growth of *Salmonella* at 37°C. Of note, the ideal growth temperature for this formulation of RV, like most RV formulations, is 41.5°C ± 5.

RV broth media at 41.5°C significantly suppressed the growth rate of both *Salmonella* under test, however, this temperature inhibited the growth of all 10 negative controls (Figures 3.1, 3.2, and 3.3; Table 3.2). Even though all negative controls were inhibited, generation times for the *Salmonella* 028 and *Salmonella* 647 were deemed too low to successfully reach a detectable threshold of bacterial cells in a 4-8 hour timeframe. Also, this research is intended to be translatable to in-droplet growth. Incubating at 37°C already exerts physical heat stress on droplets being incubated, and it was anticipated droplet integrity could be compromised at 41.5°C.

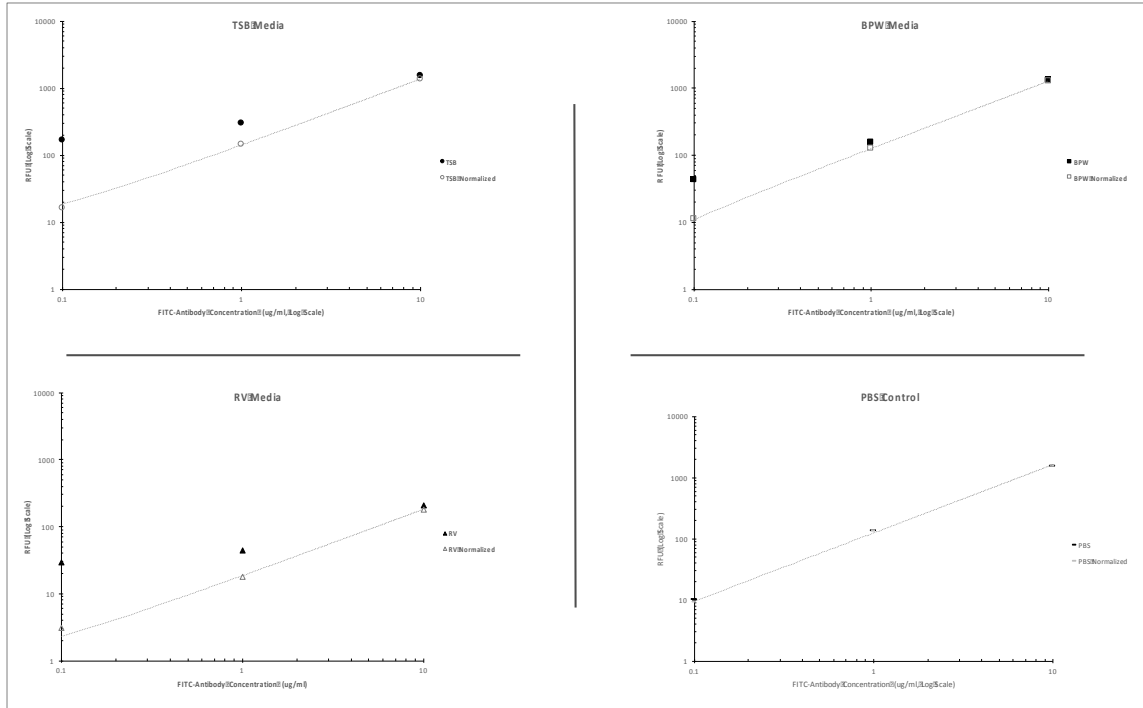
**Table 3.2:** Doubling time<sup>a</sup> and observed lag time<sup>b</sup> with a starting cell concentration of 10<sup>3</sup> CFU/ml in TSB, BPW, and RV broth media grown at 37°C or 41.5°C (RV only)

Bacterial Strain	TSB 37 C	BPW 37 C	RV 37 C	RV 41.5 C
<i>S. typhimurium</i> 028	28.6 (4)	39.8 (5)	27.5 (5.25)	210 (< 1)
<i>S. typhimurium</i> 647	37.5 (4)	51.4 (5.5)	39.8 (6)	91.2 (< 1)
<i>C. freundii</i>	39.6 (6)	66.7 (6.5)	103.5 (9.5)	No growth
<i>E. aerogens</i> *	23.1 (3.75)	35.2 (3.5)	49.2 (7.5)	No growth
<i>E. coli</i> 609	28.7 (5.5)	60.8 (5.5)	No growth	No growth
<i>E. coli</i> 706	28.8 (5)	42.5 (6)	No growth	No growth
<i>E. coli</i> 775	24 (5)	37.9 (5.75)	No growth	No growth
<i>E. coli</i> 891	24.8 (4)	38.7 (4.25)	No growth	No growth
<i>E. faecalis</i>	27.3 (4.5)	64.2 (4.75)	No growth	No growth
<i>P. hauseri</i>	38.1 (7.25)	203.9 (12.5)	No growth	No growth
<i>S. aureus</i>	35.7 (5.5)	106.6 (10.5)	No growth	No growth
<i>S. sonnei</i>	24.8 (4)	29.3 (4.75)	No growth	No growth

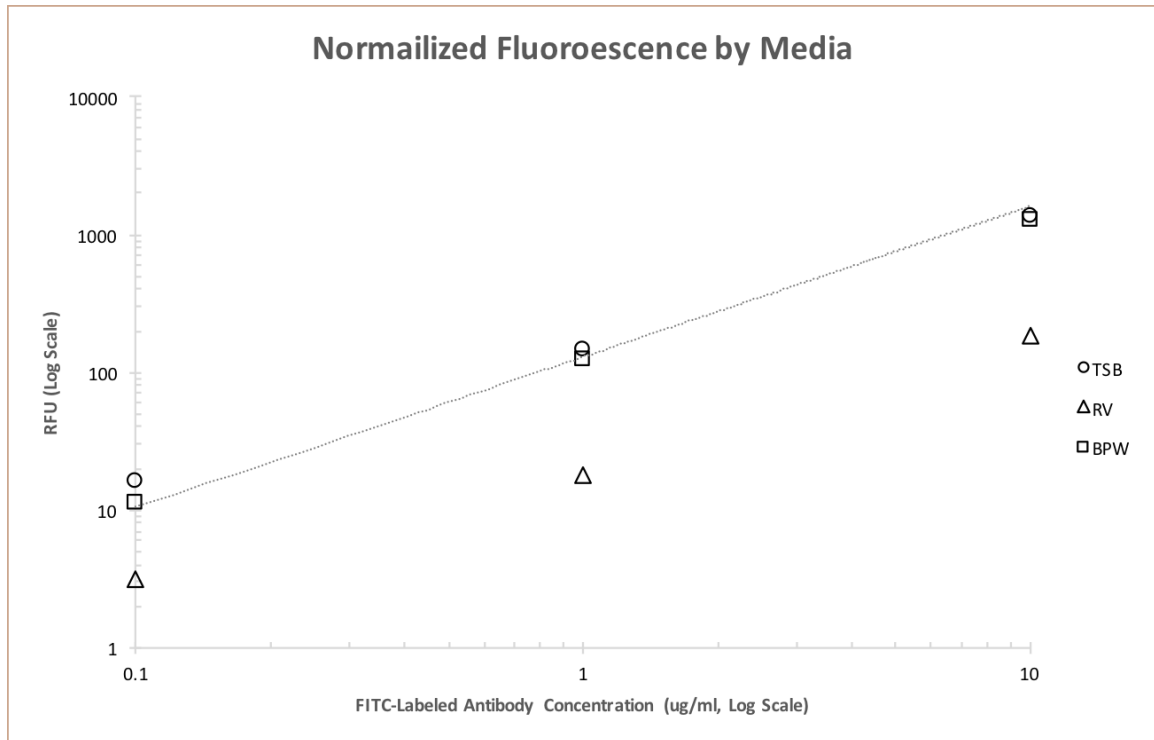
**a:** Doubling time listed in minutes; **b:** lag time listed in hours and in parentheses

\* Optimal growing temperature for *E. aerogens* is 30°C

Fluorescent contribution of the evaluated medias under test were also measured. TSB exhibited the highest level of auto-fluorescence (Table 3.3). RV broth exhibited the least fluorescence, and the intensity of fluorescence was reduced by approximately a log<sub>10</sub> RFU (Figures 3.4 and 3.5; Table 3.3). Figure 3.5 displays normalized values of the media tested. PBS served as the control.



**Figure 3.4:** TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline. Fluorescence measurements were taken at 0.1, 1, and 10 ug/ml FITC in each media with four replicates. The grey trend line represents the normalized fit for each media to better display fluorescent contribution of media.



**Figure 3.5:** TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline. Adjusted fluorescence measurements of 0.1, 1, and 10 ug/ml FITC in each media with four replicates. The grey trend line represents the PBS control to better display fluorescent contribution of media, and the fluorescent masking effect of RV and/or pH-sensitivity of FITC at low pH.

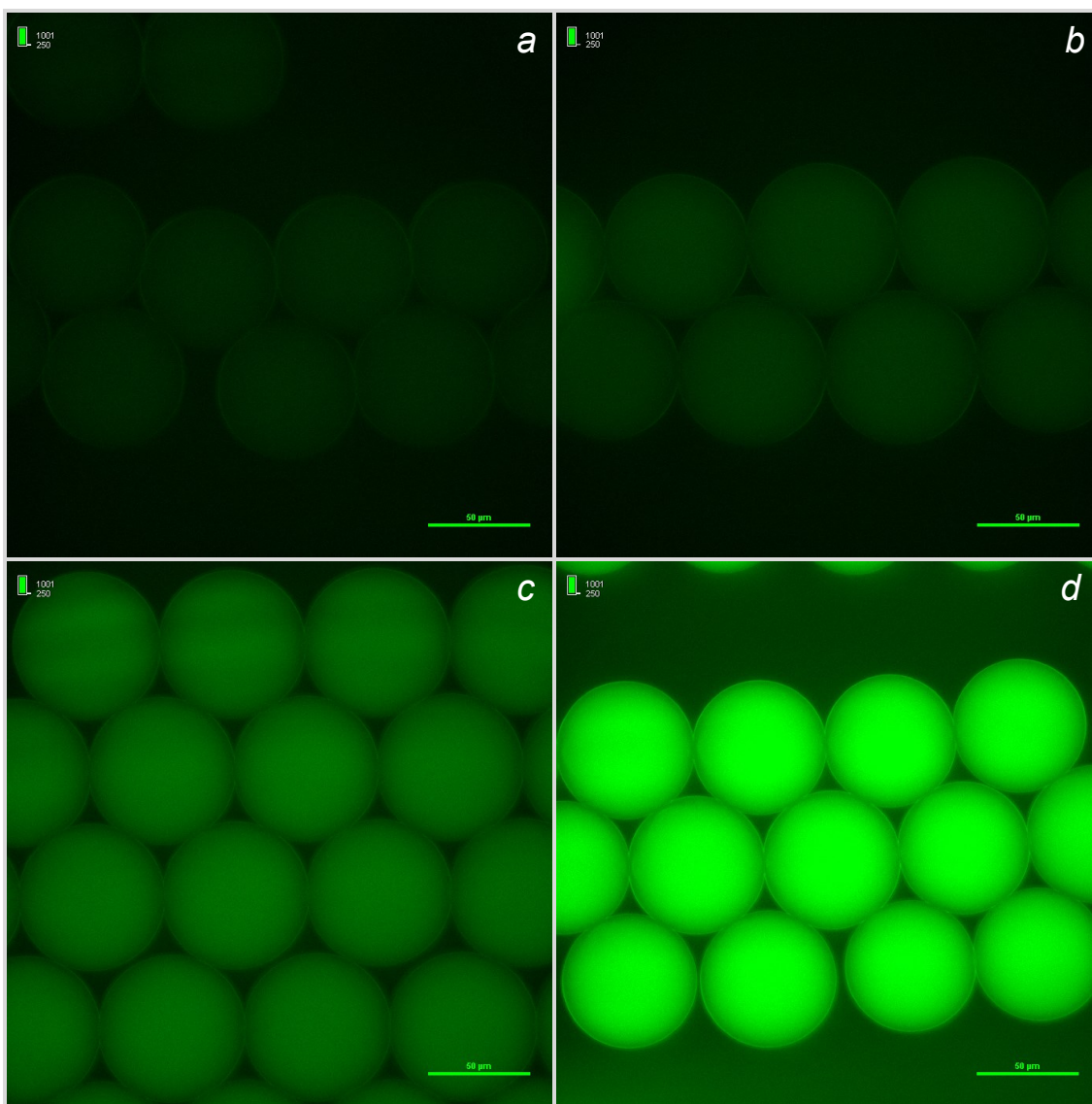
**Table 3.3:** Fluorescence and pH of Media Commonly Used for the Traditional Culture of *Salmonella*

Media <sup>a</sup>	Control [pH] <sup>b</sup>	0.1 ug/ml FITC-Ab <sup>c</sup>	1 ug/ml FITC-Ab <sup>c</sup>	10 ug/ml FITC-Ab <sup>c</sup>
TSB	151.8 [7.57]	167.9 (16.1)	296.6 (144.8)	1,527.4 (1,375.6)
BPW	31.7 [7.30]	43.1 (11.3)	157.2 (125.5)	1,306.5 (1,274.7)
RV	26.2 [5.13]	29.3 (3.1)	44 (17.8)	207.9 (181.7)
PBS	1.0 [7.75]	10.1 (9.1)	137.3 (136.3)	1,565.6 (1,564.6)

<sup>a</sup>: TSB = Tryptic Soy Broth; BPW = Buffered Peptone Water; RV = Rappaport-Vassiliadis Broth; PBS = Phosphate Buffered Saline

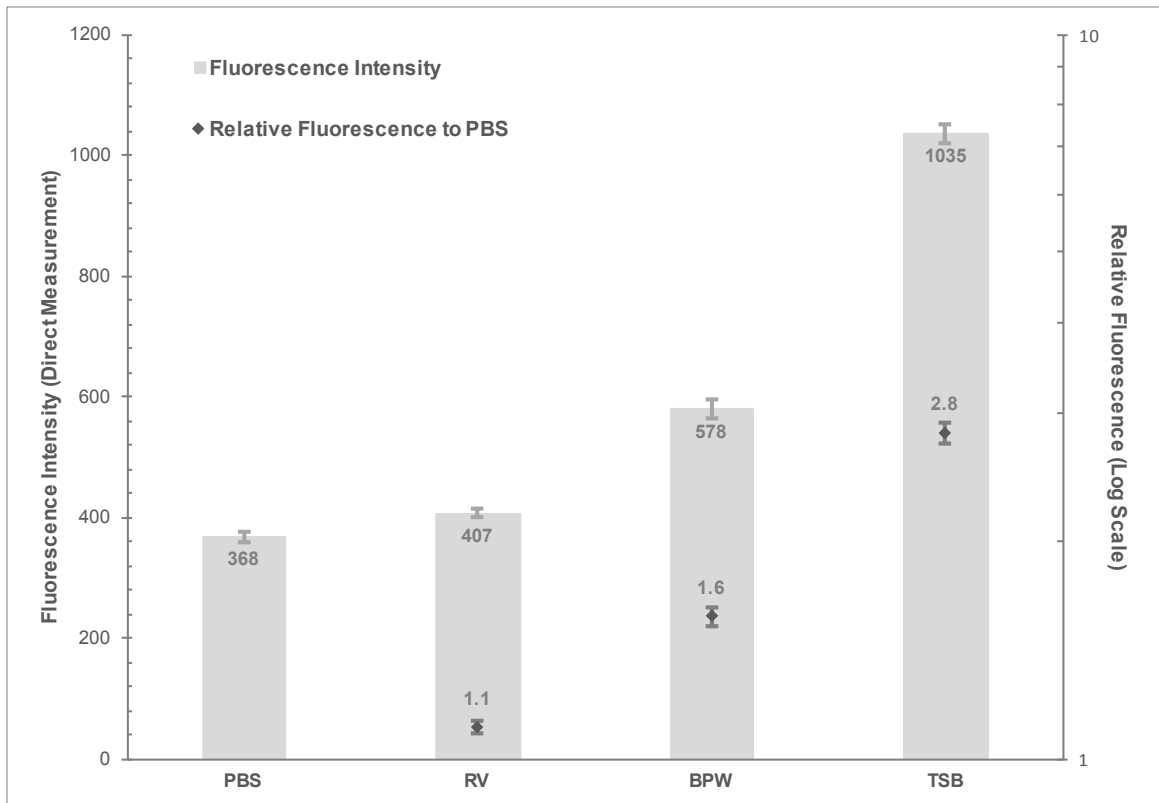
<sup>b</sup>: Control fluorescence intensities used for normalization; measured pH value of media in brackets; 0 ug/ml FITC-Ab in Control

<sup>c</sup>: Mean Relative Fluorescence Units of four replicates represented; Normalized values in parentheses



**Figure 3.6:** Media and FITC-Ab were encapsulated in 50-70 µm diameter droplets (1x media and 0.83 µg/ml FITC-Ab in-droplet) to visually assess media contribution to fluorescence. Fluorescence exposure and look-up-table (LUT) settings are standardized across all four medias evaluated. The LUT values can be found in the upper left-hand corner of each image. Medias under test included phosphate buffered saline (a), Rappaport-Vassiliadis (b), buffered peptone water (c), and tryptic soy broth (d).





**Figure 3.7:** Direct fluorescence intensity measurements and relative fluorescence to phosphate buffered saline (PBS) of each media-FITC-Ab data image in Figure 6. A total of five fluorescence values ( $n=5$ ) were analyzed for each media and the PBS control. Medias include Rappaport-Vassiliadis broth (RV), buffered peptone water (BPW), and tryptic soy broth (TSB). Direct fluorescence measurements correspond with the left y-axis, and relative fluorescence measurements correspond with the right y-axis. Error bars represent the 95% confidence interval.

### 3.5: DISCUSSION

The intent of choosing this panel of twelve bacteria is to serve as a representative sample of an agricultural or environmental water research scientists or agricultural industry regulators could encounter when performing detection and isolation for *Salmonella* (Table 3.1). RV broth, at both temperatures under test, performed very well in either eliminating or suppressing the growth of non-*Salmonella* species (Table 3.2). Tetrathionate (TT) broth is also identified as a selective broth for *Salmonella* by the FDA BAM, however, TT broth was not examined in this study. TT broth was excluded from study due to the presence of a

calcium carbonate precipitate that does not fully dissolve when the media is prepared. It was assessed that the calcium carbonate precipitate from TT broth would clog or foul microfluidic channels on-chip. In conventional microbiological culturing and confirmation, RV broth at 41.5°C would be superior at confirming *Salmonella* among the panel tested. Even at the sub-optimal temperature of 37°C, *Salmonella* likely would be able to out-compete the only other bacteria species identified in this study that grow at a suppressed rate in RV broth, *Enterobacter* and *Citrobacter*. However, the objective of this study was to assess feasibility for RV broth use in a microfluidic droplet. Therefore, the need for complete, or near complete, suppression is critical for isolating, detecting, and confirming *Salmonella* with a high degree of specificity. Only the two *Salmonella* species under test successfully grew in RV broth at 41.5°C, preventing growth of all non-*Salmonella* under test. At first glance, 41.5°C would be the temperature of choice given complete suppression of all non-*Salmonella* species. However, the growth rate of *Salmonella* was substantially restricted, when compared to *Salmonella* grown in RV at 37°C (Figure 3.1; Table 2). Using *Salmonella* 647 with a generation time of 91.2 minutes at 41.5°C as an example (Table 3.2), and assuming each droplet is starting with one *Salmonella* cell, it would take 9 hours to reach approximately 60 *Salmonella* cells per droplet and 12 hours to reach approximately 250 *Salmonella* cells per droplet. For overnight culture purposes, the reduced generation time of RV at 41.5°C is not an issue of concern, since 18- to 24-hours is utilized with likely a higher inoculation than one *Salmonella* cell. However, this rate of growth is unacceptable when the objective is to reach detectable amounts from a single cell in 4-8 hours inside a droplet. For these reasons, RV broth at 37°C would be the best choice, as the objective is to detect *Salmonella* growth in 4-8 hours. Although, media alone would not provide the optimal level of specificity for

detection of *Salmonella*. The results from these data show promise for in-droplet microfluidic use, but would need to be paired with an additional identifying factor in order to achieve optimal specificity.

These data indicate RV at 37°C has a superior growth rate, when compared to RV at 41.5°C. However, RV at 37°C does not suppress growth of *Citrobacter* and *Enterobacter*. In order to maximize selective growth rates in RV at 37°C, a fluorescently-labeled antibody specific for *Salmonella* to increase specificity could be utilized with RV broth to screen out *Citrobacter* and *Enterobacter*. Given fluorescence has the potential to create a relatively high detectable signal in the presence of *Salmonella*, this method may prove to be the ideal way to move forward in creating a double positive indicator for *Salmonella*. Masking of fluorescence must be taken into account, as these data indicate fluorescent intensities exhibit a  $\log_{10}$  reduction in RFU when diluted in RV broth (Table 3.3). The reduced fluorescence intensity observed in RV broth is likely due to a pH of 5.3 (Table 3.3). Published findings have shown FITC to be pH-sensitive, exhibiting reduced fluorescence signal at a low pH (Diehl and Markuszewski, 1989; Ma et al., 2004).

There are other fluorescent options commercially available for *Salmonella* detection, however, not all options are applicable inside a droplet. Two of the more applicable fluorescent methods would be either a fluorescent media or fluorescently-labeled antibody for *Salmonella*. There is one fluorescent substrate, 4-methylumbelliferyl caprylate (MUCAP), for *Salmonella* (Al-Kady et al., 2011). However, MUCAP is non-soluble in water, and thus is

not compatible for in-droplet applications. Therefore, a fluorescently-labeled antibody specific for *Salmonella* would be a better course of action when using in tandem with RV broth in-droplet. A direct binding antibody, such as an antibody targeting *Salmonella*'s common surface antigen, could prove effective for detection purposes. A fluorescent indicator for detection purposes would be an ideal indicator for *Salmonella* detection in-droplet, and a FITC-Ab could be applied in future microfluidic applications. It would be necessary to evaluate media contributions to fluorescence in-droplet to best assess potential uses in the future. This research evaluated the impact media can have on overall fluorescence (Figures 3.6 and 3.7).

An additional aspect to consider when developing a method to rapidly detect *Salmonella* would be incubation in-droplet. Some incubation would be necessary in order to reach optically detectable levels of colorimetric or fluorescence indicators. Current enumerating technologies are incapable of reliably detecting a single *Salmonella* cell encapsulated inside a droplet on an enumeration or sorting microfluidic chip. This may seem to be a limiting factor, and in some ways, it is not ideal. However, using the selective properties of the RV broth can further help specificity of differentiating *Salmonella* from non-*Salmonella* in mixed samples. Current capabilities of the in-droplet microfluidic system necessitate an incubation time to achieve a detectable in-droplet bacterial cell concentration to for enumeration or potentially sorting of the target bacterial organism in the future. By determining how many hours it will take to achieve a detectable number of *Salmonella*, in comparison to the non-*Salmonella*, increased specificity can be achieved. For example, according to these data, *Citrobacter* doubling time in RV broth is approximately 1/3<sup>rd</sup> of the

two *Salmonella* strains under examination in this study. The specificity of isolation and detection of *Salmonella* can potentially be achieved given the suppressed growth state of *Citrobacter* and relatively high replication rate of *Salmonella* at 37°C.

Finally, the FDA BAM states RV broth is selective for *Salmonella*, and the data provided in this study reaffirms FDA guidance. The results also show RV broth has a precise temperature window and can be less selective if used at the incorrect temperature, i.e. less than 41.5°C. When used as directed at 41.5°C for incubation, RV broth is effective at suppressing non-*Salmonella* growth. However, generation times are increased greatly for *Salmonella*. It has already been noted *Salmonella* would likely out compete another strain of bacteria in a mixed culture – at least the bacterial strains researched in the study. However, *Enterobacter* performed considerably well in the RV broth at 37°C, and only had doubling times approximately 10 to 20 minutes longer than the two *Salmonella* strains under test in this study (Table 3.2). Therefore, given the narrow temperature window between 37°C and 41.5°C, it would be worthwhile for future research to address varying concentrations of mixed cultures with *Salmonella* and non-*Salmonella*, such as *Citrobacter* and *Enterobacter*. A more accurate determination for the concentration of *Salmonella* can be achieved by carrying out future experiments of mixed cultures, and more effective selective medias for *Salmonella* could potentially be formally tested, certified, and eventually used by the scientific community.

### 3.6: CONCLUSION

Bacterial foodborne outbreaks from agricultural water sources are postured to remain a significant issue of public health concern for the foreseeable future in the U.S. and around the world (Scharff, 2015). Developing novel methodologies to isolate, detect, and prevent these outbreaks are essential to reduce mortality, illness, hospitalization, associated cost to taxpayers and overall international burden (Tauxe et al., 2010). FSMA, signed into law in 2011, sets the standard and provides guidance for how the U.S. should respond and address the critical public health threat posed to the U.S. population by contaminated agricultural products. A critical control point for detection of pathogen bacteria in the farm-to-table chain is the washing step for ready-to-eat leafy greens and fresh produce. This study is a fundamental first-step in developing methods to rapidly detect pathogenic bacteria from agricultural and environmental waters by optimizing media used in-droplet. By assessing the performance and ability of candidate medias to specifically culture target bacteria in 50-70  $\mu\text{m}$  diameter droplets, such as *Salmonella*, microfluidics has the potential to achieve the goal of one bacterial cell per unit volume of agricultural or environmental water. Additional research needs to be carried out in order to fill gaps in this capability, such as pairing colorimetric or fluorescent detection and incubation optimums, to rapidly detect target bacterial pathogens in near real-time. Leveraging this information, and future investigations into paired capabilities, the U.S. food supply can be made safer, and could set the standard for other international agricultural industries.

**Acknowledgements:** Applied Biological Sciences Group, Johns Hopkins University Applied Physics Laboratory; ERC Grant; The Osprey Foundation of Maryland.

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# Chapter 4: In-Droplet Microfluidic Growth Dynamics and Detection Using a FITC-Labeled Anti-*Salmonella* Antibody – Applications for Fresh-Cut Produce Wash Waters

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## 4.1: ABSTRACT

Foodborne contamination and associated illness in the United States is responsible for an estimated 48 million cases per year. Increased food demand, global commerce of perishable foods, and the growing threat of antibiotic resistance are driving factors for elevated concern regarding food safety. Foodborne illness is often associated with fresh-cut, ready-to-eat produce due to the perishable nature of the product and minimal processing from farm to the consumer. This research evaluates the utility of microfluidics for in-droplet detection of *Salmonella* in a shredded lettuce wash water acquired from a major Mid-Atlantic produce processing facility. Using a FITC-labeled anti-*Salmonella* antibody and relative fluorescence intensities, *Salmonella* was detected and identified with 100% specificity within four hours. The relative fluorescence intensity of *Salmonella* was approximately twice as much as the

observed intensities of five non-*Salmonella* negative controls at four hours incubation in-droplet with Rappaport-Vasiliadis (RV) broth and sterile deionized water at 37°C: *Salmonella* = 2.36 (95% CI: 2.15-2.58), *Enterobacter* = 1.12 (95% CI: 1.09-1.16), *Escherichia coli* (*E. coli*) 700609 = 1.13 (95% CI: 1.09-1.17), *E. coli* 13706 = 1.13 (95% CI: 1.07-1.19), and *Citrobacter* = 1.05 (95% CI: 1.03-1.07). When incubated four hours in-droplet at 37°C with RV broth and the shredded lettuce wash water acquired from industry, the observed relative fluorescence of *Salmonella* was statistically significantly higher than that of *Enterobacter*, 1.56 (95% CI: 1.42-1.71) and 1.10 (95% CI: 1.08-1.12), respectively. Applications for microfluidics in the food industry show great promise in dramatically shortening the time necessary to confirm viable bacterial contamination in fresh-cut produce wash waters.

## 4.2: INTRODUCTION

Foodborne illness is a significant public health concern worldwide, with a global burden of disease comparable to HIV/AIDS, malaria and tuberculosis (Havelaar et al., 2015). The World Health Organization estimated there were approximately 2 billion cases and over 1 million deaths associated with foodborne illness in 2010 (Kirk et al., 2015). The United States (U.S.) Centers for Disease Control and Prevention (CDC) estimates there are approximately 48 million cases of foodborne illness in the U.S. each year (Gould et al., 2013). Trend analyses for reported foodborne illness in the U.S. from 1996 to 2013 suggests infections from bacterial pathogens commonly associated with foodborne illness are remaining relatively constant, i.e. there is a lack of evidence showing reduction (Powell, 2016). While Norovirus is the leading cause of foodborne illness in the U.S. with over 5 million cases per year, non-typhoidal *Salmonella* is the second leading cause of foodborne

illness in the U.S. with over 1 million cases per year and is estimated to cause more hospitalizations and deaths than any other foodborne pathogen (Scallan et al., 2011). The ready-to-eat food industry is particularly susceptible to pathogen contamination. Contamination risk along the farm-to-consumer route is more prevalent in the ready-to-eat industry due to minimal processing, plant tissue damage encouraging microbial growth, and the perishable nature of fresh produce forcing rapid delivery to the consumer (Olaimat and Holley, 2012). Elevated incidence of foodborne illness linked to produce has been reported in the U.S., Canada, and European Union (Callejón et al., 2015; Kozak et al., 2013). During the 1998 to 2008 reporting period, nearly half (46%) of all foodborne illnesses in U.S. were attributable to produce, with 22% attributable to leafy greens alone (Painter et al., 2013). Given the global threat foodborne contamination presents, the food industry must continually evaluate critical control measures for its most vulnerable crops, improve upon current detection methods, and maintain a collaborative relationship with surveillance networks (Bonilauri et al., 2016; Iwamoto et al., 2015; Panisello et al., 2000).

The food industry utilizes the hazards analysis and critical control point (HACCP) framework as a tool to reduce foodborne hazards, analyze ways and means to counter the hazard, identify critical control points (CCPs), and routinely evaluate the effectiveness of the control strategies to prevent outbreaks (Mortimore, 2001). Key principles to every HACCP plan implemented in the food industry include: 1) Conduct a hazards analysis; 2) Identify CCPs; 3) Establish critical limits for preventative measure associated with each CCP; 4) Establish CCP monitoring requirements and procedures for using monitoring results to adjust processes and maintain control; 5) Establish corrective action to be taken when

monitoring indicates there is a deviation from an established critical limit; 6) Establish effective record-keeping procedures and document the HACCP plan; and 7) Establish systems to verify the HACCP system is working correctly (Hulebak and Schlosser, 2002).

The ready-to-eat, fresh-cut produce industry has a multitude of CCPs from farm-to-consumer, such as irrigation water, handling, and shelf-life (Decol et al., 2017; Francis et al., 2012; Jensen et al., 2017). A HACCP-based plan, utilizing foodborne outbreak surveillance data, to ensure microbial food safety should be an integral part of local, regional, and national produce processing facilities (Panisello et al., 2000; Tauxe, 2002).

Fresh-cut and leafy green produce wash waters are a well-documented CCP in the ready-to-eat produce industry, and have the potential to cross-contaminate otherwise safe produce when not managed correctly (Banach et al., 2015; Gil et al., 2009; López-Gálvez et al., 2009; Munther et al., 2015). These waters play a significant role in the industrial processing of ready-to-eat vegetables, including removing debris from plant surfaces and providing microbial disinfection by chlorination (Van Haute et al., 2013). Potential for cross-contamination is a notable hazard at this CCP as chlorine residual can decay or become inactivated by organic material in the wash water (Munther et al., 2015; Weng et al., 2016). Given the challenges posed and risks associated with contamination from wash waters, researchers are actively exploring methods to increase food safety associated with fresh-cut produce wash waters. Using a semi-commercial pilot scale system, researchers at the U.S. Department of Agriculture explored the use of an additive, T128, that was added to chlorinated produce wash water and was designed to increase antimicrobial efficacy and further reduce cross-contamination, as well as discourage bacterial pathogen survival (Luo et

al., 2012). The researchers reported that by adding T128 to produce wash water, survival of *Escherichia coli* (*E. coli*) O157:H7 was decreased, concluding that addition of T128 can increase the margin of safety in a chlorine-based produce sanitation system. In addition to improving antimicrobial activity of the wash waters, researchers and food scientists have explored improved detection strategies of microorganisms in these waters. For instance, a procedure using propidium monoazide real-time polymerase chain reaction (PMA-qPCR) was developed and evaluated for its capability to detect viable *E. coli* O157:H7 in vegetable wash water following a sanitizing ultrasound treatment (Elizaquível et al., 2012). The PMA-qPCR method was compared to traditional culture and plating techniques to evaluate both the ability to detect viable cells and effectiveness of the treatment. The PMA-qPCR method was comparable to traditional culturing techniques, and the ultrasound sanitation method was able to achieve a 4.4 log<sub>10</sub> reduction in *E. coli* O157:H7 (Elizaquível et al., 2012).

There are a number of detection methods used by researchers and food industry regulators to ensure food safety and mitigate outbreaks. These detection methods include traditional culture, immunoassays, PCR, genetic markers, and biosensors (Priyanka et al., 2016). Recently, local and regional public health laboratories have been shifting away from traditional culture isolates and to culture-independent diagnostic tests, such as multiplex PCR assays. This shift away from culture isolates, and the associated risk to foodborne outbreak surveillance, was highlighted by the CDC in a November, 2015 letter to territorial epidemiologists and public health directors (Shea et al., 2017). In this letter, the CDC characterized the marginalization of traditional culture methods of isolation and preference toward culture-independent methods as “a serious and current threat to public health

surveillance, particularly for Shiga toxin-producing *E. coli* and *Salmonella*.” The move to culture-independent methods is understandable. Though identified as critical by the CDC for isolation and source tracking, traditional culturing methods can take up to a week to confirm, and novel PCR applications, such as PMA-qPCR, allow for more timely confirmation of viable pathogen cell count (Zeng et al., 2016).

Applying in-droplet microfluidic technologies can bridge the gap between traditional culture methods and rapid culture-independent methods. Micro droplets have a volume of only pico- to nano-liters, and can form a miniature bioreactor for a single bacterial cell (Hamon and Hong, 2013). Researchers have already utilized in-droplet microfluidics to isolate *E. coli* in a pico-liter water-in-oil bioreactor and used time-lapse fluorescent imaging to study metabolic activity using 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG), a fluorogenic reporter (Marcoux et al., 2011). Marcoux and colleagues reported fluorescent confirmation of encapsulated *E. coli* growth and metabolism in less than two hours (Marcoux et al., 2011). This study showcases the potential for microfluidic droplets to serve both as a means of rapid detection and culturing capability. Recently, chemotactic microbes, such as *E. coli*, were sorted from both mixed and pure cultures using a chemoeffector concentration gradient and manipulating the bacterial cell motility away from the gradient (Dong et al., 2016). Innovative microfluidic technologies have also been used to assess and identify antibiotic resistance among bacterial populations (Aroonnuan et al., 2017). An in-droplet microfluidic platform was developed to screen bacteria for antibiotic resistance in clinically-relevant isolates, and responses from the bacteria and rapid characterization was achieved within one hour (Keays et al., 2016).



This study reports on the evaluation of in-droplet microfluidics as a tool for rapidly and specifically identifying *Salmonella* from microbe-spiked produce wash water supplied from a major, Mid-Atlantic produce processing facility. First, specificity of the fluorescein isothiocyanate- (FITC)-labeled antibody targeting the common surface antigen of *Salmonella* was evaluated. In-droplet growth experiments with *Salmonella*, and five negative controls, were then conducted in Rappaport-Vassiliadis (RV) selective broth media with *Salmonella*-specific FITC antibody (FITC-Ab). Once specificity and growth were characterized, a shredded lettuce wash water from the regional produce supplier was spiked with  $10^6$  CFU/ml *Salmonella*, filtered to remove plant debris, and encapsulated in-droplet for fluorescent detection in RV broth media selective for *Salmonella*.

#### **4.3: MATERIALS AND METHODS**

Bacterial Strains Used, Preparation, and Protocols:

A total of six ATCC bacterial strains, one *Salmonella* positive control and five negative controls, were utilized (Table 4.1). All strains used were Biosafety Level 1 due to the microfluidic platform location outside of a biological safety containment hood. Stocks were generated from overnight culture and stored at  $-20^{\circ}\text{C}$ . Working stocks at  $10^9$  colony forming units (CFU)/ml in phosphate buffered saline (PBS) were stored at  $5^{\circ}\text{C}$ . Streak plates for isolated colonies were performed for each experiment on tryptic soy agar plates (bioMerieux) and incubated overnight at  $37^{\circ}\text{C}$ . Isolated colonies from overnight incubation were then diluted into PBS until an absorbance of  $0.05 \pm 0.005$  at 660 nm, equating to  $10^9$

CFU/ml. This absorbance value was determined to provide a  $10^9$  CFU/ml suspension in a preliminary spread plating of 1:10 dilutions and CFU/ml counts for the six bacterial species under test (data not shown). Following an absorbance confirmation of  $0.05 \pm 0.005$  at 660 nm, a 1:10 dilution series was carried out to reach  $10^8$  and  $10^6$  CFU/ml, the concentrations ideally suited for microfluidic flow rates of encapsulation for control panel and Rappaport-Vassiliadis (RV) growth experiments, respectively. Control panel experiments utilized a bacterial input of  $10^8$  CFU/ml. This bacterial input concentration, paired with microfluidic flow rates used, yielded approximately 100 bacterial cells encapsulated per droplet. RV broth growth kinetic experiments utilized a bacterial sample input of  $10^6$  CFU/ml. This bacterial input concentration, paired with microfluidic flow rates used, yielded approximately one bacterial cell encapsulated every two-to-ten droplets. Bacterial dilutions and spread plate counts on tryptic soy agar plates (bioMerioux) were performed in shredded lettuce wash water growth experiments with *Salmonella* and *Enterobacter* to identify length of lag phase, initiation of log growth phase, and generations time in minutes. Droplets with encapsulated bacteria were incubated at 37°C for the desired time and then droplets were lysed using 1H, 1H, 2H, 2H-Perfluoro-1-Octanol (PFO) (Sigma). Approximately 10-20 ul of PFO was used per sample with brief centrifugation. The amount of PFO used varied slightly based on volume of droplets captured. Once popped, the bacterial cells in PFO form an immiscible layer on top of the media. An initial 1:100 dilution was performed due to the small volume of cells in PFO, and subsequent 1:10 dilutions were performed to achieve the desired spread plating CFU/ml concentration. Samples from time points zero and one hour were assayed by spread plate in duplicate, and time points two through five were assayed by spread plate in triplicate.

**Table 4.1:** Bacterial Strains Utilized in this Study

Bacterial Strain	ATCC ID	Other Designation(s)	Biosafety Level
<i>Citrobacter freundii</i> (Braak) Werkman and Gillen	8090	NCTC 9750	1
<i>Enterobacter aerogenes</i> Hormaeche and Edwards	13048	NCDC 819-56, NCTC 100X	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	13706	CIP 104337, NCIB 12416	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	700609	CN13	1
<i>Escherichia coli</i> (Migula) Castellani and Chalmers	700891	HS(pFamp)R	1
<i>Salmonella enterica</i> subsp. <i>enterica</i> (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium	53647	Chi4062	1

#### Media and Antibody Preparation, and pH Measurements:

RV broth was prepared at 2x concentration by adding double the manufacturer-directed amount in grams per unit volume (Beckton-Dickinson). Once mixed in filtered deionized water, the 2x RV broth mixture was autoclaved at 121°C for 15 minutes. An affinity purified polyclonal fluorescein isothiocyanate- (FITC)-conjugated antibody (Ab) targeting the common surface antigen- (CSA)-1 of *Salmonella* was utilized for activated fluorescence detection (BacTrace, Anti-*Salmonella*, CSA-1 Antibody, FITC-Labeled, SeraCare). FITC-Ab from stocks (0.5 mg/ml in PBS, 10 ul aliquots) were added to 2x RV broth to prepare a 20 ug/ml FITC-Ab concentration in 2x RV broth. A 2x RV broth with 20 ug/ml FITC-Ab was used for input on-chip as the concentration per droplet would be reduced by half upon droplet formation and bacterial encapsulation using the co-flow on-chip device, i.e. 1x RV broth with 10 ug/ml FITC-Ab upon droplet formation and bacterial encapsulation. The pH of RV broth media was measured using a pH meter (VWR Symphony SB70P Digital, Bench-model pH Meter).

#### Shredded Lettuce Wash Water Collection, Characterization, and Preparation:

Wash water from a shredded lettuce processing line was acquired from a major ready-to-eat vegetable processing facility on the East Coast. Initial samples were divided into 500 ml aliquots and stored at -80°C. Working stocks were prepared in aliquots of 5 ml and stored at -20°C. Collected wash waters for all vegetable samples were analyzed for free chlorine and organic chloramines by the N,N-diethyl-*p*-phenylene diamine colorimetric method (APHA-AWWA- WEF, 2012.). Turbidity, conductivity, and pH were also measured by Hach 2100N Turbidimeter (Hach Company, CO), Hach Sension5 portable conductivity meter (Hach Company, CO) and accutupH<sup>+</sup> probe (Fisher Scientific), respectively. Once thawed from stocks, pH of the shredded lettuce wash water was measured using a VWR pH meter (VWR Symphony SB70P Digital, Bench-model pH Meter). For microfluidic experiments, the shredded lettuce wash water was filtered through a 0.22 um syringe filter (Durapore PVDF Membrane, MILLEX GV) and spiked with bacteria to an approximate concentration of 10<sup>6</sup> CFU/ml. Residual chlorine was quenched with 1 mM sodium thiosulfate (Sigma)

#### Microfluidic Consumables, Equipment, and Procedure:

The co-flow microfluidic device utilized in this research was designed at Harvard University, and published in Nature Protocols (Mazutis et al., 2013). Microfluidic chips used in this research were fabricated by the Research and Exploratory Development Department (REDD), Johns Hopkins Applied Physics Laboratory (JHUAPL). Microfluidic equipment, consumables, and technical support were supplied by the Applied Biological Sciences Group, JHUAPL. The microfluidic droplet system utilizes a proprietary surfactant (Phasex Corp.) at a concentration of 646 mg per 40 ml 7500 Novec Oil (3M). This surfactant-oil mixture aided in oxygen transfer, served as the carrier medium and facilitated uniform

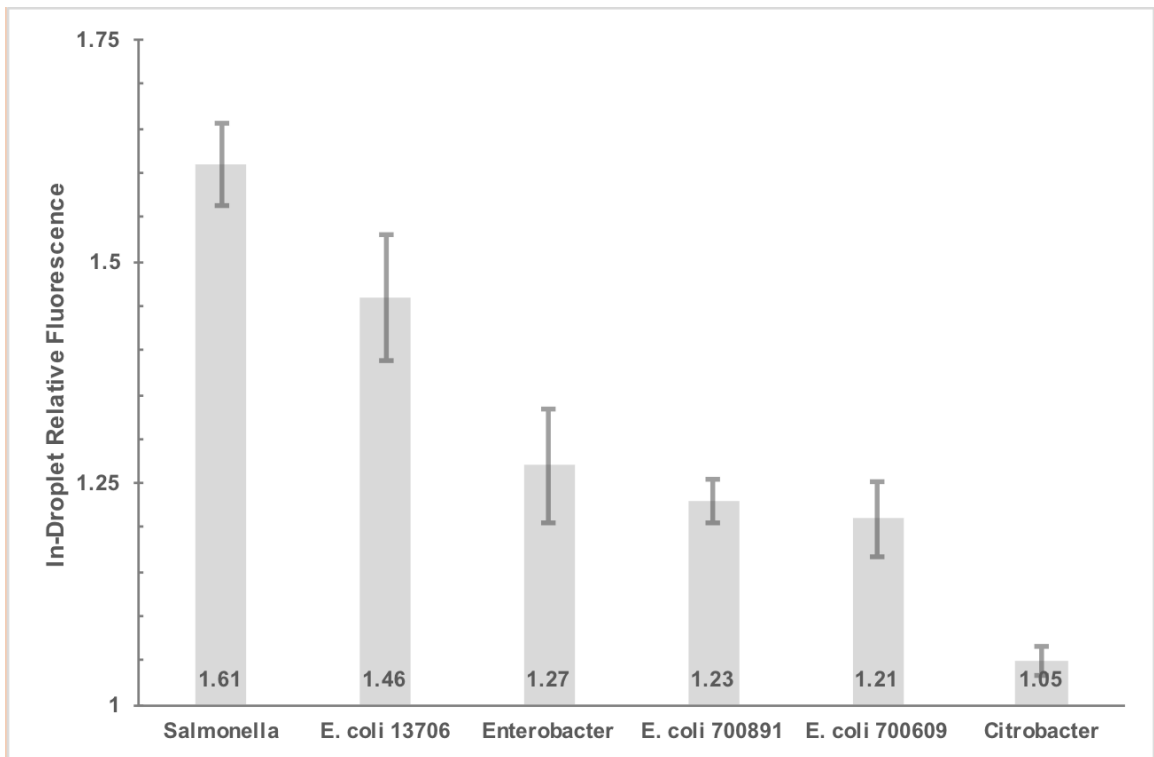
droplet formation of the sample input(s) on-chip. Flow rates reported in the Nature Protocol were 180:90:90 ul/hr (2:1:1 flow rate ratio) for the surfactant-oil mixture and the two sample inputs (Mazutis et al., 2013). Flow rates utilized in this research were modified and were 2:0.5:0.5 (surfactant-oil to sample(s) input flow rate). Slight modifications were made for uniformity of observed droplet diameter upon formation during experiments. The on-chip device used in this research had one surfactant-oil input and two sample inputs. If two sample inputs are utilized, the overall input flow rate of the samples must be split between the two inputs. For example, if flow rate of the surfactant-oil input is 2,000 ul/hr, sample input A will be 500 ul/hr and sample input B will be 500 ul/hr. Flow rates for this research were typically 1,000 ul/hr surfactant-oil input, and 250 ul/hr for each sample input. Samples were loaded into a syringe using a blunt fill needle (BD Blunt Fill Needle). The fill needle was then removed and replaced with a precision needle (BD Precision Glide). Tubing was connected between the sample syringe and the microfluidic device on-chip (95 Durometer LDPE, Scientific Commodities Micro Medical Tubing, 0.015" I.D x 0.043" O.D.). Pumps designed for syringe (1 ml to 30 ml volume BD Luer Lock Tip) use were used to regulate flow rates (Harvard Apparatus). Pump flow rate was managed via a software program compatible with Harvard Apparatus (LabVIEW 16, National Instruments). Droplet generation was monitored in real-time using Nikon objectives equipped with a high-speed camera and complimentary software (FASTEC IL5). Droplets were captured in 1.5 ml experimental tubes (Eppendorf).

Microscopy Equipment and Procedures:

Formed droplets were drawn into 50 um diameter glass capillary tubing for visualization and measurement (0.05 x 0.5 mm ID, VitroTubes). Filled capillaries were then fixed to a microscope slide (Fischer) by first sealing the loading end and elevating the slide at a 45 degree angle for 1-5 minutes. This allowed the droplets to spread out equally and stack for imaging. The non-loading end was then sealed before microscopy. The Eclipse Ni-E motorized microscope system and Ni-E Analysis Elements software (Nikon) equipped with a digital camera (ORCA-Flash 4.0 LT, Hamamatsu) were used for fluorescent images and data analysis. Permission to utilize the Eclipse Ni-E motorized microscope system and software was granted by REDD, JHUAPL. Z-stack data at 5 um intervals was collected throughout entirety of the droplet using the ND Acquisition tool in Ni-E Analysis Elements. Droplet diameter ranged from approximately 50-70 um. Z-stack data were then merged into flat, data-rich images using the EDF tool in General Analysis, Ni-E Analysis Elements. Merging Z-stack data allowed for maximum image capture of encapsulated bacteria by creating a two-dimension data image of bacteria at differing focal planes. Point estimates of fluorescence intensity were gathered and a complimentary fluorescence intensity of background was paired with each measurement. Fluorescence measurements were normalized by reporting relative fluorescence. Relative fluorescence was calculated by dividing bacterial fluorescence by background fluorescence ( $Fluorescence_{bacteria} / Fluorescence_{background}$ ). A sample size of five (n=5) relative fluorescence measurements were used for all mean, standard deviation, and 95% confidence interval calculations. Fluorescent data images were smoothed and normalized using Ni-E Elements software in order to report comparable, high resolution images.

#### 4.4: RESULTS

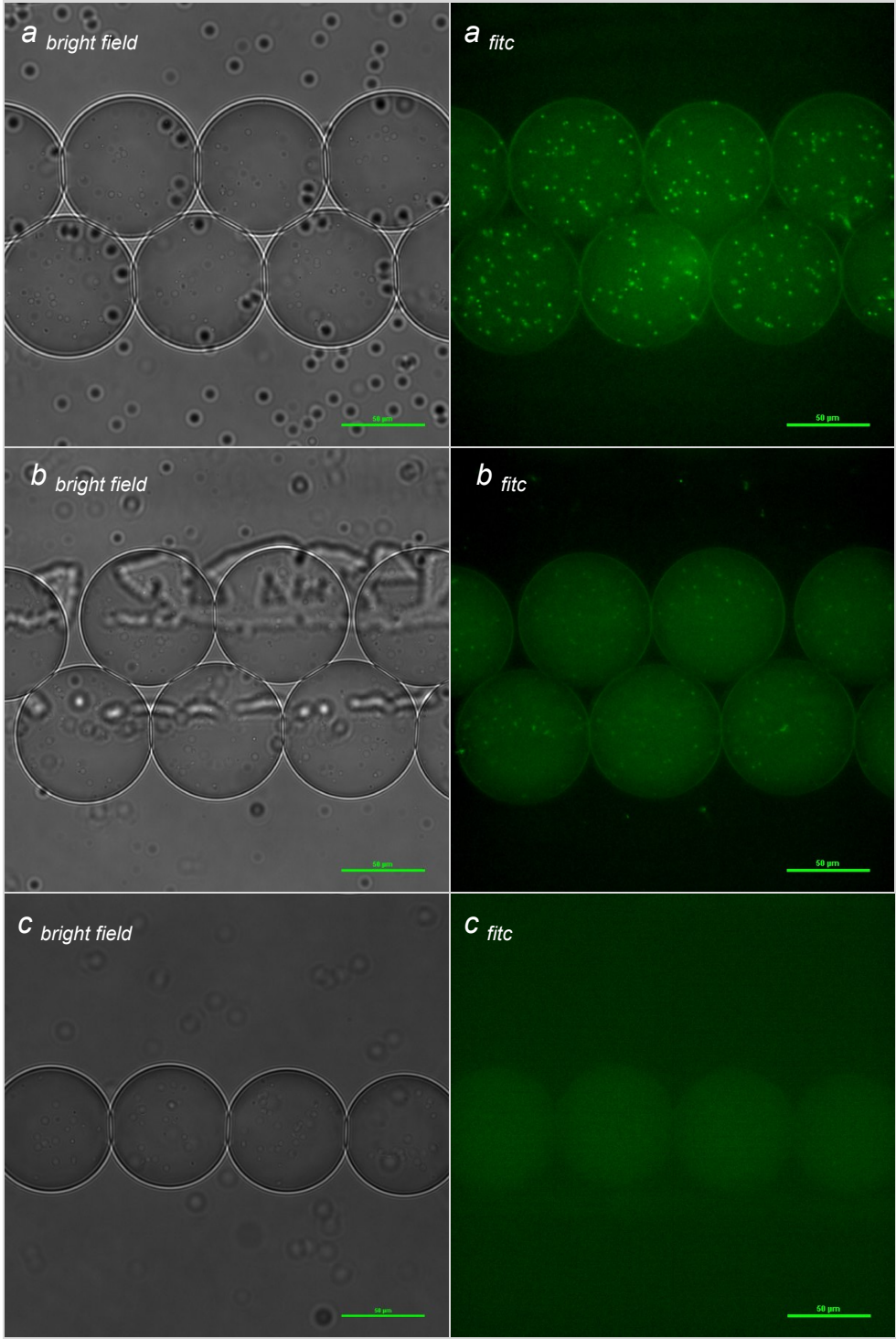
Bacteria at a  $10^8$  CFU/ml concentration were encapsulated in-droplet in combination with a FITC-Ab concentration of 1.66 ug/ml. Using 1,000:250:250 ul/hr flow rates and 50/50 merging parameters, each droplet contained approximately 100 bacterial cells and 0.83 ug/ml FITC-Ab. Using this approach ensured each droplet had ample bacteria to assess the degree of specificity for *Salmonella* and cross-reactivity with negative controls. Both sample inputs utilized PBS as the dilution solution in order to best assess FITC-Ab specificity for *Salmonella* and potential cross-reactivity, or lack of specificity, with non-*Salmonella* species. *Salmonella* had the highest RFU measured, however, *E. coli* 13706 exhibited a high propensity for cross-reactivity. The two other *E. coli* strains, 700609 and 700891, showed moderate cross-reactivity. *Citrobacter* had minimal cross-reactivity with the FITC-Ab (Figure 4.1).



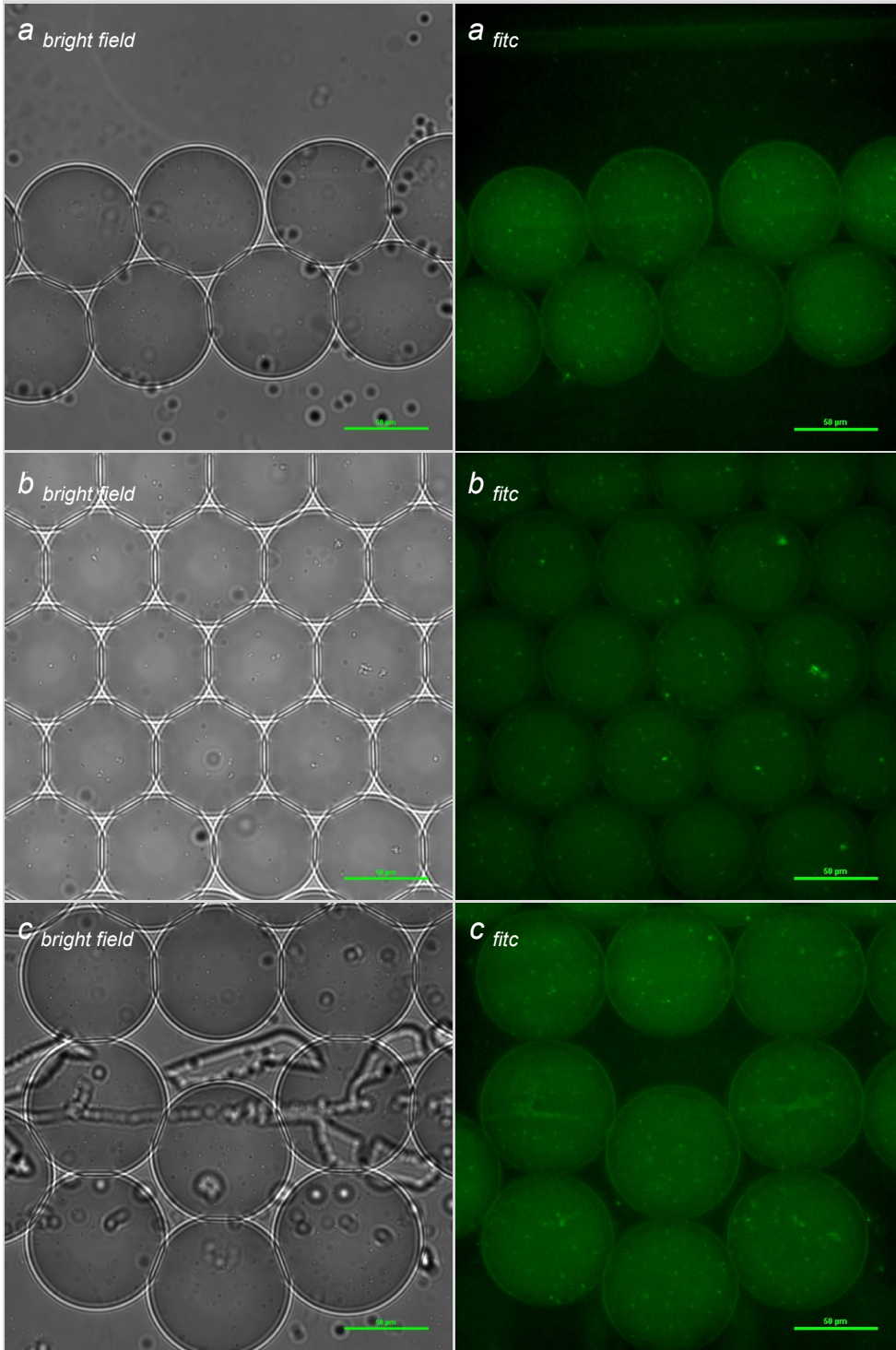
**Figure 4.1:** Fluorescence of bacteria relative to background fluorescence in-droplet of six bacterial species encapsulated in droplet with 0.83 ug/ml FITC-Ab per droplet in phosphate buffered saline. Five fluorescence measurement replicates were performed per bacterial species/strain. Error bars represent the 95% confidence interval.

Microscopic fluorescent images, with embedded metadata for fluorescent intensity, were performed through the entirety of the droplet at 5 um intervals. Once merged into a two-dimensional image, these data were used as the raw data for relative fluorescence measurements (Figures 4.2 and 4.3).





**Figure 4.2:** Bacterial species (**a:** *Salmonella*; **b:** *Enterobacter*; **c:** *Citrobacter*) with 0.83 µg/ml FITC-Ab in phosphate buffered saline.) Each droplet is approximately 50-70 µm in diameter (scale on image is 50 µm). Bright field images are presented complimentary to FITC images and visually show bacterial concentration at an ideal focal plane (5 µm interval stacking and merging of images into two dimensional images is not optimal for bright field images). FITC images are merged 5 µm interval focal plane images and represent a two-dimensional image of the entire droplet.



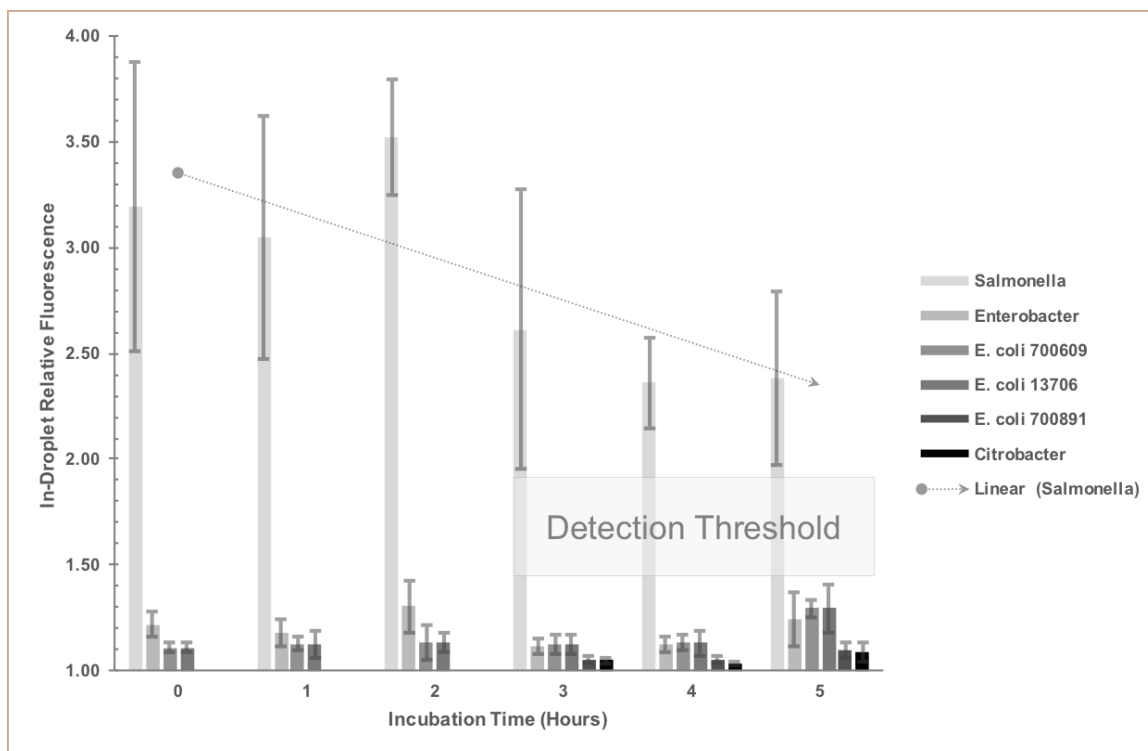
**Figure 4.3:** Bacterial strains (**a:** *E. coli* 700609; **b:** *E. coli* 13706; **c:** *E. coli* 700891) with 0.83 µg/ml FITC-Ab in phosphate buffered saline.) Each droplet is approximately 50-70 µm in diameter (scale on image is 50 µm). Bright field images are presented complimentary to FITC images and visually show bacterial concentration at an ideal focal plane (5 µm interval stacking and merging of images into two dimensional images is not optimal for bright field images). FITC images are merged 5 µm interval focal plane images and represent a two-dimensional image of the entire droplet.

Once a baseline for *Salmonella* specificity and potential cross-reactivity of the FITC-Ab was achieved in PBS (Figures 4.1, 4.2, and 4.3), Rappaport-Vassiliadis (RV) broth was evaluated in-droplet for growth suppression of non-*Salmonella* bacteria. Results discussed in Chapter 3 of this dissertation identify RV broth at 37°C to be the ideal conditions for evaluation. Bacteria at a 10<sup>6</sup> CFU/ml concentration in autoclaved deionized water were encapsulated in-droplet along with a FITC-Ab concentration of 20 ug/ml. Using 1,000:250:250 ul/hr flow rates and 50/50 merging parameters, one in every two-to-ten droplets contained a single, isolated bacterial cell and 10 ug/ml FITC-Ab. Using this approach ensured growth in-droplet originated from a single bacterial cell to assess the observed growth kinetics and changes in relative fluorescence over time. Data for each time point, stratified by bacterial species/strain, are reported in Table 4.2. Relative fluorescence data for the five-hour incubation are also presented in Figure 4.4, and a trend line was fit to *Salmonella* to display effects growth can have on relative fluorescence inside a droplet.

**Table 4.2:** Relative Fluorescence of Bacteria to Background In-Droplet Grown in Rappaport-Vassiliadis Broth Over 5-Hour Incubation at 37°C

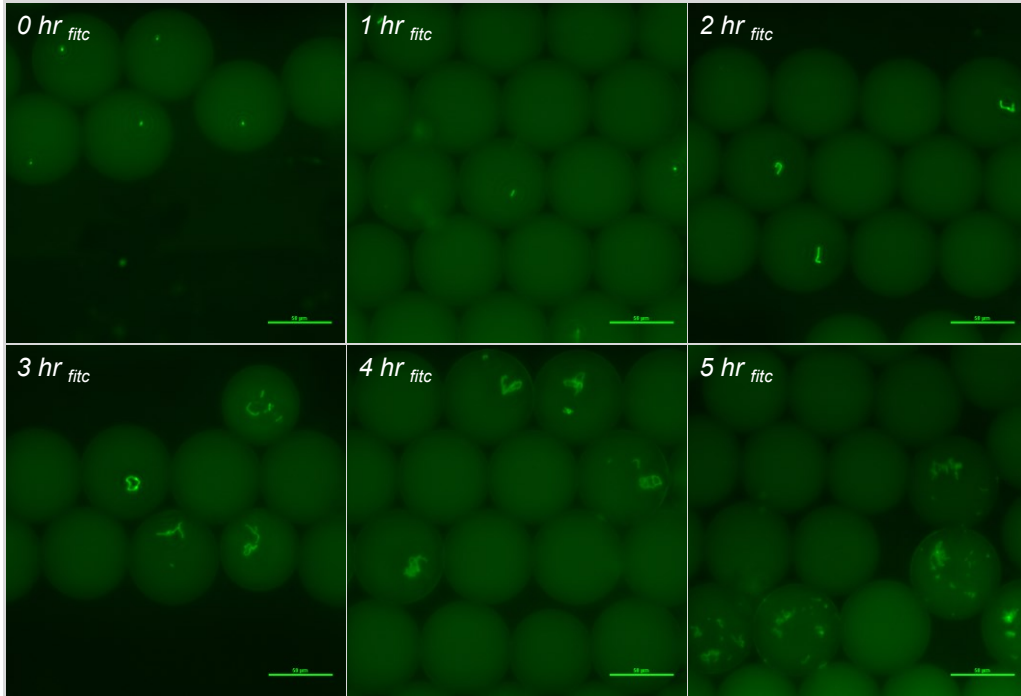
Bacteria	0 (hr)	1 (hr)	2 (hr)	3 (hr)	4 (hr)	5 (hr)
<i>Salmonella</i>	3.20 <sup>a</sup> (0.78) <sup>b</sup> , 2.51-3.88 <sup>c</sup>	3.05 (0.65), 2.48-3.62	3.52 (0.31), 3.25-3.80	2.61 (0.76), 1.95-3.28	2.36 (0.24), 2.15-2.58	2.38 (0.47), 1.97-2.80
<i>Enterobacter</i>	1.22 (0.06), 1.16-1.28	1.18 (0.07), 1.12-1.24	1.30 (0.14), 1.18-1.42	1.12 (0.04), 1.08-1.16	1.12 (0.04), 1.09-1.16	1.24 (0.15), 1.11-1.37
<i>E. coli</i> 700609	1.11 (0.03), 1.08-1.13	1.13 (0.04), 1.10-1.16	1.13 (0.09), 1.05-1.08	1.13 (0.05), 1.08-1.17	1.13 (0.04), 1.09-1.17	1.29 (0.05), 1.25-1.34
<i>E. coli</i> 13706	1.11 (0.03), 1.08-1.13	1.13 (0.07), 1.06-1.19	1.13 (0.05), 1.09-1.18	1.13 (0.05), 1.08-1.17	1.13 (0.07), 1.07-1.19	1.29 (0.13), 1.18-1.41
<i>E. coli</i> 700891	NFD <sup>*</sup>	NFD	NFD	1.05 (0.02), 1.04-1.07	1.05 (0.02), 1.03-1.07	1.10 (0.04), 1.06-1.13
<i>Citrobacter</i>	NFD	NFD	NFD	1.05 (0.02), 1.04-1.06	1.04 (1.01), 1.03-1.05	1.09 (0.05), 1.04-1.13

a: Mean (n=5); b: Standard Deviation; c: 95% Confidence Interval; \* No Fluorescence Detection (NFD)

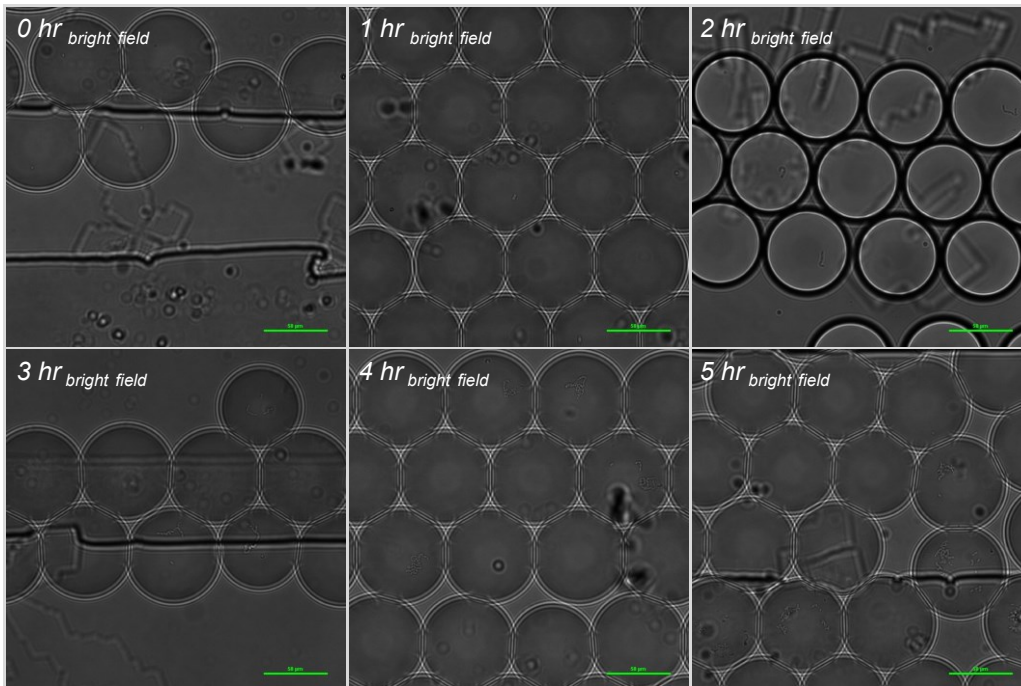


**Figure 4.4:** Relative fluorescence of bacterial species (*Salmonella*, *Enterobacter*, and *Citrobacter*) and strains (*E. coli* 700609, 13706, and 700891) incubated in Rappaport-Vassiliadis broth for five hours at 37°C with a FITC-Ab concentration of 10 ug/ml in-droplet. The trend line represents a decrease in relative fluorescence of *Salmonella* over the time course. A detection threshold region is identified on the figure. No measurable relative fluorescence was identified in time points 0 through 2 for both *Citrobacter* and *E. coli* 700891. Five replicates were measured for each bacterial species/strain.

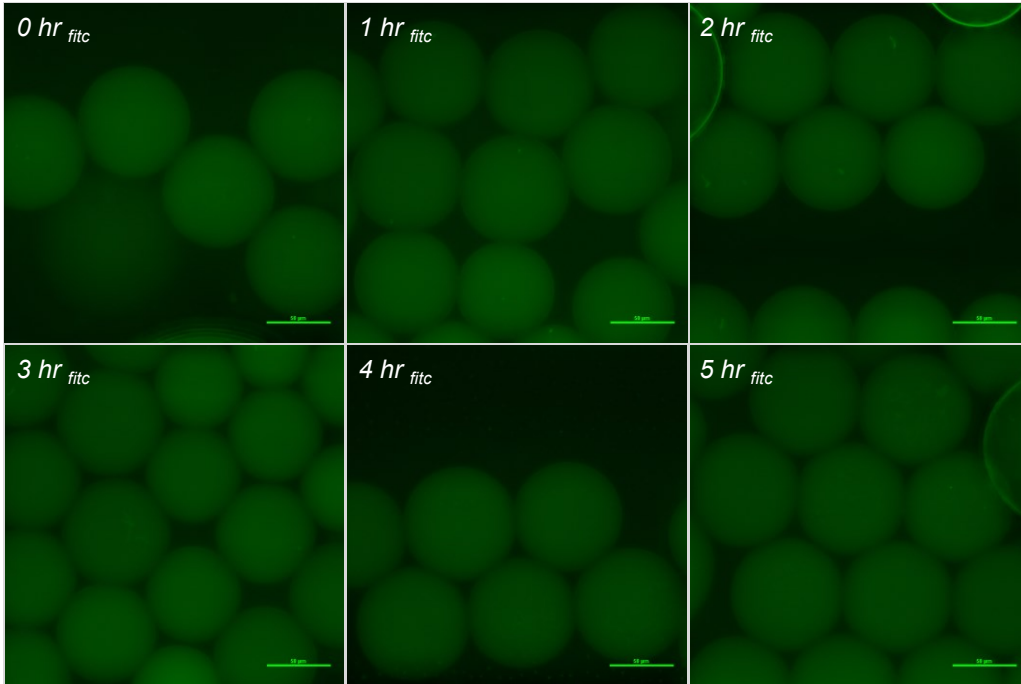
Microscopic fluorescent images, with embedded metadata for fluorescent intensity, were performed through the entirety of the droplet at 5 um intervals. These data were collected at each time point of incubation for each bacterial species/strain under test. Once merged into a two-dimensional image, these data were used as the raw data for relative fluorescence measurements in Ni-E Elements software (Figures 4.5 through 4.14).



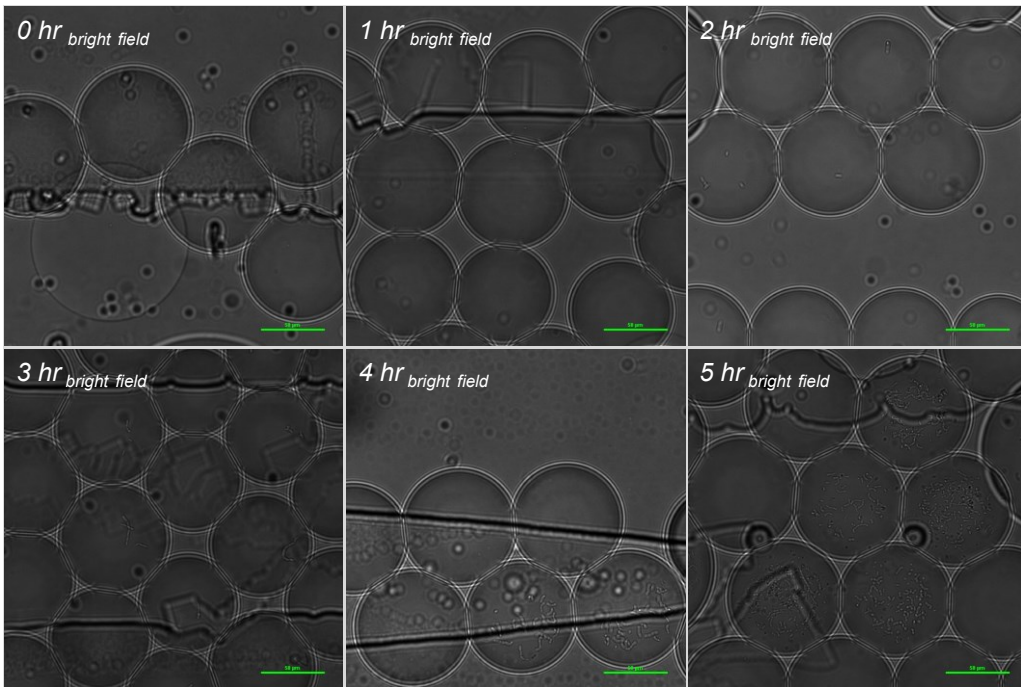
**Figure 4.5:** Fluorescent images of *Salmonella* incubation in-droplet with sterile deionized water, and 10 µg/ml FITC-Ab in 1x RV broth at 37°C.



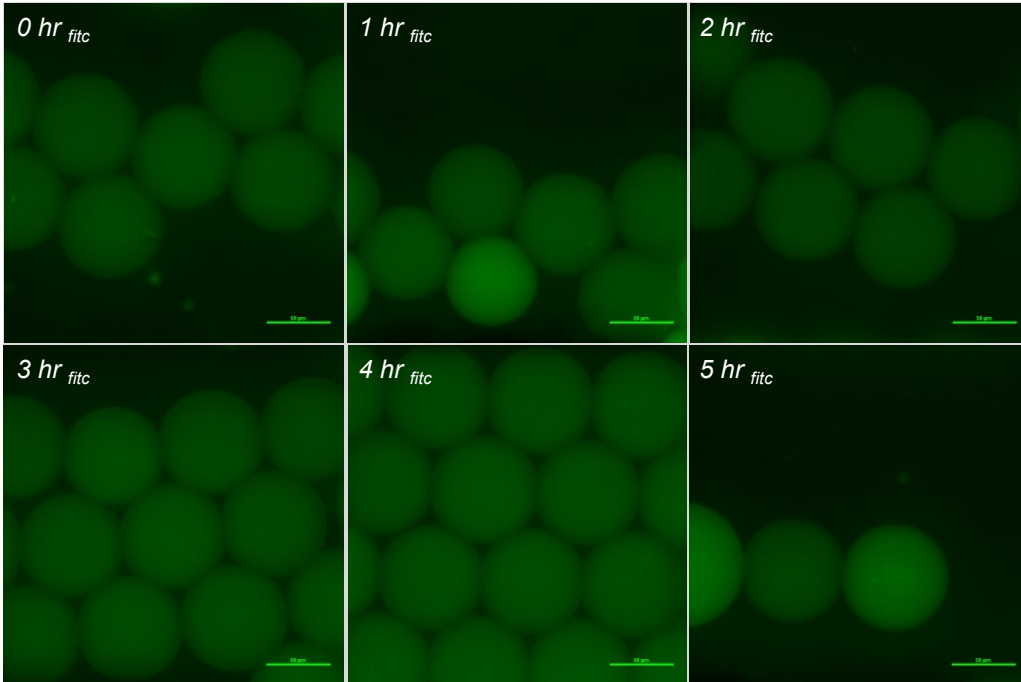
**Figure 4.6:** Bright field images of *Salmonella* incubation in-droplet with sterile deionized water, and 10 µg/ml FITC-Ab in 1x RV broth at 37°C.



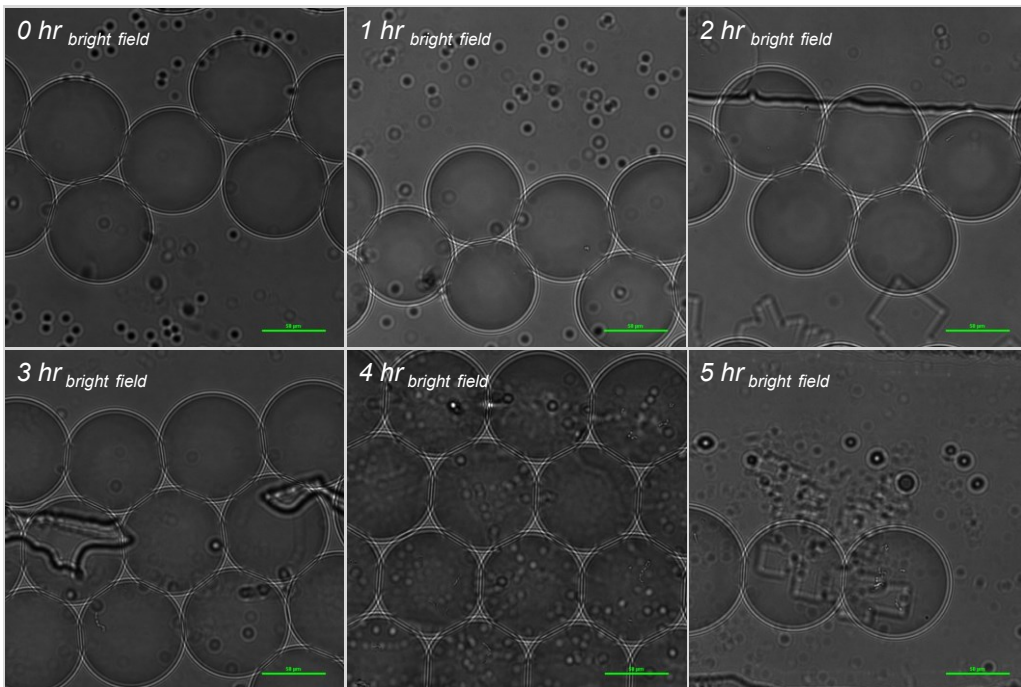
**Figure 4.7:** Fluorescent images of *Enterobacter* incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



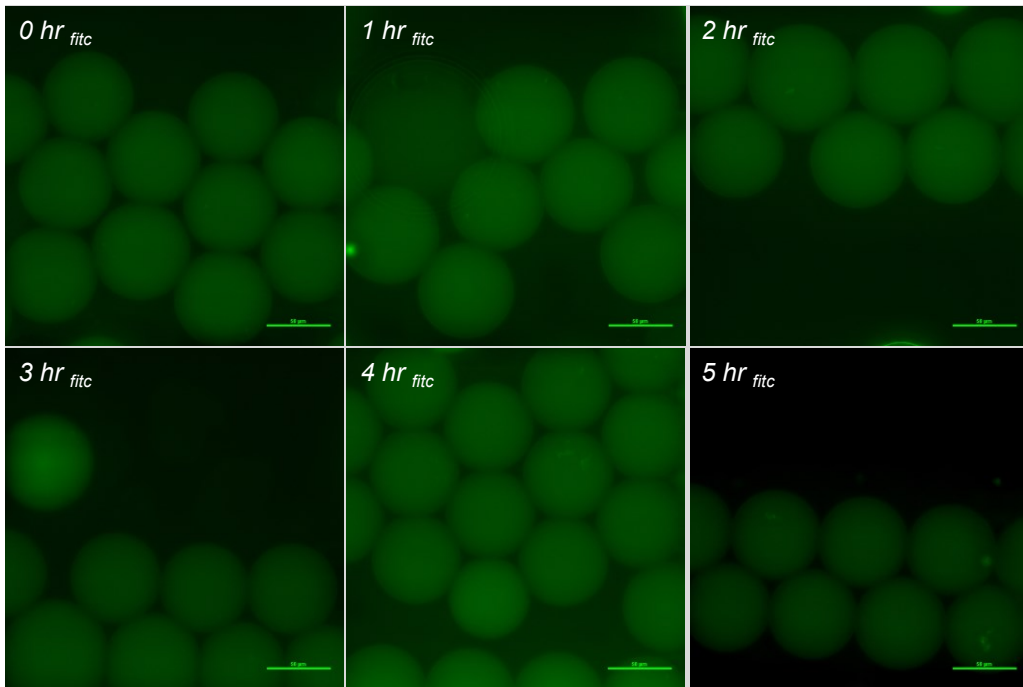
**Figure 4.8:** Bright field images of *Enterobacter* incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



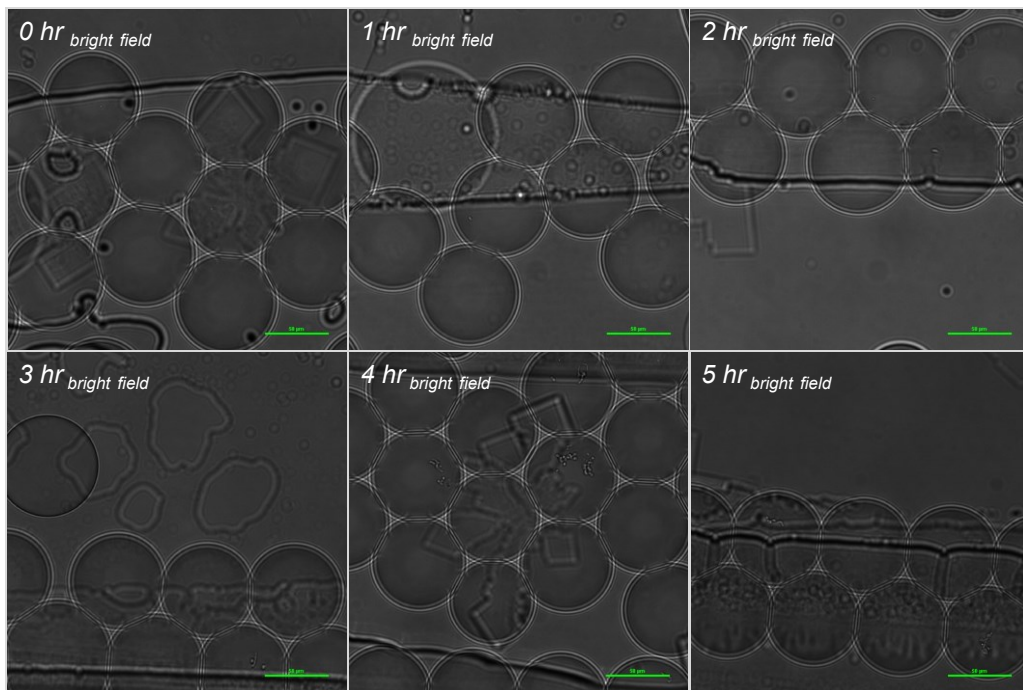
**Figure 4.9:** Fluorescent images of *E. coli* 700609 incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



**Figure 4.10:** Bright field images of *E. coli* 700609 incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.

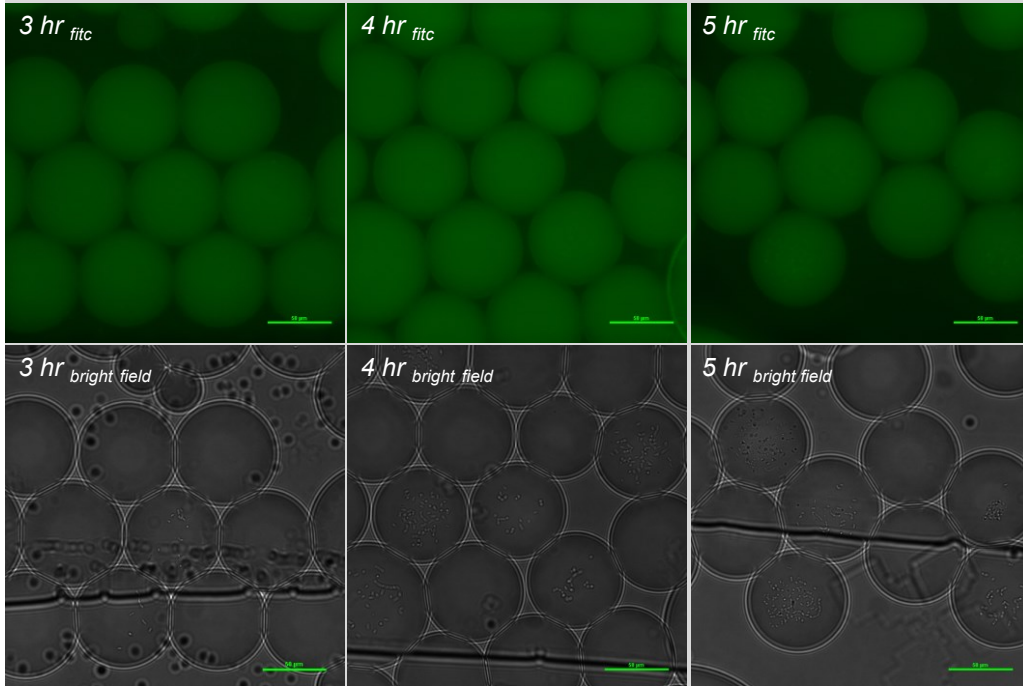


**Figure 4.11:** Fluorescent images of *E. coli* 13706 incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.

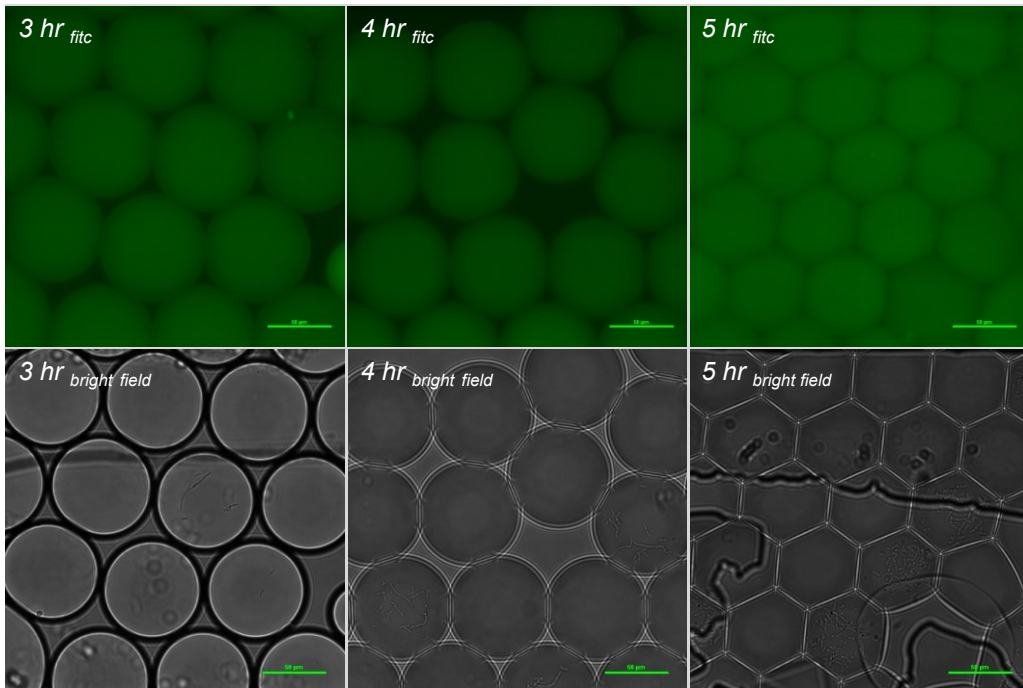


**Figure 4.12:** Bright field images of *E. coli* 13706 incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



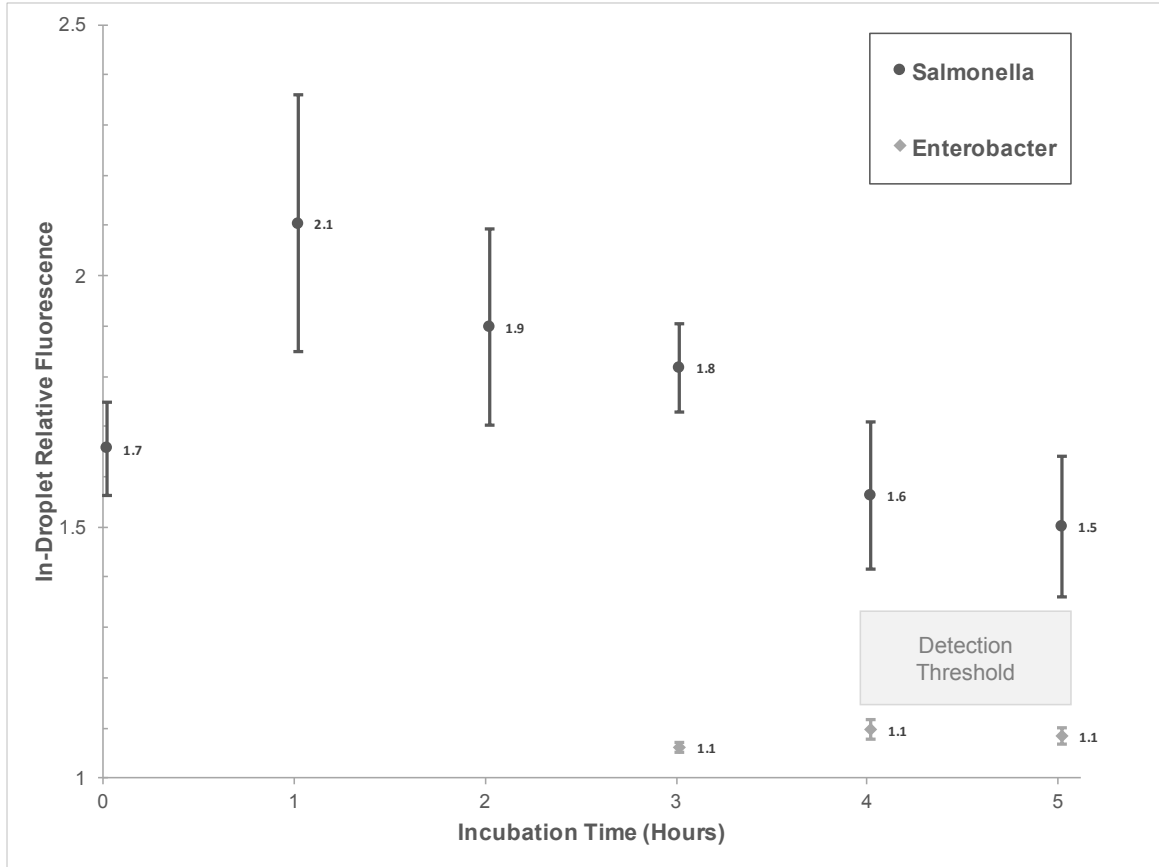


**Figure 4.13:** Images of *E. coli* 700891 incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C. No relative fluorescence detection at hours 0 through 2.

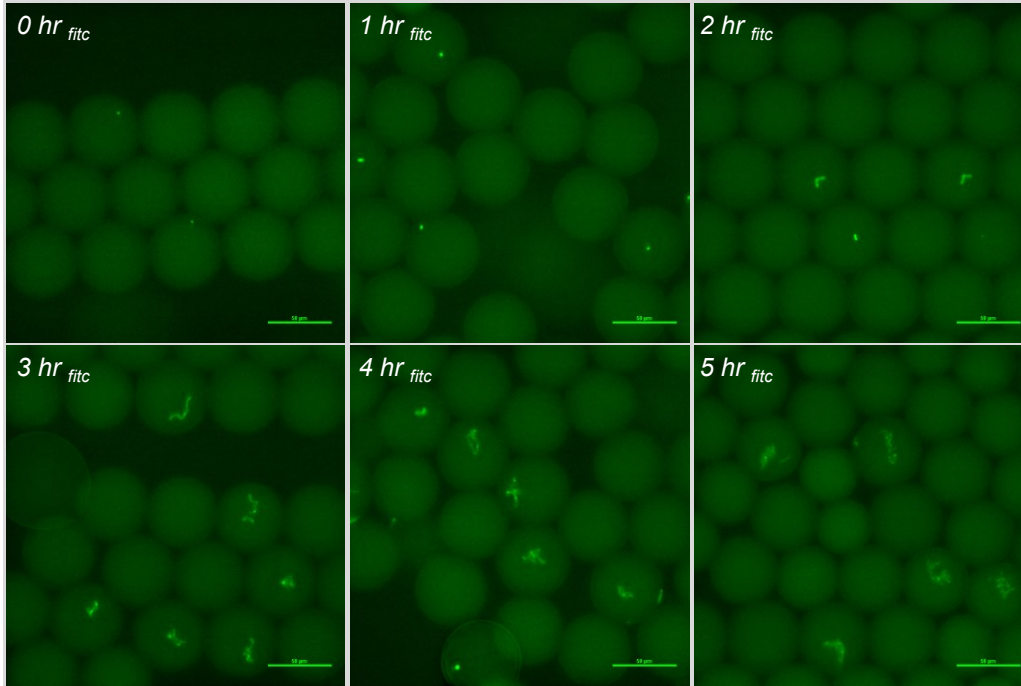


**Figure 4.14:** Images of *Citrobacter* incubation in-droplet with sterile deionized water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C. No relative fluorescence detection at hours 0 through 2.

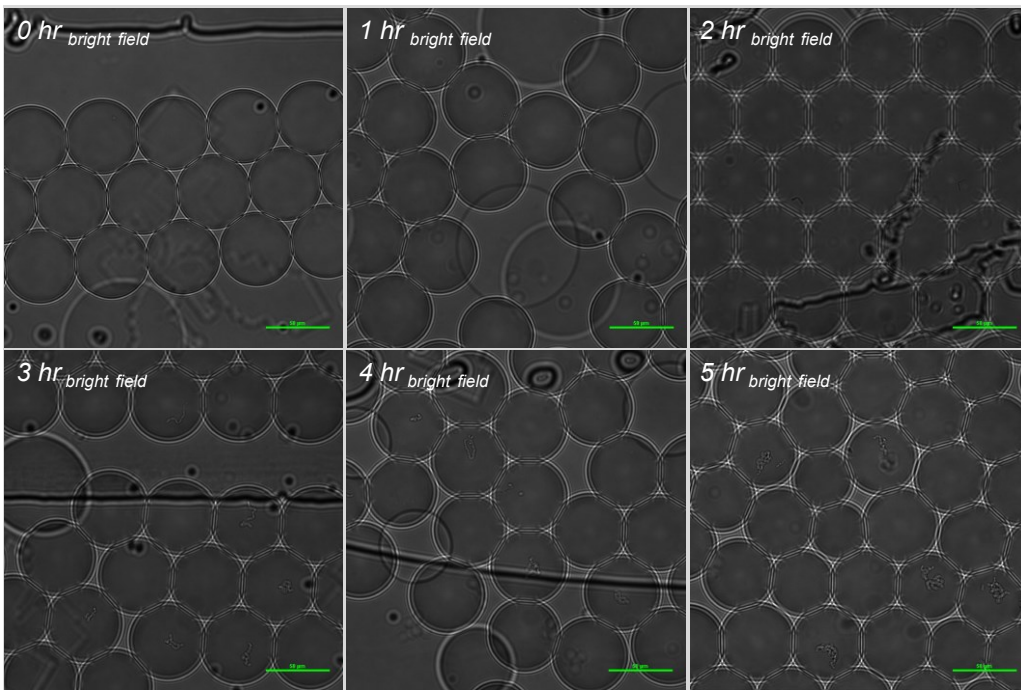
*Salmonella* and *Enterobacter* were then spiked into shredded lettuce wash water collected at a major ready-to-eat produce processing facility on the East Coast. The wash water physiochemical properties at time of collection were 17.05 mg/L organic chloramine, 50.5 nephelometric turbidity units, 4.37 pH (after thawing from stock, 5.97 pH), and a conductivity of 1,008 (mS/cm). Any remaining chlorine (organic chloramines) were quenched by 1 mM sodium thiosulfate and the wash water was filtered to remove any debris 0.22  $\mu\text{m}$  or larger. The target bacterial concentration was  $10^6$  CFU/ml for both *Salmonella* and *Enterobacter*. This bacterial cell concentration ensures there is approximately one bacterial cell encapsulated every two to ten droplets formed. Relative fluorescence of the encapsulated bacteria and growth kinetics at 37°C from zero to five hours incubation were measured (Figures 4.15). Fluorescent images with embedded metadata were used to acquire relative fluorescence via Ni-E Elements software and visual confirmation of growth detection for *Salmonella* and *Enterobacter* (Figures 4.16 through 4.19).



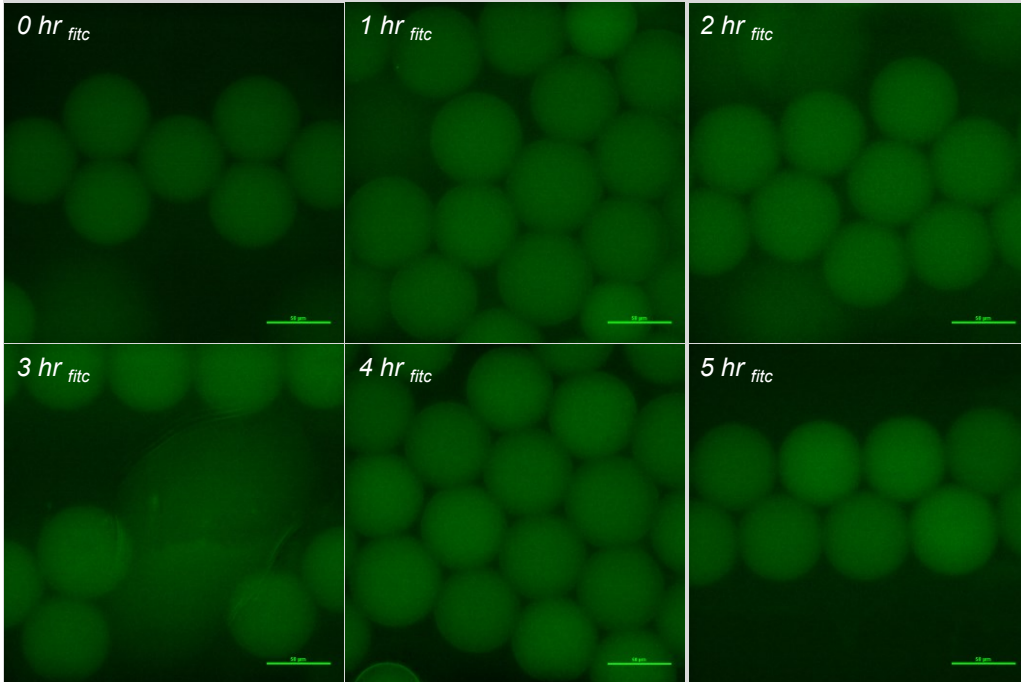
**Figure 4.15:** Relative fluorescence of encapsulated *Salmonella* and *Enterobacter* in shredded lettuce wash water (0.5x in-droplet) and incubated in Rappaport-Vassiliadis broth (1x in-droplet) for five hours with a FITC-Ab concentration of 10 ug/ml in-droplet. The trend line represents a decrease in relative fluorescence of *Salmonella* over the time course. A potential detection threshold region is identified on the figure. No measurable relative fluorescence was identified in time points 0 through 2 for both *Enterobacter*. Five replicates were measured for each bacterial species at each time point.



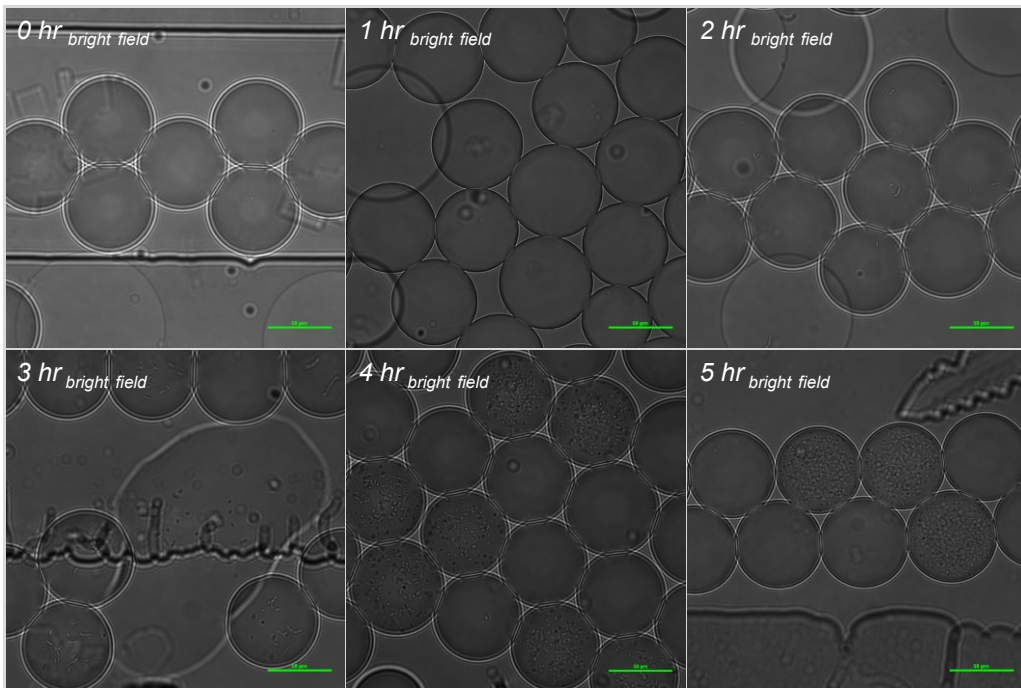
**Figure 4.16:** Fluorescent images of *Salmonella* incubation in-droplet with 0.5x shredded lettuce wash water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



**Figure 4.17:** Bright field images of *Salmonella* incubation in-droplet with 0.5x shredded lettuce wash water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



**Figure 4.18:** Fluorescent images of *Enterobacter* incubation in-droplet with 0.5x shredded lettuce wash water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.



**Figure 4.19:** Bright field images of *Enterobacter* incubation in-droplet with 0.5x shredded lettuce wash water, and 10 ug/ml FITC-Ab in 1x RV broth at 37°C.

Published research has reported faster bacterial growth rates for bacteria incubated inside droplets, when compared to growth rates using traditional methods (Churski et al., 2012). Separate tubes (one tube per each time point) were also collected in parallel for both *Salmonella* and *Enterobacter* incubation experiments in order to perform aerobic spread plate counts and analyze observed growth rate in-droplet. Droplets were lysed using PFO, diluted accordingly, spread to TSA plates, and incubated over night at 37°C. Aerobic plate counts indicated both *Salmonella* and *Enterobacter* were in lag phase from zero to approximately two hours in-droplet. Both *Salmonella* and *Enterobacter* initiated exponential growth at two hours and replicated exponentially until hour four of incubation. Observed lag times and log<sub>10</sub> growth phase generation time in-droplet using aerobic plate counts are summarized and compared to observed lag times and log<sub>10</sub> growth measured in Chapter 3 (Table 4.3).

**Table 4.3:** Comparison of Observed Lag and Generation Times for *Salmonella* and *Enterobacter* Outside and Inside Droplet

Bacteria	Generation and Lag Time, Non-Droplet <sup>a</sup>	Generation and Lag Time, In-Droplet <sup>b</sup>
<i>Salmonella</i>	40 <sup>c</sup> (6) <sup>d</sup>	27 (1-2)
<i>Enterobacter</i>	49 (7.5)	21 (1-2)

<sup>a</sup>: Turbidity/absorbance used for observed lag time and generation time, 200 ul of 10<sup>3</sup> CFU/ml starting concentration

<sup>b</sup>: Aerobic spread plate count used for observed lag time and generation time, 1 bacterial cell per 50-70 um droplet

<sup>c</sup>: Generation time in minutes

<sup>d</sup>: Observed lag time in hours

## 4.5: DISCUSSION

Initial experiments focused on assessing specificity of the FITC-Ab for *Salmonella*, and the cross-reactivity of with non-*Salmonella* species. Potential media contribution to fluorescence were eliminated by utilizing PBS for these experiments. Moreover, by suspending both bacterial cells and FITC-Ab in PBS, a more precise ratio of bacterial fluorescence to

background could be assessed. A relatively high concentration of bacterial cells (approximately 100 cells) was generated in-droplet to better visualize cross-reactivity of the FITC-Ab with negative controls, and to provide ample sites for relative fluorescence measurements. The anti-*Salmonella* FITC-Ab exhibited good sensitivity by binding to all encapsulated *Salmonella* via *Salmonella's* CSA-1 (Figure 4.2), and displayed robust relative fluorescence values (Figure 4.1). Deficiencies in specificity, however, are exposed by evaluating calculated relative fluorescence values and fluorescence images for the three *E. coli* strains and *Enterobacter* (Figures 4.1, 4.2, and 4.3). Cross-reactivity with the FITC-Ab and members of the negative control panel was anticipated as *E. coli*, *Enterobacter*, and *Salmonella* are similar in many ways. All three are gram-negative, from the *Enterobacteriaceae* family, and can be found in the gastrointestinal tract of an animal host (Winfield and Groisman, 2003). For this reason, use of a *Salmonella* selective broth, RV, was then utilized to suppress growth of non-*Salmonella* species in-droplet.

Assessment and evaluation of medias identified by the U.S. Food and Drug Administration Bacteriological Analytical Manual was carried out, and RV broth was identified as the best candidate for in-droplet use to detect *Salmonella* (Chapter 3). Two key aspects must be noted for the use of RV broth in this study. RV broth exhibits fluorescence masking of the FITC-Ab, and this masking reduces relative fluorescence units by approximately one  $\log_{10}$  (Figure 3.5, Chapter 3). Therefore, FITC-Ab concentrations were increased from 0.83 ug/ml to 10 ug/ml for in-droplet RV broth experiments carried out in this study. Also, *Salmonella* had a much higher generation rate in RV broth at 37°C instead of

the manufacturer-directed incubation temperature of 41.5°C (RV R10 Broth, Becton Dickinson) (Figure 3.1 and Table 3.2, Chapter 3).

RV broth performed as expected in suppressing most non-*Salmonella* species under test, based on experiments outside of droplets. While growth was not completely inhibited, all *E. coli* species were noticeably suppressed by the presence of RV broth (Figures 4.9 through 4.13). One *E. coli* strain evaluated, ATCC 700891, was particularly suppressed. For instance, relative measurements were unattainable at time points zero through two hours (Table 4.2; Figures 4.4 and 4.13). The FITC-Ab cross-reacted minimally with *Citrobacter* in both PBS and RV broth with sterile deionized water. This is a promising observation as *Citrobacter* is one of two bacterial species evaluated capable of growing in RV broth at 37°C.

In experiments utilizing PBS and RV broth in sterile deionized water, the FITC-Ab had moderate-to-high cross reactivity with *Enterobacter* (Table 2; Figure 1). Moreover, *Enterobacter* grows well in RV broth at 37°C (Figures 4.4, 4.7, and 4.8). For this reason, *Enterobacter* was selected to be tested as the negative control when spiking the real-world shredded lettuce wash water acquired from a major ready-to-eat produce processing facility. Fluorescent images with embedded metadata were captured every hour and analyzed for a five-hour incubation of both *Salmonella* and *Enterobacter* (Figures 4.16 through 4.19). The wash water conditions under test were intended to simulate a highly contaminated sample, and encapsulate one bacterial cell per two-to-ten droplets generated. This allowed for monitoring of growth kinetics in-droplet over the five-hour time course. Both *Salmonella* and



*Enterobacter* had reduced relative fluorescence values when compared to experiments utilizing RV broth and sterile deionized water (Figures 4.4 and 4.15).

The manufacturer of the RV broth formulation utilized in this study states the pH is 5.1 at 25°C (Becton Dickinson). Moreover, it has been reported that FITC fluorescence intensity has a direct correlation with pH gradients. Ma et al. explored the relationship between decreasing FITC intensity with decreases in pH (Ma et al., 2004). Utilizing the figures reported by Ma et al., there is nearly a  $\log_{10}$  reduction in fluorescence intensity of FITC between a 7.5 pH solution and a 5.0 pH solution. This fluorescence relationship with pH could help explain the masking effect observed from RV broth when compared to other medias (Chapter 3). A pH-related FITC intensity relationship is also something to consider in relation to the shredded lettuce wash water. The pH of the shredded lettuce wash water was 4.37 upon collection, and measured to have a pH of 5.97 after thawing from stock. A 20 ml volume of 50/50 2x RV broth and shredded lettuce wash water mixture (simulating in-droplet concentrations) was measured and had a pH of 5.26. This low pH likely explains the reduction in fluorescence intensity observed in the wash water experiments.

Bacterial growth in-droplet was also evaluated and compared to growth outside of droplets. Both *Salmonella* and *Enterobacter* exhibited faster generations times, both were reduced by 32% and 57%, respectively (Table 4.3). Droplet-based incubation of bacteria is thought to produce faster growth rates than more traditional culturing techniques due to rapid mixing, reduced contaminants, reproducibility of droplets, and small volumes (Churski et al., 2012). Lag times were minimal in-droplet as well. *Salmonella* and *Enterobacter* initiated

log phase growth between one and two hours of incubation. These observed increased growth rates, and decreased lag times, further showcase microfluidic droplets as a rapid application for viable bacterial detection. It should be noted growth rates outside of droplets were calculated using turbidity measurements for growth, and these samples under test did not contain FITC-Ab.

#### **4.6: CONCLUSION**

Foodborne illness is an ever-present international threat to public health (Havelaar et al., 2015). In the U.S. alone, 1 in 6 people will acquire foodborne illness annually and this national burden is estimated to cost between \$55.5 and \$93.2 billion (Scharff, 2015). Fresh-cut produce and leafy greens are often implicated in foodborne outbreaks, and it has been estimated leafy greens are the most common cause of foodborne outbreaks in the U.S. (HERMAN et al., 2015). Developing cutting-edge methods for identification of food contamination that can be implemented in food industry HACCP control strategies are critical to ensure a safer food supply in the future (Hyde et al., 2016; Mortimore, 2001). Applications with in-droplet microfluidics show great promise for rapid, same-day (or same-shift) and viable bacterial detection at CCP's, such as fresh-cut produce wash waters.

**Acknowledgements:** Dr. Shih-Chi Weng, Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health; Applied Biological Sciences Group, Johns Hopkins University Applied Physics Laboratory; ERC Grant; The Osprey Foundation of Maryland.

#### 4.7: CHAPTER 4 REFERENCES

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## Chapter 5: Conclusion and Next Steps

Foodborne outbreaks and associated illnesses will remain a persistent threat to public health and a chief biosecurity concern for the food industry, particularly fresh-cut and ready-to-eat produce industries (Crim et al., 2015; Crowe et al., 2015; Doyle et al., 2015). It is critical for the research and regulatory communities to continually improve upon current detection methods and work to develop improved technologies that can be implemented at critical control points (CCP's) along the farm-to-consumer route (Arora et al., 2011; Bonilauri et al., 2016; Priyanka et al., 2016). Fresh-cut produce wash waters are a critical CCP in the route of the ready-to-eat produce chain due to minimal processing and potential for cross-contamination (Rajwar et al., 2016).

Molecular detection using qPCR is a method that can be used by the food industry to identify if produce wash waters are contaminated (Bhagwat, 2004). This dissertation utilized produce wash waters acquired from a major, Mid-Atlantic produce processing facility to explore inhibition challenges for direct detection of *Salmonella* using qPCR. Data analyzed in this dissertation suggest organic chloramine concentrations are the primary physiochemical constituent contributing to qPCR inhibition. Common chlorine quenchers, such as sodium ascorbate and sodium thiosulfate, were found to effectively remove inhibition and did not impact sensitivity of the qPCR reaction.

Microfluidic droplet-based detection was also explored in this dissertation. This developing technology shows much promise for rapid isolation and detection of ready-to-eat wash water bacterial contaminants, such as *E. coli* or *Salmonella* (Bridle et al., 2014; Gil et al., 2009). Microfluidic-related research here evaluated traditional medias commonly used for *Salmonella*, optimized fluorescently-labeled antibody concentrations, and isolated and detected *Salmonella* in both sterile deionized water and a real-world shredded lettuce wash water spiked with a known concentration of *Salmonella*.

This research utilized a fluorescein- (FITC)-conjugated anti-*Salmonella* antibody. FITC performed adequately in generating detectable and measureable intensities to compare relative fluorescence values from *Salmonella* and non-*Salmonella* negative controls. However, FITC does exhibit pH sensitivity by way of a reduced fluorescence intensity at lower pH, such as 4-5 (Ma et al., 2004). This low pH property of FITC best explains the reduction in fluorescence intensity observed when using Rappaport-Vassiliadis (RV) broth in-droplet to select for *Salmonella* and suppress non-*Salmonella* growth. There are a number of modern fluorophores available with comparable excitation and emission wavelengths that are pH insensitive, such as Oregon Green or Alexa Fluor 488 (Invitrogen, 2010; Mottram et al., 2006). Substituting an alternative fluorophore antibody conjugate for FITC could minimize fluorescent intensity degradation due to a low pH, as well as improve photostability and initial brightness observed (Lavis, 2017; Wysocki and Lavis, 2011).

This dissertation performed in-droplet microfluidic media, fluorescently-labeled antibody, microfluidic flow rate, and microscopic relative fluorescent signal optimization for



detection of *Salmonella* in produce wash waters. This work is intended to be utilized for future automated optical enumeration. A critical aspect of optimization for optical enumeration in future studies will be the combination of an in-line laser on the microfluidic platform and fluorophore utilized. In order to maximize fluorescent intensity efficiency and relative fluorescence detection, the optimal fluorophore for the in-laser utilized must be selected and conjugated (or purchased already conjugated) to the antibody utilized for detection. The lasers currently available for in-line excitation on the microfluidic platform in the Applied Biological Science Group (QPA), Johns Hopkins University Applied Physics Laboratory (JHUAPL), are listed in Table 5.1 and paired with readily available fluorophores for antibody conjugation from ThermoFischer Scientific.

**Table 5.1:** Readily Available Fluorescent Dyes for Antibody Conjugation\*

Laser Source (nm)	Fluorescent Dye	Excitation (laser-line, nm)	Emmision (max, nm)	Initial Brightness <sup>a</sup>	Photostability <sup>b</sup>	pH Sensitive
360	Alexa Fluor 350	350	442	1	1	No
473	Alexa Fluor 488	488	525	4	2	No
	Oregon Green	488	526	4	3	No <sup>c</sup>
	FITC <sup>d</sup>	488	525	3	1	Yes
532	Alexa Fluor 532	532	554	3	2	No
	TRITC <sup>e</sup>	488/532	576	4	4	No
	Cy3	488/532	568	3	4	No
635	Cy5	633/647	666	5	3	No

\* Technical data summarized from ThermoFisher Scientific

a: scale of 0 to 5; 0 = no fluorescence and 5 = the highest initial brightness

b: scale of 0 to 5; 0 = no photostability in buffer and 5 = the highest photostability in buffer

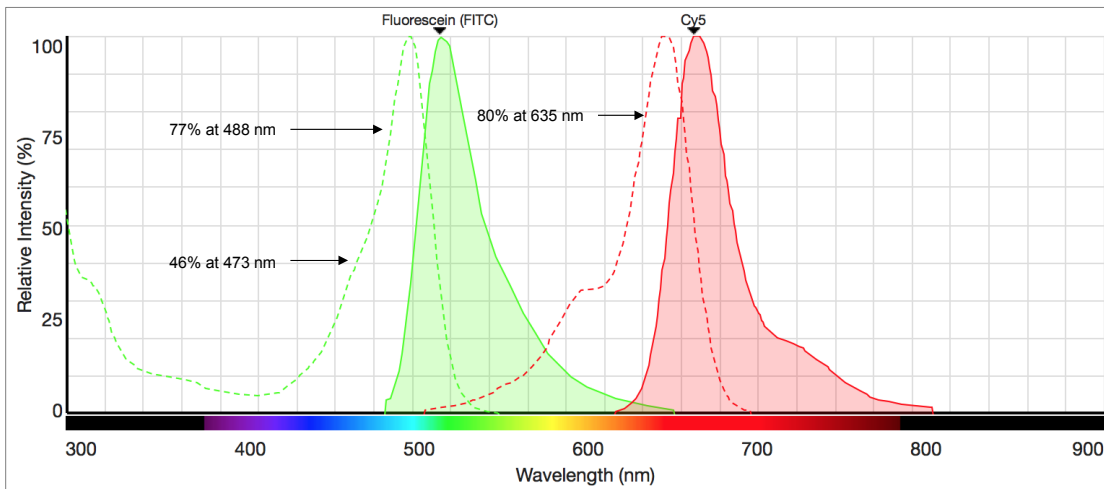
c: pH insensitive in the physiological range of 7-9 pH; pH sensitive in acidic conditions

d: fluorescein isothiocyanate

e: tetramethylrhodamine

Using available laser resources available at QPA, JHUAPL and technical data summarized in Table 5.1, a fluorophore better aligned with in-line laser excitation, such as TRITC, Cy3, or Cy5, is better suited for automated enumeration by relative fluorescence. This dissertation explored the potential for automated enumeration using a FITC-labeled anti-*Salmonella* antibody, however, observed relative fluorescence to background in-droplet

was not optimal. This was likely due to non-optimal in-line laser excitation (473 nm) and pH sensitivity of FITC (Diehl and Markuszewski, 1989; Shapiro, 1983). For instance, relative fluorescence of FITC at 473 nm is only 46% efficient, compared to 77% efficiency at 488 nm. Using Cy5, relative fluorescence is 80% efficient with a 635 nm excitation (Figure 5.1).



a: Figure and corresponding data generated using Fluorescence SpectraViewer, ThermoFisher Scientific

**Figure 5.1:** Impact Laser-Line Excitation has on Relative Fluorescence Intensity (%)<sup>a</sup>

This dissertation characterized qPCR inhibition challenges, evaluated medias, fluorescently-labeled antibody concentration, and microscopically confirmed specific detection of *Salmonella* in fresh-cut produce wash waters. *Salmonella*, and other foodborne bacterial contaminants, will remain a principle public health concern for the foreseeable future. Developing and refining technical applications to detect and counter pathogenic contamination at CCPs throughout the farm-to-consumer route is a key principle of food industry hazard analysis and critical control point programs. Using research and lessons

learned in this dissertation, researchers and food industry regulators can further improve capabilities to rapidly detect viable bacterial foodborne pathogens within 4 to 5 hours.

Improved food safety is critical moving forward, and can mitigate an annual disease burden of 1 in 6 Americans at an estimated cost of \$1,068 to \$1,626 per episode – a total health-related cost of \$51 to \$76.1 billion per year in the U.S. (Cody and Stretch, 2014; Scharff, 2012). From a public health standpoint, the up-side to improved detection and prevention of foodborne outbreaks is clear by potentially lessening the burden of foodborne illness. It is important to consider food safety-related policy implications, implementation, and the ultimate cost to farmers at the local, regional, and national scale. Prior to implementation of the 2011 FSMA, a 1998 survey of New York fruit and vegetable growers analyzed the management strategies for manure, compost, and water quality as they relate to food safety risk (Rangarajan et al., 2002). Rangarajan and colleagues found 92% of growers washed produce on the farm, although, only 16% reported using sanitizers (Rangarajan et al., 2002). At the time, the researchers identified the need for small farms (less than 100 acres) to improve training in three specific areas: 1) record keeping of manure applications, 2) composting processes to achieve pathogen kill, and 3) sanitization of produce wash waters (Rangarajan et al., 2002).

A recent survey of Mid-Atlantic leafy green and tomato growers investigated the prevalence and cost of produce safety practices under the Produce Rule of FSMA, and found safety practices for water testing, soil amendments, and product sampling varied by farm size (Lichtenberg and Tselepidakis Page, 2016). Lichtenberg and Tselepidakis report

the burden of complying with the FSMA Produce Rule increased with decreasing farm size (Lichtenberg and Tselepidakis Page, 2016). In another recent study, The Pennsylvania State University Extension Service collected empirical data from Pennsylvania-based produce growers participating in the Extension Service's on-farm food safety program for knowledge, attitudes, and skill in good agricultural practices (GAP) (Tobin et al., 2013). Tobin and colleagues found increased technical information among produce growers did not necessarily translate to execution of GAP fundamentals (Tobin et al., 2013). However, the researchers did identify farm size and the overall desire for growers to contribute to a safe food supply as motivating factors to practice sound GAP (Tobin et al., 2013).

In 2015, implementation of the FSMA directives, such as mandatory prevention-based controls and recall authority, increased facility inspections, and increased consumer communication, was estimated to cost the U.S. tax payer a minimum of \$1.4 to \$1.6 billion over the next five years (Drew and Clydesdale, 2015). Drew and Clydesdale also identify increased food prices as a downstream consequence of FSMA (Drew and Clydesdale, 2015). Projected cost to the U.S. tax payer for improved, long-term food safety is substantial, but should be more palatable when weighed against prospective reductions in health-related costs and overall burden of foodborne illness in the U.S. Ensuring improved food safety in the future will require continued effort from researchers, regulators, and policy makers to work together on addressing diverse aspects of the complex foodborne illness paradigm.

## CHAPTER 5 REFERENCES

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# Curriculum Vitae

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Clearance: Top Secret/Sensitive Compartmentalized Information

## Research and Professional Experience

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### Johns Hopkins University School of Public Health and Applied Physics Laboratory

*Ph.D. Candidate:* Johns Hopkins University, 2010 to present (with a two-year leave of absence for Active Duty mobilization). Develop robust, deployable and potentially on-site methods for food-processing facilities and on-site field investigations to prevent Salmonella outbreaks. Research has focused on evaluating quantitative polymerase chain reaction inhibitive properties and practical methods to overcome inhibition in molecular analysis. Research also aims to rapidly culture, identify, and differentiate *Salmonella* from mixed cultures in agricultural waters by merging on-chip microfluidic droplet and activated fluorescence high-throughput technologies. This detection method for *Salmonella* has the potential to reduce viable culture confirmation by more than two and half days.

### Joint Exercise Deployment Detachment, Alexandria, VA (Army Reserves)

*Intelligence Officer in Charge:* Joint Exercise Deployment Detachment's Exercise Element, from 2014 to present, conducting and leading low-visibility ground and aerial intelligence, advanced geospatial intelligence, and computer network operations in response to validated national-level requirements. Trains, deploys, and employs specialized intelligence skills gaining access to environments to conduct intelligence operations achieving decisive effects for decision makers. Contributes to the recruitment, assessment, selection, and training of candidates for the unit. Participates in full mission profile training missions with other team members to provide the commander with feedback to determine if candidates are able to detect surveillance, portray a non-alerting profile, and are prepared to conduct worldwide operations. Redacted Officer Evaluation Reports available upon request.

### Department of Defense Activity, United States Army, Fort Meade, MD (Active Duty)

*Intelligence Officer in Charge:* Department of Defense Activity, from July 2012 to July 2014. Active duty mobilization from reserve status to serve as the lead intelligence officer for a battalion-sized activity within a brigade-level unit conducting worldwide ground- and air-based, multi-disciplined intelligence collection for validated national requirements. Provide physical, information, automation, and communications security to the unit's multiple sensitive compartmented information facilities. Responsible for the management of signature reduction programs, intelligence, and counterintelligence support. Serve as the principle Special Access Program manager, Special Security Officer, Intelligence Oversight Officer, Information Assurance Officer, and Signature Reduction and Management Officer. Redacted Officer Evaluation Reports available upon request.

**Johns Hopkins University Bloomberg School of Public Health in Baltimore, MD**

*Teaching Assistant*, Issues for Water and Sanitation in Tropical Environmental Health, Spring 2012

**Johns Hopkins University Bloomberg School of Public Health in Baltimore, MD**

*Teaching Assistant*, Food- and Water-Borne Diseases, Spring 2012

**Johns Hopkins University Bloomberg School of Public Health in Baltimore, MD**

*Teaching Assistant*, Principles of Environmental Health Sciences, Fall 2011

**Joint Intelligence Task Force–Combating Terrorism, DIA, Washington, DC (Active Duty)**

*Counterterrorism Intelligence Officer*, Chemical, Biological, Radiological and Nuclear Branch, August 2006 to August 2010. Senior Analyst and researcher for all-source intelligence assessments, counterterrorism operational support and collection development products for strategic, operational, and tactical counterterrorism missions. Focused on in-depth pre-operational indicators of terrorist activity and modus operandi. Participated in 24/7 crisis support at the Defense Intelligence Analysis Center, Pentagon, Intelligence Agencies and Combatant Commands. Provided tailored counterterrorism briefings and augmented special mission units with analysis to support operations. Deployed to both Afghanistan and Iraq to provide counterterrorism analysis and targeting support to conventional forces, joint special operations forces and special mission units

**Rochester Institute of Technology, Rochester, NY**

*Graduate Student*, Clinical Chemistry, December 2003 to December 2005, Researched the identification, origin, and application of naturally-occurring organic halocarbons for use in pharmaceuticals, and the overall impact naturally-occurring organic halocarbons, such as methane and chloroform, have on the environment.

**Education and Certifications**

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**Johns Hopkins Bloomberg School of Public Health, Baltimore, MD**

Ph.D. Candidate, Environmental Health Engineering, In-Progress

Dissertation: “*Rapid Detection of Viable Bacteria in Agricultural Wash Waters – A Droplet-based Approach*”

**Johns Hopkins Bloomberg School of Public Health, Baltimore, MD**

Certificate in Risk Sciences and Public Policy, May 2011

**Rochester Institute of Technology, Rochester, NY**

M.S. Clinical Chemistry, December 2005

## **Clemson University, Clemson, SC**

B.S. Plant Sciences, December 2002

### **Military Education**

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Military Intelligence Captains Career Course, Fort Huachuca, AZ 2014

Military Intelligence Officer Basic Course, Fort Huachuca, AZ 2006

Joint Operations Support Course, Washington, DC 2012

U.S. Army Airborne Course, Fort Benning, GA 2013

### **Joint Intelligence and Interagency Education**

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Nuclear Awareness and Technical Response to Nuclear Threats, Fort Story, VA 2008

Chemical and Biological Warfare Intelligence Course, JMITC, Washington, DC 2007

Asymmetric Warfare Intelligence Analysis Course, JMITC, Washington, DC 2007

Adversarial Deception Analysis Course, JMITC, Washington, DC 2006

Counterterrorism Analysis Course, JMITC, Washington, DC 2006

### **Presentations**

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Oral presentation: Department of Clinical Chemistry Seminar Defense “Naturally-Occurring Organohalogens” J. Brian Harmon and Jim J. Worman. Rochester Institute of Technology, Rochester, NY. November, 2005.

Poster presentation: Regional Seminar “Naturally-Occurring Organohalogens” J. Brian Harmon and Jim J. Worman. American Chemical Society, Rochester, NY. June, 2005.

Poster presentation: Seminar Series “Naturally-Occurring Organohalogens” J. Brian Harmon and Jim J. Worman. Rochester Academy of Sciences, Rochester, NY. April, 2005.

### **Professional Memberships**

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- Reserve Officers Association of America

### **Collaborations**

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- PhD Research Advisor, Dr. Kellogg Schwab, Professor, Department of Environmental Health Sciences, Johns Hopkins University

- PhD Research Co-Advisor, Dr. Charles Young, Senior Scientist, Applied Physics Laboratory, Johns Hopkins University
- Mr. Rich Mahone, Naval Special Warfare Development Group, U.S. Navy, Department of Defense

#### **Additional Skills and Activities**

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- Find, Fix, Finish, Exploit and Analyze Targeting Methods and Tactics
- All-Source Intelligence Collection Management and Targeting

#### **References**

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Available upon request