Novel approaches to the low-cost, portable and rapid detection of bacterial pathogens in foods and food-processing environments

by

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DEDICATION

This dissertation is dedicated to my parents, Keith and Denise Hice, for their encouragement and words of wisdom during the completion of my PhD. I also dedicate this dissertation to Elliot Bauman, for his continued love and support during our extended time apart.

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ABSTRACT

Continued outbreaks of foodborne illness involving dairy products in the United States stress the importance for rapid methods of detection of pathogenic microorganisms in food processing environments. Pathogenic microorganisms, such as *Salmonella* are widespread, and can be found in a variety of foods, ingredients and in industrial environments. The presence of pathogens in dairy products constitutes great risk for increased exposure, illness and reduces overall quality of the foodstream. As a result, emphasis has been placed on adapting or developing sensitive techniques to rapidly detect notable pathogens, such as *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in both contaminated foods and industrial environments. Common assays employed in the detection of pathogenic microorganisms, though effective in identification, are time consuming and may require several days for processing. The necessity to quickly screen food products and industrial environments has led to an emphasis to develop rapid, sensitive, automated techniques in food processing operations. Numerous methods of identification and detection have been implemented in food processing environments.

An optimal approach to the rapid detection of microbial pathogens would incorporate several advantages including: **1)** improved time-to-result, **2)** low-cost, **3)** ease of operation and **4)** simple interpretation. Such an approach may enable simple and cost-effective sampling of pathogenic microorganisms, which can be used to improve industrial efficiency. As a possible alternative to existing detection efforts, low-cost diagnostic (LCD) tools, particularly paper-based analytical devices (PADs), may be employed for rapid, sensitive and selective detection. PADs are frequently combined with colorimetric detection, in which chromogenic substrates are used to yield a visual representation of detection. Different enzyme-substrate pairs may be employed to

accomplish various goals—from simple "presence/absence" to species-specificity. While "presence/absence" is limited, the use of shared enzymes is advantageous during detection and identification of metabolic state. Depending upon environmental factors, bacteria may exist in active or dormant states; reversion of a pathogen from dormancy to a metabolically active state may result in rapid growth and instances of illness.

As the level of enzymatic expression varies between metabolic states, oxidoreductases and alkaline phosphatases (ALP) were investigated as vehicles for colorimetric detection. Oxidoreductases are present in greater amounts in metabolically active bacteria, and are capable of reducing 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) to formazans. Nitrophenyl phosphate (PNPP) is present in dormant bacteria, and cleaves phosphate groups from para-nitrophenyl-phosphate salts, resulting in para-nitrophenol. Combined use of enzymatic substrates, including INT and 5-methylphenazin-5-ium methyl sulfate (PMS) for metabolically active bacteria, and INT and PNPP for dormant bacteria, yielded an improved colorimetric readout visible by eye within 30 min. With detection achieved within 30 min, the two assays, INT-PMS and INT-PNPP, decrease time-to-result, are portable and may be amenable to on-site detection in agricultural, environmental and industrial settings.

While the use of non-specific bacterial enzymes may limit some applications, immobilization of bacteria-specific bacteriophage (P22, T4) onto paper can provide an additional layer of specificity. Bacteriophage are robust, and may be easily absorbed onto paper. In this work, immobilized bacteriophage facilitated specific capture of *Salmonella* Typhimurium on paper, followed by detection of metabolic state with either the INT-PMS or INT-PNPP assay. This combined approach can be applied to the analysis of mixed cultures, given the generally genera-specific nature of the selected bacteriophages. Moreover, the use of chromogenic substrates

simplifies assay design, as color change is easily interpreted by the eye or with basic instrumentation. However, despite these advantages, the requirement for a 48-hour absorption period represents a drawback, lengthening time-to-result.

An alternative to the use of bacteriophage for cell capture are magnetic ionic liquids (MILs). MILs are magnetoactive "molten salt" solvents, containing a paramagnetic component integrated into the cation or anion moiety of the salt. MILs are considered "green" solvents, and are nonvolatile, nonflammable, with tunable physicochemical properties. Due to their hydrophobic and liquid nature, MILs can be quickly be distributed with agitation (stirring or vortexing) throughout aqueous food samples as liquid micro- or nanodispersions. After encountering and binding bacterial cells, cell-MIL complexes can then be collected magnetically or after density-driven sedimentation for further processing. MIL-based capture of bacteria has been previously combined with real-time polymerase chain reaction (qPCR) for the rapid detection of *E. coli*. While use of qPCR obviates the need for time-consuming steps such as gel electrophoresis, its inherent complexity and cost may prohibit its use in point-of-care or resource-limited settings. Isothermal methods for nucleic acid amplification, such as recombinase polymerase amplification (RPA), may have considerable advantages as alternatives to PCR. RPA results in exponential amplification of nucleic acids and operates at a constant, near-physiological temperature (~40°C), eliminating the need for a thermocycler, generating target-specific amplicons in less than 20 min.

The combined use of MIL-based extraction and rapid, streamlined pathogen detection using RPA was investigated. The ability of MIL solvents to quickly extract *Salmonella* Typhimurium was first examined by dispersing MIL into an aqueous suspension, followed by rapid (~30 s) physical enrichment (concentration) and extraction using an applied magnetic field. Following extraction, viable bacteria were desorbed from the MIL extraction phase with exposure

to a nutrient-rich broth (Luria Bertani medium), referred here to as a "back-extraction" step. In efforts to improve back-extraction, recovery of the model Gram-negative bacterium *Serratia marcescens* from the MIL extraction phase was investigated using several back-extraction media varying in ionic strength and nutrient composition. The highest recovery of cells was obtained using a nutrient-rich tryptone medium supplemented with NaCl. This modification of the extraction protocol enabled improvement in MIL-based bacterial concentration, enriching cells by a factor of 5 - 6X within 3–5 min.

The improved MIL assay was then examined in conjunction with RPA for rapid detection of *Salmonella* Typhimurium. MIL-based sample preparation was compared with use of a commercial sample preparation solution, PrepMan® Ultra Sample Preparation Reagent (PMU), for detection of *Salmonella* Typhimurium in artificially-contaminated pasteurized foods. PMU is commonly coupled with PCR to eliminate or inactivate PCR inhibitors and uses both heating and centrifugation steps. As an established method for sample preparation, use of PMU served as a benchmark method against which our MIL-based process was compared. In aqueous suspensions of *Salmonella* Typhimurium, detection was achieved as low as 10^3 CFU mL^{-1} using the combined MIL-RPA approach, which is equivalent to the previously investigated MIL-qPCR method, and, in our hands, outperformed the PMU method by an order of magnitude. Visualization of amplified products was achieved using gel electrophoresis or lateral flow readouts. Nucleic acid lateral flow immunoassays (NALFIA) require less than 5 min for amplicon visualization, are portable, require minimal technical expertise during interpretation and are easy to implement outside of laboratory settings. The need for electric-based heating elements for RPA incubation was eliminated through the use of low-cost, portable, supersaturated sodium acetate heat packs. This repurposing of

consumer-grade hand warmers for nucleic acid amplification is a novel approach and easily incorporated into the MIL-RPA scheme.

While MILs have been successfully used for capture and concentration of bacteria from foods prior to culture- or nucleic acid-based detection, little is known about their interactions with bacteria—including modes of physical association or potential antimicrobial activities. Further understanding these interactions may facilitate optimization of MIL-based capture in challenging food matrices, as well as modification of downstream procedures to mitigate the impacts of potential bacterial injury during extraction and concentration. To begin this work, a series of multi-strain panels, including seven representative *Salmonella* DNA subgroups and eight strains of *E. coli* O157:H7, were exposed to the Ni(II) MIL and plated in parallel on non-selective and selective media. Calculated enrichment factors (E_F) were similar between media types, while individual cell counts were nearly identical, suggesting that the Ni(II) MIL, as applied during our capture and concentration assay, does not cause assay-limiting cellular injury in these two pathogens. Observed variability between E_F values may result from differences in the extraction efficiency of the MIL, with some strains exhibiting weaker affinity for the MIL compared to other strains tested, which is an area of ongoing research. Importantly, our results demonstrate capture and recovery of strains representative of all seven *Salmonella* DNA subgroups and all eight strains of *E. coli* O157:H7 tested, with comparable recovery on non-selective and selective media. This initial and ongoing research on characterization of MIL-bacterial interactions establishes the foundation for further evaluation of new MIL structures for improving the preconcentration and recovery of viable microorganisms from complex food matrices.

CHAPTER 1

A SURVEY OF RAPID METHODS OF DETECTION OF FOODBORNE PATHOGENS IN DAIRY PRODUCTS AND PROCESSING ENVIRONMENTS

1.1 Introduction

Salmonella is a ubiquitous, Gram-negative genus of bacteria that is widespread in the environment, and can be found in many different foods, food ingredients and within industrial food processing environments (El-Gazzar & Marth, 1992). Infection frequently results from ingestion of tainted food products, or the fecal-oral route. Illness is often observed in children under the age of five, and adults over age 60—commonly referred to as "at risk populations." Foodborne illness typically results from the consumption of contaminated poultry, eggs and dairy products. Foods prepared on dirty surfaces, in industrial environments and restaurants, are vectors for illness (Murray, Rosenthal, & Pfaller, 2009). According to the Centers for Disease Control and Prevention (CDC) *Salmonella* is estimated to cause 1.2 million cases of illness in the United States. Of that, nearly 19,000 individuals are hospitalized, and 380 cases of death are reported annually (Centers for Disease Control and Prevention [CDC], 2019). Although mortality from infection is low, morbidity is high, as *Salmonella* is amongst the top five foodborne pathogens responsible for domestically acquired foodborne illness, and first for number of cases resulting in either hospitalizations or death (CDC, 2018).

The most common form of salmonellosis in the US is *gastroenteritis* (Murray *et al*., 2009). Onset of symptoms can occur 12 to 72 hours following infection, and include diarrhea, nausea, vomiting, fever and abdominal cramps (CDC, 2019; Murray *et al*., 2009). Symptoms are frequently self-limiting, and can last as little as two days, or as long as one week (Murray *et al*., 2009). According to the CDC, within the last five years several outbreaks of salmonellosis were

reported. Among the food items recalled due to contamination were fresh produce, such as cucumbers and bean sprouts, poultry products and raw-cashew cheese (CDC, 2019). On average, between 20 and 30 percent of those affected were hospitalized, with no reported deaths. Similar food products are often involved in outbreaks nearly every year; common foodstuffs include alfalfa sprouts, nut products (cashews, pistachios, peanuts) and poultry. The CDC advises that, fresh produce—such as bean or alfalfa sprouts—is a known source of foodborne illness and should be handled in compliance with common food safety practices (CDC, 2019).

Although outbreaks of foodborne illness involving dairy products seldom occur, when they do, they can be quite detrimental, causing severe illness and affecting many (Modi, Hirvi, Hill & Griffiths, 2001). Wood, Collins-Thompson, Irvine and Myhr (1984) describe one such case in which the shedding of 200 CFU/mL of *Salmonella* Muenster directly into the milk by one dairy cow lead to an outbreak of salmonellosis in Ontario, Canada. In 1985, more than 180,000 individuals in the Midwest were afflicted with salmonellosis (El-Gazzar & Marth, 1992). The outbreak was traced to samples of 2% milk, contaminated following pasteurization (El-Gazzar & Marth, 1992). Transmission of *Salmonella* from an animal reservoir can cause substantial outbreaks of illness—most commonly when unpasteurized, or raw, milk is consumed (Wood *et al.*, 1984). Despite the risk, proponents of raw milk continue to campaign for its legalization in states that prohibit intrastate sale, as the demand for raw milk is on the rise (Mungai, Behravesh, & Gould, 2015). Regulations regarding the sale of raw milk differ state-by-state, and include complete bans (as in Iowa), to limited farm sales (Mungai *et al*., 2015). The risks associated with consumption of raw milk are high and can result in hospitalization from a wide variety of microorganisms, such as *Brucella*, *Campylobacter*, *Listeria* and *Salmonella*, to name a few (CDC, 2016a). In fact, between 2007 and 2012, 81 reported outbreaks were attributed to raw milk, across

26 states. These outbreaks resulted in 979 illnesses and 73 hospitalizations, with most illnesses attributed to *Campylobacter*, Shiga toxin-producing *E. coli* and *Salmonella* (Mungai *et al*., 2015). On average, the number of outbreaks involving raw milk during this period was four times higher than the number of outbreaks that occurred between 1993 and 2006 (Mungai *et al*., 2015). This is due to increased consumption of raw milk, as well as a decrease in the number of states that prohibit its sale. Nonetheless, proponents of raw milk believe that the consequences are outweighed by the benefits—many of which have not been scientifically validated (Denny, Bhat, & Eckmann, 2005).

Fortunately, due to proper pasteurization techniques, outbreaks involving dairy products other than milk are less common. However, in some cases of salmonellosis, contamination can be attributed to improper pasteurization of milk or contamination during the post-pasteurization dairy production process, including milk used to produce cheese (El-Gazzar & Marth, 1992; Modi *et al*., 2001; Wood *et al*., 1984). Despite such cases, *Salmonella* species are not generally heat resistant—species commonly grow between 35 and 37 °C—and would not easily survive pasteurization (Modi *et al*., 2001). Such examples indicate the ability *Salmonella* has to survive the cheese manufacturing process, particularly if raw milk is used during production (Wood *et al*., 1984). That said, an organism more commonly attributed to outbreaks involving cheese and dairy products is *Listeria monocytogenes*, a ubiquitous Gram-positive rod-shaped bacterium that is widespread within the environment, from soils to water to feces (Ryser & Donnelly, 2001). Contamination of produce and other foods is attributed to its presence in soils, which serves as a reservoir for contamination during processing (Kozak, Balmer, Byrne, & Fisher, 1996; Ryser & Donnelly, 2001). However, it should be noted that cattle can shed *L. monocytogenes* if infected with the organism, which can result in either mastitis or encephalitis (Kozak *et al*., 1996). Shedding

can continue undetected long after the cow has returned to health (Kozak *et al*., 1996; Marth, 1994). Naturally, this is disconcerting, as seemingly healthy cattle can contaminate raw milk; in fact, *L. monocytogenes* is commonly present in two to four percent of the raw milk supply (Kozak *et al*., 1996; Ryser & Donnelly, 2001).

Aside from the aforementioned problems associated with raw milk, *L. monocytogenes* is notably associated with cheese, as it is able to grow at refrigeration temperatures while maintaining survivability during the processing and storage of most cheeses (Ryser & Donnelly, 2001). While *L. monocytogenes* is well-known for its association with soft cheeses made with raw milk, outbreaks have been associated with pasteurized cheeses, contaminated during processing (CDC, 2016b). In 2016, a variety of cheeses—including cotija, queso fresco, feta and mozzarella—were associated with a multistate outbreak of *L. monocytogenes*, which resulted in 30 illnesses, 28 hospitalizations and three deaths (CDC, 2015a). The disease resulting from infection by *L. monocytogenes* is referred to as listeriosis and can take as long as one or two months to develop, which makes outbreaks involving this organism difficult to trace back to a particular product (Ryser & Donnelly, 2001). Although morbidity is low, with an estimated 1600 infections per year, mortality is high, with 16 percent of incidences resulting in death (CDC, 2016b). High-risk groups, such as the immunocompromised, pregnant women and newborns, are most at-risk for infection and death, while pregnant women can pass the infection to unborn infants, which can result in miscarriages and stillbirths (CDC, 2016b; Ryser & Donnelly, 2001).

Another case worth discussing is the highly publicized multistate outbreak linked to Blue Bell Creameries, manufacturers of ice cream, sherbet and other frozen dairy treats. Despite only 10 cases, the outbreak was notable as each case resulted in hospitalization, and—of those—three deaths. Onset of illness spanned from January 2010 to January 2015 (CDC, 2015b). Following

subsequent investigation, Blue Bell Creameries voluntarily recalled products implicated in the outbreak, and suspended production at its production facility in Broken Arrow, OK, where multiple contaminated products were manufactured (CDC, 2015b; Food and Drug Administration [FDA], 2016). However, the recall was expanded to include all products available for retail sale when *L. monocytogenes* was detected in additional products manufactured elsewhere (FDA, 2016). Investigations ongoing, the company explained that extensive cleaning, sanitizing and training program would be introduced across all manufacturing facilities (FDA, 2016).

An additional bacterium of interest to the dairy foodstream is *Escherichia coli,* specifically *E. coli* O157:H7. *E. coli* is a Gram-negative rod-shaped bacterium that exists as part of the normal flora of the intestinal tract in humans and some animals (Meng, Feng, & Doyle, 2001). Classification is based on both serotyping and virotyping, where serological classification is achieved via differences amongst the O and H antigens (Meng *et al*., 2001). The former corresponds to the O antigen of the lipopolysaccharide (LPS) core, LPS being the chief component of the outer membrane in Gram-negative bacteria, while the latter refers to the flagella (Meng *et al*., 2001). Virotyping refers to various virulence factors, such as attachment to intestinal epithelium cells, invasion, toxin production and so forth—pathogenicity is arbitrated by these virulence factors, as well as the ability of the organism to multiply within the host (Meng *et al*., 2001). That said, most *E. coli* do not cause illness, and are considered non-pathogenic; however, six virotypes of pathogenic *E. coli* exist, including: enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC), and are known to cause severe diarrheagenic illness in humans (Farrokh *et al*., 2012; Meng *et al*., 2001). Classified as a Shiga toxin-producing *E. coli* (STEC) serotype, *E. coli* O157:H7 is a member of the

enterohemorrhagic *E. coli* (EHEC) sub-group and is highly virulent—doses as low as five cells have the potential to cause illness in human hosts (Farrokh *et al*., 2013). Because of this, coupled with its frequent association with ruminant animals (notably cattle), *E. coli* O157:H7 has been linked to outbreaks involving dairy products in the past, and is considered of concern to the dairy industry (Farrokh *et al*., 2013).

In 2005, four cases of reported illness associated with *E. coli* O157:H7 infection were linked to consumption of raw milk obtained from a local dairy (CDC, 2007; Denny *et al*., 2005). The dairy participated in a cow-share agreement, allowing invested shareholders to obtain raw milk from the farm. Although cow-sharing programs are legal in certain states, a license and proper documentation must be obtained prior to distribution—the farm linked to the reported cases was unlicensed yet distributed raw milk to participating shareholders regardless (CDC, 2007; Denny *et al*., 2005). This resulted in a retrospective cohort study, in which 43 of the 45 families involved in the cow-sharing program were interviewed, while information was collected from 157 individuals (CDC, 2007; Denny *et al*., 2005). Amongst those interviewed, 18 cases of *E. coli* O157:H7 infection were identified, with nearly half confirmed by diagnostic laboratory testing (CDC, 2007; Denny *et al*., 2005). Inspection reports identified accumulation of mud and manure outside *and* within the milking parlor itself—locations that were frequently in contact with buckets used for milk collection (CDC, 2007). Moreover, reports identified insufficient hand-washing stations, inappropriate cleaning and sanitation of equipment and poor handling of fresh milk were commonplace within the dairy, any of which could facilitate contamination of the raw milk product (CDC, 2007).

It is worth noting that, STECs are widespread amongst dairy cattle, with some researchers noting worldwide prevalence rates for *E. coli* O157:H7 as high as 48.8%, based on fecal testing

(Hussein & Sakuma, 2005; Lim, Yoon & Hovde, 2010). Contamination of raw milk with STEC is attributed to fecal shedding, however, some authors note that an additional source of contamination is possibly intra-mammary, such as mastitis resulting from STEC infection (Farrokh *et al*., 2013). Several authors remain in dispute over this, with most arguing that feces remains the most likely source of contamination. As STECs, quite possibly *E. coli* O157:H7, are commensals within the intestines of dairy cattle, it is possible that fecal shedding results in soiling of the teats, which in turn can facilitate contamination of raw milk during milking (Farrokh *et al*., 2013). Unlike *L. monocytogenes*, *E. coli* O157:H7 is not as prevalent in raw milk—about zero to two percent between 2003 and 2013 (Farrokh *et al*., 2013). However, it is important to note that outbreaks associated with *E. coli* O157:H7 can affect dairy products, including cheese. *E. coli* O157:H7 maintains survivability during cheese production, with low levels of viable cells present following lengthy storage (six weeks)—to recapitulate, *E. coli* O157:H7 infection requires doses as low as five cells (Farrokh *et al*., 2013; Maher, Jordan, Upton, & Coffey, 2001).

In 2010, Bravo Farms recalled all its cheese products, including Gouda, pepper jack and cheddar, following FDA investigation into a multistate outbreak of *E. coli* O157:H7 infection that affected 38 individuals—15 of whom were hospitalized (CDC, 2010). No deaths were associated with the incident, however, one case of hemolytic uremic syndrome (HUS) was reported (CDC, 2010). Common symptoms of *E. coli* O157:H7 infection include hemorrhagic colitis, with 10 percent of cases possibly resulting in HUS (Farrokh *et al*., 2013; Meng *et al*., 2001). The consequence of HUS is renal failure, and it is more common in children under five years of age (CDC, 2010; Farrokh *et al*., 2013; Hussein & Sakuma, 2005). Because STECs are omnipresent amongst cattle, it is important that preventative measures, such as heard management (probiotic use, vaccinations, feed and lifestyle changes) and cross-contamination prevention, are in place to

ensure that reasonable levels are present on site (Farrokh *et al*., 2013). It is important to note that, as outlined within the *Pasteurized Milk Ordinance*, Grade "A" raw milk and milk products for pasteurization, ultra-pasteurization, aseptic processing or retort processing following packaging, cannot surpass bacterial limits of 100,000 CFU per mL for any single producer (FDA, 2015). While for Grade "A" pasteurized milk and milk products, bacterial limits must be no more than 20,000 CFU per mL (FDA, 2015). However, despite such preventative control measures, contamination can occur, resulting in adulterated products.

As a result, these examples of prevalence, contamination and outbreaks attributed to *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7, emphasize the need for inexpensive, rapid and sensitive methods of detection in both dairy products and processing environments (Goodridge & Griffiths, 2002). Use of low-cost techniques is crucial and can be used to improve industrial efficiency and in underdeveloped countries where foodborne illness is a common problem. Though previous reviews emphasizing the relationship between *Salmonella*, *L. monocytogenes*, *E. coli* O157:H7 and dairy products have been conducted in the past (El-Gazzar & Marth, 1992; Farrokh *et al*., 2013; Marth, 1969), this review will evaluate current, established and developing research in this field, in order to provide food processing technicians, public health officials, food microbiologists, academicians and the general public with information focused on the detection of these microorganisms and the growing concern of dairy-related foodborne illness. Specifically, this review will assess methods of rapid detection of *Salmonella* and other notable foodborne pathogens in dairy products and food processing environments. These methods have the advantage of requiring less time for preparation, sampling and processing, while maintaining low cost, ease of operation and simple interpretation of results (Vasavada, 1993). The limitations of established detection techniques are discussed throughout this review. Emphasis is given to developing

methods of rapid detection and how such methods can be adapted for use in the field. Detection methods that are rapid, inexpensive and user-friendly (ease of operation, simple interpretation) are desirable.

There is an increasing need for sensitive and rapid detection methods of foodborne pathogens. The aforementioned outbreaks of foodborne illness involving contaminated dairy products represent a small sample of cases. Within the last 20 years, the incidence of outbreaks of foodborne illness has increased (Oliver, Jayarao, & Almeida, 2005; Omiccioli, Amagliani, Brandi, & Magnani, 2009). The prevention of illness and death as a result of foodborne pathogens is a major concern for most public health officials (Oliver *et al*., 2005). As Omiccioli *et al*. (2009) note, between 1998 and 2005, the CDC identified 45 outbreaks of foodborne illness attributed to raw, unpasteurized milk or cheese produced from unpasteurized milk. Nearly 1,007 cases of illness were reported; of these cases, 104 individuals were hospitalized, and two deaths occurred (Omiccioli *et al*., 2009). Safety of food products is a global concern and the identification of sensitive and rapid detection techniques of foodborne pathogens in both dairy products and production facilities is critical, as it will ensure the safety and quality of the dairy foodstream and enable public health officials to adopt suitable precautionary measures of *preventing* the spread of illness should an outbreak occur (Bhagwat, 2003; Oliver *et al*., 2005; Omiccioli *et al*., 2009).

1.2 Detection of Foodborne Pathogens Using Paper-Based Analytical Devices

Due to the continued presence of notable foodborne pathogens in a variety of food products and processing environments, the need to rapidly detect these organisms—while maintaining low cost—is on the rise. Standard culture methods, often considered the "gold standard" of detection and enumeration, require several days before a confirmed result can be identified (Jokerst *et al*., 2012). The length of time is often dependent upon pre-enrichment, enrichment and selective

plating. The lengthy timeline is problematic as the product may already be in consumer hands before a definitive result is obtained (Jokerst *et al*., 2012). As a possible solution to this problem, researchers have looked towards low-cost diagnostic (LCD) tools as methods of rapid, sensitive and selective detection. One example includes paper-based analytical devices (PADs). Although there are several ways to produce PADs, wax printing is both the quickest and easiest method currently employed (Jokerst *et al*., 2012). Wax printed paper maintains many advantages, including cost, which is estimated as \$0.001 per cm^2 for a single 8.5 X 11-inch sheet of Whatman® #1 filter paper. As many as 275 devices can be printed on a single sheet, resulting in an approximate cost of \$0.002 per device—much less expensive than the cost of detection, enumeration and identification methods currently employed in industry (Jokerst *et al*., 2012).

Paper-based analytical devices are commonly combined with a colorimetric read-out to achieve a visual representation of detection. In this case, an enzyme will react with a chromogenic substrate, consequently producing a way to visually identify bacterial presence (Jokerst *et al*., 2012). A variety of chromogenic substrates can be used for such assays, and range in selectivity. For example, for the detection of *E. coli*, *β*-galactosidase interacts with chlorophenol red *β*-galactopyranoside (CPRG) to produce a red-violet colorimetric product once CPRG is hydrolyzed. In the detection of *L. monocytogenes*, phosphatidylinositol-specific phospholipase C (PI-PLC) interacts with 5-bromo-4-chloro-3-indolyl-*myo*-inositol phosphate (X-InP) to produce a blue product, while in the detection of *Salmonella*, esterase interacts with 5-bromo-6-chloro-3-indolyl caprylate (magenta caprylate) to produce a purple product (Jokerst *et al*., 2012). PI-PLC maintains a high degree of specificity with *L. monocytogenes*, as it is important in the expression of virulence in pathogenic strains of *Listeria*, allowing the organism to escape host cell vacuoles (Jokerst *et al*., 2012; Notermans, Dufrenne, Leimeister-Wachter, Domann, & Chakraborty, 1991; Wei, Schreiber,

& Baer, 2005). However, *E. coli* O157:H7 is not the only *E. coli* serovar to produce *β*-galactosidase, which can result in non-specificity of the assay (Jokerst *et al*., 2012). *β*-galactosidase acts as an important facilitator in the hydrolysis of *β*-galactosides into simple sugars and is common amongst multiple serovars of *E. coli*. As a result, further study is required for the detection and identification of additional serovars (Jokerst *et al*., 2012).

Fortunately, Jokerst *et al*. note that—for the purposes of their study— the enzyme-substrate pairs maintained specificity for each organism under investigation, and a colorimetric product was only produced when the correct pair of enzyme and substrate was present (Jokerst *et al*., 2012). This is advantageous, as both detection *and* identification can be achieved in as little as eight to 12 hours, with a limit of detection (LOD) of 10^1 CFU/cm^2—keep in mind that such an assay is inexpensive, especially when compared to methods of detection discussed later in this review. Furthermore, it is important to note that detection on solid substrates, such as paper has additional advantages, aside from cost. Firstly, PADs are portable, and can be used in the field. Secondly, unlike detection in solution, detection on paper facilitates *localization* of the chromogenic substrate, allowing for simple identification and interpretation of results. Because of this, paper is of renewed interest as a vehicle for detection, as it is easily modified for use in a variety of low-cost methods of detection (Yetisen, Akram, & Lowe, 2013). Finally, due to their simple design, and wide range of uses, PADs can be coupled with imaging using smart phones to monitor the color change as a function of time, as opposed to a single measurement at the end of the reaction (Adkins *et al*., 2017). The use of a light box prevents interference from background or environmental lighting conditions and can help to ensure standardization.

Specific enzymes, such as those previously discussed are important when particular bacterial species are of interest. However, if simple presence or absence is required, as in

environmental testing of lake waters, the use of shared, related enzymes can be of use during detection. It is important to note that bacteria are capable of existing in active or dormant states, depending on a variety of environmental factors, including nutrient availability and growth conditions (temperature, microbial competition). However, should conditions improve, bacteria are able to revert to a metabolically active state. Their sometimes rapid growth can lead to bacterial blooms, which have been attributed to both environmental and manufacturing problems—including death of wildlife and complications during industrial fermentation (Hallegraeff, 1993; Murphree, Heist, & Moe, 2014). Aside from issues affecting the environment and industry, reversion from a dormant to an active state can also be problematic should pathogenic bacteria begin rapidly reproducing—the resulting outcome of which could be onset of illness, oftentimes including outbreaks (Coates, 2003). Not surprisingly, the level of enzymatic expression can differ between metabolic states. For example, *E. coli* that exists in a dormant state exhibits greater expression of alkaline phosphatase (ALP)—especially during levels of phosphate starvation—and nitrate reductase, which may confer a selective advantage during periods of arrested growth (Clegg, Jia, & Cole, 2006; Yang & Metcalf, 2004). On the other hand, it has been observed that metabolically active *E. coli* express greater numbers of oxidoreductases, such as dehydrogenases for use in the citric acid cycle, and less ALP—which again is encoded during periods of phosphate starvation (Achbergerová & Nahalká, 2011; Almaas, Kovács, Vicsek, Oltval, & Barabási, 2004).

Previous research has indicated the potential ALP and other non-specific enzymes have as vehicles for detection, including colorimetric detection (Blake, Johnston, Russell-Jones, & Gotschlich, 1984). Their research employed the use of ALP-bound anti-antibody during Western blots. Detection of ALP was achieved using tetrazolium salts, reduced to diformazan once the ALP is cleaved and the hydroxyl group dimerizes. This release of hydrogen ions facilitates the reduction

of the tetrazolium salt to diformazan, producing a strong blue color (Blake, *et al*., 1984). Examples include the reduction of tetrazolium salts to formazans in the presence of oxidoreductases, such as dehydrogenases, as well as reduction to *p*-nitrophenyl-phosphate in the presence of ALP (Berridge, Herst, & Tan, 2005; Bessey, Lowry, & Brock, 1946). In the presence of these non-specific enzymes, onset of color change occurs within min, lending strength to their use as tools for the rapid detection of microorganisms. That said, due to their wide variety of uses in microbial detection, PADs have been coupled with these tetrazolium salts to *non-specifically* detect bacteria, including the detection of various metabolic states (Hice, Santoscoy, Soupir, & Cademartiri, 2018). Researchers have taken advantage of the use of these non-specific enzymes to rapidly detect a variety of bacterial species at differing metabolic states. This is achieved using tetrazolium salts, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and 5-methylphenazin-5-ium methyl sulfate (PMS) for metabolically active bacteria, and a mixture of INT and nitrophenyl phosphate (PNPP) for dormant bacteria, coupled with PADs (Hice *et al*., 2018). Although specificity in identification of microorganisms is of interest throughout this review, it is important to address rapid methods that may not employ specific tools of detection, yet are critical for the identification of other important aspects of detection, such as the determination of metabolic states of bacteria.

1.3 Detection of Foodborne Pathogens Using Bacteriophages

At first glance, the use of non-specific enzymes in the detection of foodborne pathogens appears at a disadvantage when compared to more explicit methods of microbial detection yet to be discussed. These methods, such as nucleic-based amplification, are typically as or more sensitive, and can be highly specific in the detection of a specific organism of interest. However, such methods have several disadvantages: they require more time, are laborious and can be

expensive—especially when compared to colorimetric detection using PADs. Nevertheless, the lack of specificity using ALP and oxidoreductases can be detrimental, especially in manufacturing processes where identification *and* detection of an organism of interest is crucial, as in meat, poultry and dairy processing plants. That said, to address this lack of specificity during detection, researchers have turned to bacteriophages as a sensitive and specific low-cost alternative to microbial detection (Hice *et al*., 2018). First reported in 1915, bacteriophages are virus particles that infect, replicate and have the potential to lyse bacteria (Entis *et al*., 2001). Composed of protein and either DNA or RNA, bacteriophages are unable to replicate without a host bacterium, and instead utilize bacterial cell machinery to replicate (Entis *et al*., 2001). For detection purposes, it is important to note that—although cell contact is described as a random event—bacteriophage replication will only occur if the host cell maintains site-specific receptors for a particular bacteriophage; such receptors can include lipopolysaccharides, proteins, pili, flagella and teichoic acids to name a few (Entis *et al*., 2001). Through these site-specific receptors, and variations thereof, bacteriophages can maintain host specificity to genus, making them key components in low-cost methods of detection (Entis *et al*., 2001).

The combined use of tetrazolium salts and bacteriophages has been successfully used to detect and capture bacteria of interest, such as *E. coli* and *Salmonella* (Hice *et al*., 2018). Such methods have taken advantage of the reactivity and robustness of the bacteriophage, and have effectively adsorbed on paper to operationalize a low-cost, portable assay for microbial detection (Hice *et al*., 2018). It is important to note that previous researchers have investigated the bacterial capture ability bacteriophages possess (Anany, Chen, Pelton, & Griffiths, 2011). Specifically, bacteriophages have been used to control the presence of such notable foodborne pathogens as, *E. coli* O157:H7 and *Listeria monocytogenes* (Anany *et al*., 2011). To achieve this, bacteriophages,

specific to these organisms, were bound to positively charged cellulose membranes—the negatively charged head of the bacteriophage interacts with the positive cellulose membrane—while control and reduction in bacterial growth was observed using bacteriophage-treated ready-to-eat (RTE) meats (Anany *et al*., 2011). Aside from the aforementioned example, a variety of bacteriophage-based approaches exist, and have been actively studied for the detection of foodborne pathogens, including *Salmonella* and *E. coli* O157:H7 (Favrin, Jassim, & Griffiths, 2003). In some examples, target organisms are captured and concentrated using immunomagnetic separation (IMS), which facilitates specific binding to target cells through the use of antibody-coated magnetic beads (Entis *et al*., 2001; Favrin, Jassim, & Griffiths, 2000). The beads are then captured by a nearby magnet (usually placed on the side of the tube), effectively concentrating and purifying the cells from the food matrix (Entis *et al*., 2001). IMS is used in place of lengthy enrichment steps, reducing assay time by up to 24 hours, while the purified mixture can easily be coupled with a variety of detection methods, including enzyme-linked immunosorbent assays (ELISAs) and nucleic-based amplification methods (Entis *et al*., 2001; Favrin *et al*., 2000; Favrin *et al*., 2003).

To recapitulate, capture and concentration of target cells is achieved by IMS, while detection is facilitated using bacteriophage. In one such study, *Salmonella* and *E. coli* O157:H7 were detected in a variety of food matrixes, including skimmed milk powder, chicken rinses and ground beef (Favrin *et al*., 2003). Using anti-*Salmonella* or anti-*E. coli* Dynabeads^{TM}, target cells were captured and purified as previously described (Favrin *et al*., 2003). Concentrated volumes (10^8 PFU/mL) of either SJ2 bacteriophage (*Salmonella*) or LG1 bacteriophage (*E. coli* O157:H7) were added to the sample and incubated at 37 °C to allow attachment of the bacteriophage to the host cell (Favrin *et al*., 2003). Progeny bacteriophage were released following resuspension of the

bead-bound cells, allowing for amplification using healthy *Salmonella* or *E. coli* cells, referred to as *signal amplifying cells* (SACs). Optical density (OD) was determined before and after a brief incubation period (1.5 to 2 hours at 37 °C) when the addition of the progeny phage to the SACs occurred (Favrin *et al*., 2003). A reduction in OD was calculated as a *percentage* of the negative control value (sterile broth), where a positive result was recorded as 70 percent as or less than the average negative control (Favrin *et al*., 2000; Favrin *et al*., 2003). Using data collected from artificially inoculated food samples, 70 percent generated consistent discernment between positive and negative samples (Favrin *et al*., 2000). Salmonella, LOD was identified as 2 to 3 CFU/ 25 mL in skimmed milk powder, chicken rinses and ground beef (Favrin *et al*., 2003). It is important to note that *Salmonella* was detected in all three food types, which is impressive considering that both chicken rinses and ground beef contain substantial amounts of background flora (Favrin *et al*., 2003). Yet, despite the assay's success in detecting *Salmonella* and *E. coli* O157:H7 individually, it was not successful in yielding reliable results when the two organisms were combined—especially when the ratio of the organisms differed (Favrin *et al*., 2003). As described, this assay relies on the characteristic infection cycle and specificity of the bacteriophage towards a target host cell, and consequently does not require manipulation of the bacteriophage genome to achieve detection (Favrin *et al*., 2000).

Although effective in detecting *Salmonella* in complex food samples, it is important to note that the SJ2 bacteriophage is *not* specific for a particular *Salmonella* serovar (for example, Enteritidis); however, this bacteriophage is also *not* broad enough to be used as a tool for a generic test of *Salmonella* (Favrin *et al*., 2000; Favrin *et al*., 2003). Research suggests that better bacteriophage selection may allow for the development of a generic test for *Salmonella* using this IMS-bacteriophage assay (Favrin *et al*., 2000). That said, a possible solution would be use of the

R-core-specific Felix-O1 bacteriophage, a *Salmonella*-specific bacteriophage that relies on LPS R-core terminating in *N*-acetylglucosamine for ideal infection of the host cell (McConnell & Schoelz, 1983). The use of this bacteriophage in the detection of *Salmonella* is advantageous, as previous research indicates that 98.2 percent of tested *Salmonella* strains were successfully lysed by this bacteriophage—this includes 653 strains of *Salmonella* (Welkos, Schreiber, & Baer, 1974). A similar study using more than 5,000 *Salmonella* strains found that, of the strains tested, 98 to 99.5 percent were susceptible to lysis by the Felix-O1 bacteriophage (Favrin *et al*., 2000; Gunnarsson, Hurvell, & Thal, 1977; Hirsh & Martin, 1983a). Use of the Felix-O1 bacteriophage can be coupled with several methods of detection, including high-performance liquid chromatography (HPLC), where HPLC was used to detect *Salmonella* via an increase in the number of bacteriophage associated with host cell lysis and replication of bacteriophages (Entis *et al.*, 2001; Hirsh & Martin, 1983a).

Following a two hour incubation period where interaction between the bacteriophage and host cells occurred, the mixture was treated with chloroform, centrifuged and subsequent cell debris was discarded (Entis *et al.*, 2001; Hirsh & Martin, 1983a). The remaining solution, referred to as the bacteriophage supernatant, was injected into the HPLC column, where a detector response was recorded at 3.2 min (Entis *et al.*, 2001; Hirsh & Martin, 1983a). For *Salmonella*, LOD was identified as 10^6 CFU/mL. Moreover, detection was only achieved using *Salmonella*, and was not obtained when various Gram-negative and positive organisms were used—including closely related members of the *Enterobacteriaceae*, such as *E. coli*, *Klebsiella pneumoniae* and *Serratia marcescens* (Hirsh & Martin, 1983a). However, despite its selectivity in detection, this method has a significant disadvantage when compared to rapid methods yet to be discussed; namely, it requires high concentrations of bacteria to achieve detection (Entis *et al.*, 2001; Hirsh & Martin, 1983a).

Modifications to the design of this method (incorporation of large pore electropositive filters and overnight incubation) allowed for the detection of less than five cells per mL in artificially contaminated milk samples (Entis *et al.*, 2001; Hirsh & Martin, 1983b). The use of the filters facilitated the removal of bacteria from the milk samples, while overnight enrichment in brain heart infusion (BHI) broth with added brilliant green dye suppressed the growth of coliforms—ensuring that 75 to 100 percent of the total population of bacteria consisted of *Salmonella* (Entis *et al.*, 2001; Hirsh & Martin, 1983b). Subsequent detection was achieved as previously described (Hirsh & Martin, 1983a).

1.4 Multiplex Detection of Foodborne Pathogens by Real-Time PCR

Common assays employed in the detection of foodborne pathogens—though effective in identification—are often time consuming, requiring up to seven days for processing, disrupting high capacity processes, resulting in higher cost and increased waste (Omiccioli *et al*., 2009). As a result, research focuses on developing rapid analytic methods of detection (Omiccioli *et al*., 2009). One such method often employed in industry, is polymerase chain reaction (PCR). Developed in 1983, PCR is not in any way a "new" method of detection, however, it is the basis for many bacterial detection methods used for both industrial and research purposes (Entis *et al.*, 2001; Lazcka, Del Campo, & Muñoz, 2006). PCR is a rapid DNA-based detection method focused on the isolation, amplification and quantification of short DNA sequences in periods of five to 24 hours, depending upon the variation employed (Lazcka *et al.*, 2006). PCR works by *denaturing* the target double stranded DNA (dsDNA) using high heat (94–98 °C), which results in separation of the two strands (Entis *et al.*, 2001; Lazcka *et al.*, 2006). This separation facilitates *annealing* of specific primers to complementary target DNA strands at lower temperatures (48–72 °C), now serving as templates for *extension* (68–72 °C), where the enzyme, DNA polymerase, subsequently

adds nucleotide bases (dNTPs) onto the ends of each primer (Entis *et al.*, 2001; Lazcka *et al.*, 2006). This results in copies of two new dsDNA strands, which consequently serve as templates for each additional PCR cycle, doubling each time; this allows for exponential amplification of DNA, which is then able to be detected (Entis *et al.*, 2001; Lazcka *et al.*, 2006).

Several sensitive and rapid bacterial detection methods that incorporate PCR exist, each of which are important tools for the identification of foodborne pathogens (Bhagwat, 2003; Omiccioli *et al*., 2009). The simultaneous detection of several common foodborne pathogens is advantageous, as the reduction of different analyses may bring increased economy to the process, especially when compared to conventional detection techniques (Kawasaki *et al*., 2005). In one amplification reaction, multiplex PCR (mPCR) can easily detect multiple targets, while Real-Time or quantitative PCR (qPCR) techniques can be used simultaneously with mPCR (Omiccioli *et al*., 2009). Such techniques eliminate both post-PCR processing of samples and risk of cross-contamination, significantly reducing time required for analysis (Omiccioli *et al*., 2009). As described by Bhagwat (2003), qPCR employs a fluorescent dye, such as SYBR Green I—fluorescent emission is achieved when the dye attaches to the target amplicon (Lazcka *et al.*, 2006). The fluorescent signal is measured through the optical detection of amplified targets, which consequently indicate the presence of double-stranded DNA or other dual-labeled probes, facilitating observation of the amplification of DNA in "real time" (Lazcka *et al.*, 2006; Omiccioli *et al*., 2009). By conducting qPCR, researchers can eliminate post-amplification steps, such as the laborious gel electrophoresis, which enables detection of the amplified sequence (Lazcka *et al.*, 2006). In milk, the specificity of multiplex qPCR has been evaluated, and results indicate that this assay is 100 percent inclusive for target species, and 100 percent exclusive for non-target strains (Omiccioli *et al*., 2009; Singh, Batish, & Grover, 2011). Recovery of foodborne pathogens from

dairy products is often hampered by low levels of contamination (Omiccioli *et al*., 2009). It is important to note that a major limitation of PCR-based detection methods is the inability to distinguish between living and dead cells, as DNA is present regardless of cell viability (Lazcka *et al.*, 2006). To combat this problem, Reverse Transcriptase (RT-PCR) is employed in the detection of solely viable cells (Lazcka *et al.*, 2006). The enzyme, reverse transcriptase (RT) acts by synthesizing single stranded DNA from RNA (Lazcka *et al.*, 2006). Although multiple types of RNA exist, messenger RNA (mRNA) is specifically used in RT-PCR, as it has a short half-life, and is therefore only present in living cells (Yaron & Matthews, 2002).

To reliably detect the presence of foodborne pathogens, studies have demonstrated the need for an enrichment step to improve sensitivity. Without enrichment, detection of bacterial presence in contaminated milk samples was unreliable (Omiccioli *et al*., 2009), and detection of non-specific products was observed (Singh *et al*., 2011). As observed by Omiccioli *et al*. (2009) and Singh *et al*. (2011), following the introduction of an enrichment step, LOD was respectively measured at 1 CFU per 25 mL and 1 CFU per mL for each pathogen sampled (in this case, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157). Processing time was reduced to 48 hours from seven or more days necessary for conventional culture techniques (Omiccioli *et al*., 2009). A variety of multi-pathogen enrichment broths are available, and reduce the amount of space, time, cost and labor required to culture microorganisms (Garrido *et al*., 2013a; Kim & Bhunia, 2008). Selective enrichment broths are a necessity when testing for the presence of foodborne pathogens in food and environmental samples (Suo & Wang, 2012). Such media is necessary to enrich target organisms within the sample, while aiding in recovery of stressed or injured cells (Suo & Wang, 2012; Wu, 2008). SEL, a selective enrichment broth is useful for simultaneous growth of *Salmonella enterica*, *Escherichia coli* O157:H7 and *Listeria*

monocytogenes, which can aid in regulating the testing of foods at risk for multiple-pathogen contamination in the food processing industry (Kim & Bhunia, 2008). SEL, unlike the commercially available Universal Preenrichment Broth (UPB), contains selective inhibitory agents, and may be more appropriate for samples with large amounts of background microflora, such as unprocessed, raw foods (Bailey & Cox, 1991; Kim & Bhunia, 2008; Suo & Wang, 2012).

Nonselective media, such as UPB, Buffered Peptone Water (BPW) and No. 17 Medium (also known as TA10 Broth) show promise in recovery of injured target pathogens, however, may also enrich background microflora, rendering them inadequate for use in complex food samples (Suo & Wang, 2012). The enrichment and recovery of target pathogens—especially those that may be physiologically stressed or damaged—is critical, as injured microorganisms may repair themselves under more optimal conditions, such as food and feed products meant for human or animal consumption (Wu, 2008). While some researchers have demonstrated the effectiveness of No.17 Medium in recovering target pathogens, such studies employed BPW or UPB as a control medium, rather than SEL (Garrido *et al*., 2013a; Garrido *et al*., 2013b). Furthermore, as the aforementioned studies demonstrated, SEL also supports bacterial growth at 10^1 CFU/mL (Kim & Bhunia, 2008). When coupled with mPCR, SEL exhibits low levels of detection for all three pathogens tested, indicating that it is a suitable enrichment broth for such techniques (Kim & Bhunia, 2008; Suo & Wang, 2012). In an industrial environment, the use of enrichment media is advantageous, as the same broth can be used to simultaneously enrich target pathogens, while food and environmental samples are tested for bacterial presence within 24 hours (Kim & Bhunia, 2008; Suo & Wang, 2012). This is beneficial for screening large quantities of samples for the occurrence of foodborne pathogens in food processing environments and is an improvement on traditional culturing methods that may require up to seven days for analysis (Suo & Wang, 2012).

1.5 Multiplex Detection of Foodborne Pathogens by RPA

In recent years, recombinase polymerase amplification (RPA) has demonstrated promise as a means for the detection of a variety of microbial pathogens and viruses (Murinda *et al*., 2014). During RPA, the recombinase/oligonucleotide primer complex initiates amplification through the identification of homologous target sequences within duplex DNA (Murinda *et al*., 2014; Piepenberg, Williams, Stemple, & Armes, 2006; TwistDX, 2018; Yan *et al*., 2014). Once the homologous target sequence is identified, the recombinase-primer complex separates the strands of DNA, consequently allowing the primer and sequence to hybridize (Yan *et al*., 2014). Unlike PCR which employs use of high heat to separate duplex DNA, RPA instead uses an enzyme (recombinase)—this is similar to Helicase-Dependent Amplification (HDA), which uses helicase to separate duplex DNA (Li & Macdonald, 2015). Single stranded DNA-binding proteins (SSB) aid in the hybridization process by preventing separation of the primer-sequence hybrid, and elongation occurs following the formation of a primer-DNA complex (Yan *et al*., 2014). If the target sequence is present in the sample, DNA polymerase (*Bacillus subtilis* Pol I, Bsu) proceeds with elongation and replication, using the single stranded DNA as a template (Li & Macdonald, 2015; Murinda *et al*., 2014; Yan *et al*., 2014). Much like PCR, exponential amplification is achieved as the two copies of newly synthesized duplex DNA acts as templates for subsequent cycles (Yan *et al*., 2014).

Fortunately, RPA requires only two primers (forward and reverse) for amplification, unlike other isothermal methods of detection, such as loop-mediated isothermal amplification (LAMP), which requires six primers (Euler *et al.,* 2013; Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014; Santiago-Felipe, Tortajada-Genaro, Puchades, & Maquieira, 2014). Moreover, if a fluorescent probe is used, this reaction can be monitored in real-time, as in qPCR (Piepenberg *et*

al., 2006). During this process, the probe is degraded following hybridization, subsequently releasing a fluorophore. The amount of fluorophore present increases as amplification progresses, which consequently enables real-time observation and measurement (Murinda *et al*., 2014; Piepenberg *et al*., 2006). Recently, TwistDX (www.twistdx.co.uk) developed several RPA kits for the commercial detection of common foodborne pathogens, including *Salmonella*, *Listeria monocytogenes* and *Campylobacter* species, through the amplification and detection of highly specific genes for pathogenicity, such as the *invA* gene in *Salmonella* (Murinda *et al*., 2014). The lyophilized reaction mix remains stable, even during non-refrigerated transportation (Lillis *et al*., 2016; TwistDX, 2018). To detect foodborne pathogens, a low-cost fluorescent reader device is employed to monitor the amplification of target nucleotide sequences in real-time (Murinda *et al*., 2014). Intercalating dyes or fluorescent probes can be used in order to monitor the assay in *real-time*.

RPA is advantageous as a method of detection, as it is rapid, sensitive and highly specific, much like PCR (Murinda *et al*., 2014; Yan *et al*., 2014). However, *unlike* PCR, RPA works at a constant cellular temperature (37–39 °C) and is therefore suitable for the detection of nucleic acids in live cells (Kersting *et al*., 2014; Piepenberg *et al*., 2006; Yan *et al*., 2014). Moreover, RPA is user-friendly, and does not require a thermal cycling device or a highly trained technician, consequently reducing cost (Yan *et al*., 2014). Using a small, portable heating device (such as the Twirla™, available from TwistDX) this novel, effective method of detection yields identifiable results within 10 min (TwistDX, 2018). The Twirla™ device can heat the reaction tubes to the temperature necessary for measurement (TwistDX, 2018). The portability of the device is due to its small size—this makes it a useful tool for use in the field (TwistDX, 2018). Whereas conventional thermocyclers remain laboratory-bound, the Twirla™ is both lightweight and

portable, making it an essential tool for field-work, or in environments without access to a laboratory. In addition, the cost of the Twirla™ incubator is significantly less than that of a thermocycler, a benefit to industry, as well as underdeveloped countries. As cost and analysis time are reduced, RPA is more suitable than PCR for the detection of foodborne pathogens in food processing industries, in the field or in regions where skilled technicians are not available.

Much like PCR, multiplex detection techniques can be coupled with RPA to simultaneously detect several microbial pathogens. While this method of analysis is not widely used, studies that incorporate established multiplex detection techniques into the RPA protocol have shown promise. Kersting *et al*. (2014) demonstrated the simultaneous detection of three unrelated, yet significant, microbial pathogens—*Neisseria gonorrhoeae*, *Salmonella enterica* and methicillin-resistant *Staphylococcus aureus* (MRSA), while Choi *et al*. (2016) demonstrated the use of RPA in the detection of *Salmonella enterica*, *Escherichia coli* O157:H7 and *Vibrio parahaemolyticus*—three foodborne pathogens of clinical significance. In such studies, multiplex RPA is often transferred to biochips for point-of-care testing, and analysis takes place on the surface of the chip (Kersting *et al*., 2014). Due to the addition of high heat, PCR is not optimized for biochip point-of-care testing (Kersting *et al*., 2014; Li & Macdonald, 2015; Mauk, Liu, Sadik, & Bau, 2015).

In addition, disposable lateral flow devices can also be employed (in place of biochips) in the detection of nucleic acids using RPA (Piepenberg *et al*., 2006). Nucleic acid lateral flow immunoassays (NALFIA) often use a *sandwich assay*, in which RPA forward and reverse primers are tagged with either 6-carboxyfluorescin (6-FAM) or biotin (Posthma-Trumpie, Korf, & Amerongen, 2009). The 6-FAM is recognized by an anti-fluorescein antibody, bound to the nitrocellulose membrane of the lateral flow strip, while streptavidin-labeled gold nanoparticles

preferentially bind biotin (Posthma-Trumpie *et al*., 2009). Following successful hybridization of the primers to the DNA template, and subsequent amplification, the resulting dsDNA will be "sandwiched" between the anti-fluorescein antibody and the streptavidin-labeled gold nanoparticle (Shan, Lai, Xiong, Wei, & Xu, 2015). A visualized colorimetric product is obtained due to the aggregation of the gold nanoparticles. These devices also contain an internal control, which indicates adequate flow of the sample though the strip (Posthuma-Trumpie *et al*., 2009). The internal control is commonly comprised of bound biotin, which will bind to the streptavidin-labeled gold nanoparticles, producing a colorimetric effect (Shan *et al*., 2015). In general terms, such devices use pairs of specific antibodies for the detection of target sequences containing two antigenic labels, and offer a simple, low-cost solution to visualize "presence/absence" detection of product (Mauk *et al*., 2015; Piepenberg *et al*., 2006; Posthuma-Trumpie *et al*., 2009). Biochips and lateral flow devices, much like RPA, are portable, economical and much easier to implement outside of laboratory settings—all of which are valuable assets for use in the field.

Following analysis, Kersting *et al*. (2014) demonstrated the highly specific amplification and fluorescence of all three sampled pathogens. This sensitive method was able to detect 100 CFU/mL (*N. gonorrhoeae*), 10 CFU/mL (*S. enterica*) and 10 CFU/mL (MRSA), comparable to the LOD observed using multiplex qPCR (Euler *et al.,* 2013; Kersting *et al*., 2014). Similarly, Santiago-Felipe, Tortajada-Genaro, Morais, Puchades, and Maquieira (2015) observed a LOD of less than 40 CFU/mL for the duplex detection of *Salmonella* and *Cronobacter* spp. isolated from inoculated milk. Furthermore, amplification of *Salmonella* was observed after 20 min, a reaction time that is much quicker than conventional PCR (Kersting *et al*., 2014). Combined with manual set-up, on-chip RPA analysis can be performed in 60 min—decreasing time of analysis (Kersting *et al*., 2014). If this method were fully automated, processing time and risk of contamination from

hands-on labor would be greatly reduced (Kersting *et al*., 2014; Santiago-Felipe *et al*., 2015). Overall, RPA offers the possibility of a portable, economical rapid detection technique, of use in non-laboratory, agricultural settings—such as farms and ranches, where contamination with foodborne pathogens is common.

1.6 Automated Point-of-Care (POC) Testing of Foodborne Pathogens by RPA

Kersting *et al*. (2014) concluded that the aforementioned "on-chip" analysis method of detection, yielding viable results in under 20 min, is considerably faster than conventional PCR techniques, with no detriment to sensitivity or specificity. It is possible that such a method may eventually evolve into a point-of-care testing device, however, further optimization would be required, as the system involved manual set-up, and was therefore not fully automated (Kersting *el al*., 2014). Nevertheless, without the constricting requirements of PCR, such as a thermocycling process, such a device could easily be simplified at low cost (Kersting *et al*., 2014). To achieve complete automation, researchers have proposed novel methods for *automated* point-of-care testing devices that couple RPA with micro total analysis systems, or more colloquially, "lab-on-a-chip" devices (Kim, Park, Kim, & Cho, 2014; Mauk *et al*., 2015). Such devices maintain a strong potential for point-of-care testing devices, as—much like the biochips discussed by Kersting *et al*. (2014)—their miniaturized size and reduced materials consumption may lead to enhanced performance, while reducing contamination from hands-on labor (Kim *et al*., 2014).

Unlike RPA, PCR—often considered the "gold standard" in terms of rapid detection—is not an adequate method of detection for point-of-care testing devices, much like the "lab-on-a-chip" device. To recapitulate, this is due to the need for a thermocycler during denaturing, annealing and extension of DNA during amplification (Choi *et al*., 2016). In recent years, such automated devices have gained popularity, especially the lab-on-a-disk concept, in which

necessary functions are integrated within a disc-like device (Kim *et al*., 2014). This device achieved complete automation by using a single rotor, which allows for mixing of reagents necessary for an isothermal reaction, such as RPA (Kim *et al*., 2014). Much like the aforementioned biochips, DNA extraction, isothermal methods of detection, such as RPA, and signal detection can be completely carried out on a single lab-on-a-disk. Moreover, such devices house networks of reaction chambers, allowing for simple processing and analysis of samples, while reducing contamination (Choi *et al*., 2016; Kim *et al.*, 2014; Liao *et al*., 2016; Mauk *et al.*, 2015). Unlike the 60 min necessary during preparation and detection of amplicons using biochips (Kersting *et al*., 2014), the automated method of lab-on-a-disk can be performed in under 30 min (Kim *et al*., 2014). Furthermore, such devices can also be designed to detect multiple pathogens, using several different amplification chambers as well as molecular targets (Kim *et al*., 2014; Mauk *et al*., 2015).

To determine the effectiveness of their lab-on-a-disk device using *Salmonella*-spiked milk samples, Kim *et al*. (2014) employed the previously mentioned and commercially available TwistDX amplification kit. Lysis of cells was achieved by 20 seconds of laser-assisted irradiation, in which magnetic beads were heated, consequently leading to cell lysis (Kim *et al*., 2014). Following this, lysate DNA was amplified for 20 min, and then transferred into the metering chamber, while excess liquid passed into the waste chamber. Ten microliters of RPA product was then moved into the dilution chamber, where it was mixed with Phosphate Buffered Saline with Tween 20 (PBST). Rotation of the disk allowed the metering chamber to close, while the dilution chamber opened. The solution was rapidly mixed through short, one second stop-and-rotation cycles. The mixed solution was then moved to the detection chamber, where visual detection was achieved after five minutes using a lateral flow device (Kim *et al*., 2014). LOD in the Phosphate

Buffered Saline (PBS) control, and *Salmonella*-spiked milk samples were respectively identified as 10^1 CFU/mL and 10^2 CFU/mL—comparable to the results obtained using either multiplex qPCR or RPA analysis using biochips (Euler *et al.,* 2013; Kersting *et al*., 2014; Kim *et al*., 2014).

A similar study, performed by Choi *et al*. (2016) assessed the effectiveness of an integrated centrifugal microdevice for the simultaneous detection of three foodborne pathogens (*Salmonella enterica*, *Escherichia coli* O157:H7 and *Vibrio parahaemolyticus*) in spiked milk samples. This disk-like device was designed similarly to the aforementioned device used by Kim *et al*. (2014), and contained a sample reservoir, as well as aliquoting, reaction and waste chambers (Choi *et al*., 2016). To allow for processing of samples, chambers were connected by channels and connecting microchambers, while solutions were mixed at various controlled centrifugation speeds. At 800 rpm, the mixed sample of foodborne pathogens was distributed into four separate aliquoting chambers. As speeds increased to 3000 rpm, the RPA reagents were transferred to the reaction chambers, while speeds increased even further, to 5000 rpm, as bacterial samples were loaded into each reaction chamber (Choi *et al*., 2016). Mixing of bacterial samples and RPA reagents was facilitated by shaking the chip-like device between -600 rpm and +600 rpm for 30 seconds. The mixture was heated to 39 ˚C, on-device, and fluorescence was measured every two minutes (Choi *et al*., 2016). LOD for the bacterial samples was identified as 1250 cells per mL, again, results comparable to previously described studies.

Although lab-on-a-chip devices have gained popularity among researchers, few have sought to couple POC testing with smartphones, to reduce cost by eliminating the need for a fluorescence reader device (Liao *et al*., 2016). In this design, both the smartphone's flashlight and camera are used during optical detection; the flashlight (coupled with an excitation optical filter) is used to *excite* the fluorescent dye, while the camera is used to record fluorescence emission in

real-time (Liao *et al*., 2016). Considering the prevalence of mobile phones, it is surprising that such devices are often overlooked when new diagnostic systems are implemented (Liao *et al*., 2016). To recapitulate, as mobile phones are readily available—even ubiquitous in some countries—it would not be out of the realm of possibility to infer that the consumer may already have a smartphone in their possession, and that the cost of the phone can be subtracted from the overall cost of the POC test (Liao *et al*., 2016).

1.7 Novel Sample Preparation Techniques Using Magnetic Ionic Liquids

Apart from the lab-on-a-chip devices, nucleic acid amplification can be improved though the use of modified sample preparation techniques that facilitate isolation and preconcentration of bacteria. Food matrices are often complex, and inadequate preconcentration may hamper detection limits using nucleic acid amplification, including PCR and RPA (Clark, Purslow, Pierson, Nacham, & Anderson, 2017a). Background microflora present in the food matrix may outcompete the target microorganism, while interference or inhibition may stem from the matrix itself, both of which may attribute to false-negative results and improper interpretation. Detection is often hampered by suspended sample matrix solids, viscosity and non-uniform distribution of the target organism in the sample matrix, requiring the use of laboratory equipment (vortex, centrifuge) or sample preparation reagents (PrepMan Ultra), to achieve adequate preconcentration (Clark *et al*., 2017a; Hice, Clark, Anderson & Brehm-Stecher, 2019). To remedy this, magnetic separation techniques have been used to effectively enrich target microorganisms from environmental samples. When added to a sample, magnetoactive substrates, often coated with antibodies (as with IMS), interact with target microorganisms, and are extracted using an applied magnetic field (Clark *et al*., 2017a; Entis *et al*., 2001; Hice *et al*., 2019). To recapitulate, the target microorganisms are captured and preconcentrated, facilitating simple combination with several detection methods,

including nucleic-based amplification methods (Entis *et al*., 2001; Favrin *et al*., 2000; Favrin *et al*., 2003). While selectivity is achieved using immunoaffinity, due to the use of requisite antibodies, these methods are unstable and costly (Clark *et al*., 2017a).

Magnetic ionic liquids (MIL) are non-volatile, hydrophobic solvents that exhibit susceptibility to magnetic fields. Increased susceptibility to magnetic fields may be achieved by synthetic design, as multiple paramagnetic components can be added to the cation/anion moiety (Clark *et al*., 2017a). The composition of large, flexible ions, coupled with negative Gibbs free energy of solvation, thermodynamically favors the liquid state, facilitating liquid-liquid microextraction from aqueous sample matrices (Mester, Wagner, & Rossmanith, 2010). Moreover, due to their distinctive properties (nonvolatile, nonflammable, chemically and thermally stable), MILs are environmentally attractive alternatives to many traditional organic solvents (Clark *et al*., 2017a; Mester *et al*., 2010; Pierson, Nacham, Clark, Nan, Mudryk, & Anderson, 2017). Their hydrophobicity allows for fine dispersal throughout various sample matrices, which is advantageous, considering use of magnetic beads (affinity, immunoaffinity) suffers from diminished extraction efficiencies due to settling and aggregation (Clark, Varona, & Anderson, 2017b). Use of a solid substrate (beads, nanoparticles, microarrays) adds additional complexity due to slow binding and hybridization of nucleic acids at the solid-liquid interface (Clark *et al.,* 2017b). The vulnerability and reactiveness of MILs to magnetic fields—coupled with their unique properties—is advantageous, and facilitates the possibility for simple, rapid liquid-liquid preconcentration of microorganisms from aqueous sample matrices. In fact, MILs have been successfully applied as extraction phases in liquid-liquid microextraction techniques for the capture and pre-concentration of *E. coli* from aqueous milk samples (Clark *et al*., 2017a).

Preconcentration of *E. coli* was achieved following direct addition of 15 µL of a trihexyl(tetradecyl)phosphonium nickel(II) hexafluoroacetylacetonate ($[P_{66614}^{+}][Ni(hfacac)_3^{-}]$) MIL to an artificially spiked 2 mL 2% milk sample. Through vortex agitation, microdroplets of MIL were homogenously dispersed throughout the aqueous sample, while a 0.9-T magnetic field was applied to the vial, allowing for preconcentration of the MIL-bound *E. coli*, and subsequent removal of the aqueous phase (Clark *et al*., 2017a). Following a brief wash step with deionized water, which facilitates removal of any remaining aqueous suspension, MIL-bound *E. coli* were recovered by addition of nutrient-rich media and successive vortexing steps (Clark *et al*., 2017a). Viable cells were recovered from this "back-extraction," and were analyzed via selective plating or using PCR. Using the ($[P_{66614}^{+}][Ni(hfacac)_3^{-}]$) MIL, detection was achieved as low as 10^4 CFU/mL, however, when coupled with qPCR, LOD was reported as 10^2 CFU/mL (Clark *et al*., 2017a). The use of MILs as a tool for microbial extraction is advantageous, as it facilitates simple, rapid preconcentration of target microorganisms in place of more expensive and less stable alternatives that rely on use of antibodies, while it can easily be coupled with conventional culture-based methods, PCR and qPCR, resulting in detection and identification. Yet, use of selective plating adds time-to-result, while qPCR commands significant initial investment. That said, as a potential alternative, the combined use of MIL-based sample preparation and RPA is valuable, and provides a simple, streamlined alternative to orthodox detection techniques. MIL-based extractions impart centrifuge-free, single-tube simplicity and rapidity, while RPA exhibits promise as a non-cycling rapid, sensitive and specific method of detection.

1.8 Conclusions

Conventional methods used for the detection of *Salmonella* in dairy products and in food processing environments are often slow, require highly skilled technicians or are laboratory bound.

Such methods of detection are unsuitable in agricultural settings, where access to proper laboratory equipment is not available. Because of this, the need for a sensitive, portable and rapid method of detection of foodborne pathogens is required, and several techniques have been introduced in recent years (Vasavada, 1993). Detection methods incorporating established PCR techniques are considered the "gold-standard" in the identification of foodborne pathogens. Such methods, such as qPCR and mPCR techniques, are regarded as sensitive and rapid, and reduce the expense and preparation time required by conventional detection techniques. Unfortunately, much like traditional PCR, qPCR and mPCR techniques do not eliminate the problems encountered when conventional methods of detection are employed. Although qPCR and mPCR slightly reduce cost, these techniques still require a thermal cycling device and a highly trained technician. Moreover, these methods remain laboratory-bound, and are not suitable for use in the field.

Decades of research has demonstrated that PCR is not the "end-all" solution to the establishment of a sensitive, portable and rapid detection method. Enzymatic colorimetric methods of detection, as well as use of paper-bound bacteriophages require less processing time at a lower cost. While selectivity may present as an issue, due to use of non-specific enzymes, these methods have been successfully employed in distinguishing between metabolic states (Hice *et al*., 2018). On the other hand, RPA has demonstrated promise in recent years as a method of detection, as it is rapid and maintains similar levels of specificity to the more established PCR methods. Furthermore, RPA does not require a thermal cycling device, and works at cellular temperatures. RPA offers greater portability and ease-of-use than PCR, which is advantageous for point-of-care testing in agricultural settings where contamination with foodborne pathogens commonly occurs. When combined with improved sample preparation techniques, as with the lab-on-a-chip or MILs, detection limits improve, and reduce time-to-result. The utility of MILs for rapid extraction and

concentration of pathogenic microorganisms from food samples provides a means for physical enrichment that is compatible with RPA.

While MILs suffer from innate lack of selectivity, investigation into incorporating a selective element is underway. Use of peptides or other functional groups may be used to attain selectivity, while sequence-specific capture of DNA has been achieved using ion-tagged oligonucleotides (ITOs) (Clark *et al.,* 2017b). Imidazolium-based ion tags, which facilitate interactions between the ITO probe and hydrophobic MIL, have been added to oligonucleotides; this interaction has demonstrated profound improvement of contact between the probe and the MIL (Clark *et al*., 2017b). Following hybridization between the ITO and target DNA, the MIL-supported ITO can be extracted from solution, and analyzed using qPCR. However, as MILs have been successfully combined with both qPCR and RPA, it is possible that the use of MIL-supported ITOs may demonstrate similar success in improved DNA extraction as qPCR. This combined approach would certainly maintain several advantages including: **1)** time-to-result, **2)** low-cost, **3)** ease of operation and **4)** simple interpretation. Such an approach to rapid detection may enable simple and low-cost sampling of pathogenic microorganisms, which can be used to improve industrial efficiency.

1.9 Organization of the Dissertation

Chapter 2 describes an approach to paper-based enzymatic colorimetric assays for the identification of metabolic state in *Salmonella* Typhimurium and *E. coli* from environmental samples. Oxidoreductases and alkaline phosphatases, non-specific bacterial enzymes present at varying levels depending on metabolic state, were employed. Respectively, these enzymes act by reducing tetrazolium salts to purple formazans, or cleaving para-nitrophenyl-phosphate salts to yellow para-nitrophenol. Color change occurred in less than 60 min, and was observed at 10^3 CFU

and quantifiable at 10^6 CFU. Specificity was added by immobilizing bacteria-specific bacteriophage (P22 or T4) on paper, facilitating detection as low as 10^2 CFU or 10^4 CFU, respectively.

Chapter 3 describes the combined use of MILs and RPA for the capture, concentration and detection of *Salmonella* Typhimurium in water, 2% milk, almond milk and liquid egg product samples. Two MILs were investigated for their bacterial extraction efficiencies, and viable cells were recovered from the MIL-phase using a modified Luria Bertani (LB) broth. Following extraction, a 20 min isothermal RPA assay was employed, and results were visualized using gel electrophoresis or NALFIA. Sodium acetate heat packs were used as a chemical heat source during amplification. The combined approach facilitated detection as low as 10^3 CFU mL^{-1} in 30 to 45 min.

Chapter 4 describes the investigation into the physical properties of two MILs, including potential antimicrobial effects. Here, a panel of Gram-negative bacteria were surveyed, including nine serotypes of *Salmonella* and eight strains of *E. coli* O157:H7. Non-selective and selective media were used to screen for potential deleterious effects of the MILs, including cell injury, over time. MIL-based exposure was compared to another ionic liquid, 1-ethyl-3-methylimidazolium thiocyanate ($[EMIM^+]SCN^-$), which has used for extraction of pathogens from food and has been reported to cause cellular injury. While the ($[P_{66614}^+][Ni(hfacac)_3^-]$) MIL did not possess detectable antimicrobial activity, exposure to the ($[P_{66614}^+][Dy(hfacac)_4^-]$) MIL resulted in cell death. Investigation into the individual components of the ($[P_{66614}^+][Dy(hfacac)_4^-]$) MIL suggested that the ammonium salt was largely responsible for the cytotoxic effects observed.

1.10 References

Achbergerová, L., & Nahálka, J. (2011). Polyphosphate – an ancient energy source and active metabolic regulator. *Microbial Cell Factories, 10*, 1-14. doi:10.1186/1475-2859-10-63

Adkins, J. A., Boehle, K., Friend, C., Chamberlain, B., Bisha, B., & Henry, C. S. (2017). Colorimetric and electrochemical bacteria detection using printed paper-and transparency-based analytic devices. *Analytical Chemistry*, *89*(6), 3613-3621. doi:10.1021/acs.analchem.6b05009

Almaas, E., Kovács, B., Vicsek, T., Oltval, Z. N., & Barabási, A. L. (2004). Global organization of metabolic fluxes in the bacterium *Escherichia coli*. *Nature, 427*(6977), 839-843. doi:10.1038/nature02298

Anany, H., Chen, W., Pelton, R., & Griffiths, M. W. (2011). Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat by using phages immobilized on modified cellulose membranes. *Applied and Environmental Microbiology, 77*(18), 6379-6387. doi:10.1128/AEM.05493-11

Bailey, J. S., & Cox, N. A. (1992). Universal Preenrichment Broth for the simultaneous detection of *Salmonella* and *Listeria* in foods. *Journal of Food Protection, 55*(4), 256-259. doi:10.4315/0362-028X-55.4.256

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review, 11*, 127-152. doi:10.1016/S1387-2656(05)11004-7

Bessey, O. A., Lowry, O. H., & Brock, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *Journal of Biological Chemistry, 164*, 321-329.

Bhagwat, A. (2003). Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology, 84*(2), 217-224. doi:10.1016/S0168-1605(02)00481-6

Blake, M. S., Johnston, K. H., Russell-Jones, G. J., & Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Analytical Biochemistry, 136*(1), 175-179. doi:10.1016/0003-2697(84)90320-8

Centers for Disease Control and Prevention (CDC). (2007). *Escherichia coli* O157:H7 infection associated with drinking raw milk—Washington and Oregon, November – December 2005. *Morbidity and Mortality Weekly Report (MMWR), 56*, 165-167.

Centers for Disease Control and Prevention (CDC). (2010). *E. coli* (*Escherichia coli*): Outbreaks. Retrieved June 19, 2017, from https://www.cdc.gov/ecoli/2010/bravo-farms-cheese-11-24-10.html

Centers for Disease Control and Prevention (CDC). (2015a). *Listeria* (Listeriosis): Outbreaks. Retrieved June 19, 2017, from https://www.cdc.gov/listeria/outbreaks/soft-cheeses-09-15/index.html

Centers for Disease Control and Prevention (CDC). (2015b). *Listeria* (Listeriosis): Outbreaks. Retrieved June 19, 2017, from https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html

Centers for Disease Control and Prevention (CDC). (2016a). Food safety and raw milk. Retrieved June 19, 2017, from https://www.cdc.gov/foodsafety/rawmilk/raw-milk-questions-and-answers.html

Centers for Disease Control and Prevention (CDC). (2016b). *Listeria* (Listeriosis). Retrieved June 19, 2017, from https://www.cdc.gov/listeria/index.html

Centers for Disease Control and Prevention (CDC). (2018). Burden of foodborne illness. Retrieved June 1, 2018, from https://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html

Centers for Disease Control and Prevention (CDC). (2019). *Salmonella*. Retrieved April 14, 2019, from http://www.cdc.gov/salmonella/index.html

Choi, G., Jung, J., Park, B., Oh, S., Seo, J., Choi, J., Kim, D., & Seo, T. (2016). A centrifugal direct recombinase polymerase amplification (direct-RPA) microdevice for multiplex and real-time identification of food poisoning bacteria. *Royal Society of Chemistry, 16*(12), 2309-2316. doi:10.1039/c6lc00329j

Clark, K. D., Purslow, J. A., Pierson, S. A., Nacham, O., & Anderson, J. L. (2017a). Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR amplification and culture-based diagnostics. *Analytical and Bioanalytical Chemistry, 409*(21), 4983-4991. doi:10.1007/s00216-017-0439-y

Clark, K. D., Varona, M., & Anderson, J. L. (2017b). Ion-tagged oligonucleotides coupled with a magnetic liquid support for the sequence-specific capture of DNA. *Angewandte Chemie - International Edition, 56*, 7630-7633. doi:10.1002/anie.201703299

Clegg, S. J., Jia, W., & Cole, J. A. (2006). Role of the *Escherichia coli* nitrate transport protein, NarU, in survival during severe nutrient starvation and slow growth. *Microbiology, 152*, 2091-2100. doi:10.1099/mic.0.28688-0

Coates, A. R. M. (Ed.). (2003). *Dormancy and Low-Growth States in Microbial Disease.* Cambridge, UK: Cambridge University Press.

Denny, J. Bhat, M., & Eckmann, K. (2005). Outbreak of *Escherichia coli* O157:H7 associated with raw milk consumption in the Pacific Northwest. *Foodborne Pathogens and Disease, 5*(3), 321-328. doi:10.1089/fpd.2007.0072

El-Gazzar, F. E., & Marth, E. H. (1992). Salmonellae, salmonellosis, and dairy foods: A review. *Journal of Dairy Science, 75*(9), 2327-2343. doi:10.3168/jds.S0022-0302(92)77993-4

Entis, P., Fung, D. Y. C., Griffiths, M., McIntyre, L., Russell, S., Sharpe, A. N., & Tortorello, M. L. (2001). Rapid methods for detection, identification, and enumeration. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 89-125). Washington, DC: American Public Health Association.

Euler, M., Wang, Y., Heidenreich, D., Patel, P., Strohmeier, O., Hakenberg, S., . . . Weidmann, M. (2013). Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *Journal of Clinical Microbiology, 51*(4), 1110-1117. doi:10.1128/JCM.02704-12

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., … Cerf, O. (2013). Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. *International Journal of Food Microbiology, 162*(2), 190-212. doi:10.1016/j.ijfoodmicro.2012.08.008

Favrin, S. J., Jassim, S. A., & Griffiths, M. W. (2000). Development and optimization of a novel immunomagnetic separation-bacteriophage assay for detection of *Salmonella enterica* serovar Enteritidis in broth. *Applied and Environmental Microbiology, 67*(1), 217-224. doi:10.1128/AEM.67.1.217-224.2001

Favrin, S. J., Jassim, S. A., & Griffiths, M. W. (2003). Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157:H7 in Food. *International Journal of Food Microbiology, 85*(1-2), 63-71. doi:10.1016/S0168-1605(02)00483-X

Food and Drug Administration (FDA). (2015). Grade "A" Pasteurized Milk Ordinance. Retrieved June 19, 2017, from https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/milk/ucm513508.pdf

Food and Drug Administration (FDA). (2016). FDA investigates *Listeria monocytogenes* in ice cream products from Blue Bell Creameries. Retrieved June 19, 2017, from https://www.fda.gov/food/recallsoutbreaksemergencies/outbreaks/ucm438104.htm

Garrido, A., Chapela, M., Román, B., Fajardo, P., Lago, J., Vieites, J. M., & Cabado, A. G. (2013a). A new multiplex real-time PCR developed method for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Control, 30*(1), 76-85. doi:10.1016/j.foodcont.2012.06.029

Garrido, A., Chapela, M., Román, B., Fajardo, P., Lago, J., Vieites, J. M., & Cabado, A. G. (2013b). In-house validation of a multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and *Listeria monocytogenes*. *International Journal of Food Microbiology, 164*(1), 92-98. doi:10.1016/j.ijfoodmicro.2013.03.024

Goodridge, L., & Griffiths, M. (2002). Reporter bacteriophage assays as a means to detect foodborne pathogenic bacteria. *Food Research International, 35*(9), 863-870. doi:10.1016/S0963-9969(02)00094-7

Gunnarsson, A., Hurvell, B., & Thal, E. (1977). Recent experiences with the *Salmonella*-O-1-phage in routine diagnostic work [Abstract]. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Erste Abteilung Originale. Reihe A: Medizinische Mikrobiologie und Parasitologie, 237*(2-3), 222-227.

Hallegraeff, G. M. (1993). A review of harmful algal blooms and their apparent global increase. *Wetlands Australia, 32*(2), 79-99.

Hice, S. A., Santoscoy, M. C., Soupir, M. L., & Cademartiri, R. (2018). Distinguishing between metabolically active and dormant bacteria on paper. *Applied Microbiology and Biotechnology, 102*(1), 367-375. doi:10.1007/s00253-017-8604-y

Hice, S. A., Clark, K. D., Anderson, J. L., & Brehm-Stecher, B. F. (2019). Capture, concentration, and detection of *Salmonella* in foods using magnetic ionic liquids and recombinase polymerase amplification. *Analytical Chemistry, 91*(1), 1113-1120. doi:10.1021/acs.analchem.8b04751

Hirsh, D. C., & Martin, L. D. (1983). Rapid detection of *Salmonella* spp. by using Felix-O1 bacteriophage and high-performance liquid chromatography. *Applied and Environmental Microbiology, 45*(1), 260-264.

Hussein, H. S., & Sakuma, T. (2005). Invited review: prevalence of Shiga toxin-producing *Escherichia coli* in dairy cattle and their products. *Journal of Dairy Science, 88*(2), 450-465. doi:10.3168/jds.S0022-0302(05)72706-5

Jokerst, J., Adkins, J., Bisha, B., Mentele, M., Goodridge, L., & Henry, C. (2012). Development of a paper-based analytical device for colorimetric detection of select foodborne pathogens. *Journal of Analytical Chemistry*, *84*(6), 2900-2907. doi: 10.1021/ac203466y

Kawasaki, S., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2005). Multiplex PCR for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat samples. *Journal of Food Protection, 68*(3), 551-556.

Kersting, S., Rausch, V., Bier, F. F., & von Nickisch-Rosenegk, M. (2014). Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. *Microchimica Acta, 181*(13-14), 1715-1723. doi:10.1007/s00604-014-1198-5

Kim, H., & Bhunia, A. K. (2008). SEL, a selective enrichment broth for simultaneous growth of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. *Applied and Environmental Microbiology, 74*(15), 4853-4866. doi:10.1128/AEM.02756-07

Kim, T., Park, J., Kim, C., & Cho, Y. (2014). Fully integrated lab-on-a-disk for nucleic acid analysis of food-borne pathogens. *Analytical Chemistry, 86*(8), 3841-3848. doi:10.1021/ac403971h

Kozak, J., Balmer, T., Byrne, R., & Fisher, K. (1996). Prevalence of *Listeria monocytogenes* in foods: Incidence in dairy products. *Food Control, 7*(4-5), 215-221. doi:10.1016/S0956-7135(96)00042-4

Lazcka, O., Del Campo, F. J., Muñoz, F. X. (2006). Pathogen detection: A perspective of traditional methods and biosensors. *Biosensors and Bioelectronics, 22*(7), 1205-1217. doi:10.1016/j.bios.2006.06.036

Li, J., & Macdonald, J. (2015). Advances in isothermal amplification: Novel strategies inspired by biological processes. *Biosensors and Bioelectronics, 64*, 196-211. doi:10.1016/j.bios.2014.08.069

Liao, S., Peng, J., Mauk, M., Awasthi S., Song, J., Friedman, H., Bau, H., & Liu, C. (2016). Smart cup: A minimally-instrumented, smartphone-based point-of-care molecular diagnostic device. *Sensors and Actuators B: Chemical*, *229*, 232-238. doi:10.1016/j.snb.2016.01.073

Lillis, L., Siverson, J., Lee, A., Cantera, J., Parker, M., Piepenburg, O., Lehman, D. A., & Boyle, D. S. (2016). Factors influencing recombinase polymerase amplification (RPA) assay outcomes at point of care. *Molecular and Cellular Probes, 30*(2), 74-78. doi:10.1016/j.mcp.2016.01.009

Lim, J. Y, Yoon, J. W., & Hovdel, C. J. (2010). A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *Journal of Microbiology and Biotechnology, 20*(1), 5-14.

Maher, M. M., Jordan, K. N., Upton, M. E., & Coffey, A. (2001). Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *Journal of Applied Microbiology, 90*(2), 201-207. doi:10.1046/j.1365-2672.2001.01232.x

Marth, E. H. (1969). *Salmonellae* and salmonellosis associated with milk and milk products. A review. *Journal of Dairy Science, 52*(3), 283-315. doi:10.3168/jds.S0022-0302(69)86552-5

Marth, E. H. (1994). Listeriosis. In G. W. Beran & J. H. Steele (Eds.), *Handbook of zoonoses* (pp. 321-328). Boca Raton, FL: CRC Press.

Mauk, M. G., Liu, C., Sadik, M., & Bau, H. H. (2015). Microfluidic devices for nucleic acid (NA) isolation, isothermal NA amplification, and real-time detection. *Methods in Molecular Biology*, *1256*, 15-44. doi:10.1007/978-1-4939-2172-0_2

McConnell, M. R., and Schoelz, J. E. (1983). Evidence for shorter average O-polysaccharide chainlength in the lipopolysaccharide of a bacteriophage Felix 01-sensitive variant of *Salmonella anatum* A1. *Journal of General Microbiology, 129*(10), 3177-3184. doi:10.1099/00221287-129-10-3177

Meng, J., Feng, P., & Doyle, M. P. (2001). Pathogenic *Escherichia coli*. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 331-341). Washington, DC: American Public Health Association.

Mester, P., Wagner, M., and Rossmanith, P. (2010). Use of ionic-liquid extraction for recovery of *Salmonella* Typhimurium and *Listeria monocytogenes* from food matrices. *Journal of Food Protection, 73*(4), 680-687.

Modi, R., Hirvi, Y., Hill, A., & Griffiths, M. W. (2001). Effect of phage on survival of *Salmonella enteritidis* during manufacture and storage of cheddar cheese made from raw and pasteurized milk. *Journal of Food Protection, 64*(7), 927-933.

Mungai, E. A., Behravesh, C. B., & Gould, L. H. (2015). Increased outbreaks associated with nonpasteurized milk, United States, 2007–2012. *Emerging Infectious Diseases, 21*(1), 119-122. doi:10.3201/eid2101.140447

Murinda, S. E., Ibekwe, A. M., Zulkaffly, S., Cruz, A., Park, S., Razak, N., . . . Balkcom, S. (2014). Real-time isothermal detection of Shiga toxin-producing *Escherichia coli* using recombinase polymerase amplification. *Foodborne Pathogens and Disease, 11*(7), 529-536. doi:10.1089/fpd.2013.1663

Murphree, C. A., Heist, E. P., & Moe, L. A. (2014). Antibiotic resistance among cultured bacterial isolates from bioethanol fermentation facilities across the United States. *Current Microbiology, 69*(3), 277-285. doi:10.1007/s00284-014-0583-y

Murray, P. R., Rosenthal, K. S., & Pfaller, M. A. (2009). Enterobacteriaceae. In *Medical Microbiology* (pp. 307-309). Philadelphia, PA: Mosby Elsevier.

Notermans, S., Dufrenne, J., Leimeister-Wachter, M., Domann, E., & Chakraborty, T. (1991). Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. *Applied and Environmental Microbiology, 57*(9), 2666-2670.

Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications. *Foodborne Pathogens and Disease, 2*(2), 115-129. doi:10.1089/fpd.2005.2.115

Omiccioli, E., Amagliani, G., Brandi, G., & Magnani, M. (2009). A new platform for real-time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiology, 26*(6), 615-622. doi:10.1016/j.fm.2009.04.008

Piepenburg, O., Williams, C. H., Stemple, D. L., & Armes, N. A. (2006). DNA detection using recombination proteins. *PLoS Biology, 4*(7), 1115-1121. doi:10.1371/journal.pbio.0040204

Pierson, S. A., Nacham, O., Clark, K. D., Nan, H., Mudryk, Y., Anderson, J. L. (2017). Synthesis and characterization of low viscosity hexafluoroacetylacetonate-based hydrophobic magnetic ionic liquids. *New J. Chem., 41*, 5498-5505. doi:10.1039/C7NJ00206H

Posthuma-Trumpie, G. A., Korf, J., & van Amerongen, A. (2009). Lateral flow (immuno)assay: Its strengths, weaknesses, opportunities and threats. A literature survey. *Analytical and Bioanalytical Chemistry, 393*(2), 569-582. doi:10.1007/s00216-008-2287-2

Ryser, E. T., & Donnelly, C. W. (2001). *Listeria*. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 343-356). Washington, DC: American Public Health Association.

Santiago-Felipe, S., Tortajada-Genaro, L. A., Puchades, R., & Maquieira, A. (2014). Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis. *Analytica Chimica Acta, 811(*6), 81-87. doi:10.1016/j.aca.2013.12.017

Santiago-Felipe, S., Tortajada-Genaro, L. A., Morais, S., Puchades, R., & Maquieira, Á. (2015). Isothermal DNA amplification strategies for duplex microorganism detection. *Food Chemistry, 174*(1), 509-515. doi:10.1016/j.foodchem.2014.11.080

Singh, J., Batish, V. K., & Grover, S. (2012). Simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in dairy products using real time PCR-melt curve analysis. *Journal of Food Science and Technology, 49*(2), 234-239. doi:10.1007/s13197-011-0278-3

Shan, S., Lai, W., Xiong, Y., Wei, H., & Xu, H. (2015). Novel strategies to enhance lateral flow immunoassay sensitivity for detecting foodborne pathogens. *Journal of Agricultural and Food Chemistry, 63*(3), 745-753. doi:10.1021/jf5046415

Suo, B., & Wang, Y. (2012). Evaluation of a multiplex selective enrichment broth SEL for simultaneous detection of injured *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Brazilian Journal of Microbiology*, *44*(3), 737-742.

TwistDX. (2018). Recombinase polymerase amplification, or RPA, is the breakthrough, isothermal replacement to PCR. Retrieved April 14, 2019, from https://www.twistdx.co.uk/en/rpa

Vasavada, P. (1993). Rapid methods and automation in dairy microbiology. *Journal of Dairy Science, 76*(10), 3101-3113. doi:10.3168/jds.S0022-0302(93)77649-3

Wei, Z., Zenewicz, L. A., & Goldfine, H. (2005). *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C has evolved for virulence by greatly reduced activity on GPI anchors. *Proceedings of the National Academy of Sciences of the United States of America, 102*(36), 12927-12931. doi:10.1073/pnas.0501725102

Welkos, S., Schreiber, M., & Baer, H. (1974). Identification of *Salmonella* with the O-1 bacteriophage. *Applied Microbiology, 28*(4), 618-622.

Wood, D. S., Collins-Thompson, D. L., Irvine, D. M., & Myhr, A. N. (1984). Source and persistence of *Salmonella muenster* in naturally contaminated cheddar cheese. *Journal of Food Protection, 47*(1), 20-22. doi:10.4315/0362-028X-47.1.20

Wu, V. C. H. (2008). A review of microbial injury and recovery methods in food. *Food Microbiology, 25*(6), 735-744. doi:10.1016/j.fm.2008.04.011

Yan, L., Zhou, J., Zheng, Y., Gamson, A. S., Roembke, B. T., Nakayama, S., & Sintim, H. O. (2014). Isothermal amplified detection of DNA and RNA. *Molecular BioSystems, 10*(5), 970-1003. doi:10.1039/c3mb70304e

Yang, K., & Metcalf, W. W. (2004). A new activity for an old enzyme: *Escherichia coli* bacterial alkaline phosphatase in a phosphate-dependent hydrogenase. *Proceedings of the National Academy of Sciences of the United States of America, 101*(21), 7919-7924. doi:10.1073/pnas.0400664101

Yaron, S., & Matthews, K. R. (2002). A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: Investigation of specific target genes. *Journal of Applied Microbiology, 92*(4), 633-640. doi:10.1046/j.1365-2672.2002.01563.x

Yetisen, A. K., Akram, M. S., & Lowe, C. R. (2013). Paper-based microfluidic point-of-care diagnostic devices. *Lab on a Chip, 13*(12), 2210-2251. doi:10.1039/c3lc50169h

CHAPTER 2

DISTINGUISHING BETWEEN METABOLICALLY ACTIVE AND DORMANT BACTERIA ON PAPER

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Abstract

Switching between metabolically active and dormant states provides bacteria with protection from environmental stresses, and allows rapid growth under favorable conditions. This rapid growth can be detrimental to the environment, e.g., pathogens in recreational lakes, or to industrial processes, e.g., fermentation, making it useful to quickly determine when the ratio of dormant to metabolically active bacteria changes. While a rapid increase in metabolically active bacteria can cause complications, a high number of dormant bacteria can also be problematic, since they can be more virulent and antibiotic-resistant. To determine the metabolic state of *Escherichia coli* and *Salmonella* Typhimurium, we developed two paper-based colorimetric assays. The color changes were based on oxidoreductases reducing tetrazolium salts to formazans, and alkaline phosphatases cleaving phosphates from nitrophenyl-phosphate salt. Specifically, we added iodophenyl-nitrophenyl-phenyl tetrazolium salt (INT) and methylphenazinium methyl sulfate to metabolically active bacteria on paper, and INT and para-nitrophenyl phosphate salt to dormant bacteria on paper. The color changed in less than 60 minutes, and was generally visible at 10^3 CFU and quantifiable at 10^6 CFU. The color changes occurred in both bacteria, since oxidoreductases and alkaline phosphatases are common bacterial enzymes. On one hand, this feature makes the assays suitable to a wide range of applications, on the other, it requires specific capture, if only

one type of bacterium is of interest. We captured *Salmonella* or *E. coli* with immobilized P22 or T4 bacteriophages on the paper, before detecting them at levels of 10^2 or 10^4 CFU, respectively. Determining the ratio of the metabolic state of bacteria or a specific bacterium at low cost and in a short time, makes this methodology useful in environmental, industrial and health-care settings.

2.1 Introduction

A large fraction of bacteria exist in a dormant state, including over 30% in sludge, 83% in soil, and 50% or more in lakes (Lennon & Jones, 2011). When dormant bacteria change to an active metabolic state, their sometimes rapid reproduction can lead to a bacterial bloom. In a waterbody, a bloom can cause anoxic conditions in the environment, resulting in a fish kill, while in an industrial setting it could potentially alter the yield and product properties during fermentation (Hallegraeff, 1993; Murphree, Heist, & Moe, 2014). Rapidly reproducing pathogenic bacteria can cause disease outbreaks, especially since dormancy can increase pathogenicity and antibiotic resistance (Becker, Selbach, Rollenhagen, Ballmaier, Meyer, Mann, & Bumann, 2006; Coates, 2003; Lewis, 2007; Poncet *et al*., 2009). To prevent, or at least counteract these effects, we need methods to determine changes in the ratio of dormant and metabolically active bacteria quickly, and preferably techniques that can be applied directly in the field.

When the metabolic state of bacteria changes, the type and level of enzymes expressed change (Ayyash, Wu, & Ravi Selvaganapathy, 2014). For example, dormant *E. coli* express more alkaline phosphatases (ALP) and oxidoreductases associated with survival, such as nitrate reductase, compared to metabolically active *E. coli*, which express less ALP, and oxidoreductases in their central metabolism (Almaas *et al*., 2004 Achbergerová *et al*., 2011; Clegg, Jia, & Cole, 2006; Yang & Metcalf, 2004). A range of viable but non-culturable (VBNC) bacteria show similar behavior including *Campylobacter jejuni, Helicobacter pylori*, and *Salmonella* (Becker *et al*.,

2006; Jones, 2001; Li, Mendis, Trigui, Oliver, & Faucher, 2014). Both oxidoreductases and alkaline phosphatases react with dyes, including tetrazolium salts that reduce to formazans for oxidoreductases and nitrophenyl-phosphates for ALP (Berridge, Herst, & Tan, 2005; Bessey, Lowry, & Brock, 1946; Blake, Johnston, Russell-Jones, & Gotschlich, 1984; Rodriguez, Phipps, Ishiguro, & Ridgway, 1992). Tetrazolium salts are commonly used to determine the number of viable cells in solutions, since both the kinetics of the color change and the final color intensity is related to the concentration of the enzymes (Berridge *et al*., 2005; Blake *et al*., 1984).

Colorimetric detection of bacteria in solution by simply adding relevant dyes is very common, but often requires instrumentation for the color analysis (Blake *et al*., 1984). On materials, on the other hand, the color is localized and with that more concentrated allowing for analysis by-eye and making the assays more portable (Anany, Chen, & Pelton, 2011; Pelton, 2009). Paper, an easy to modify and low-cost material, has been used to colorimetrically detect liver function enzymes and small molecules in blood, and bacteria contamination in food, beverages and water (Fiksdal, Pommepuy, Caprais, & Midttun, 1994; Hu, Wang, Wang, Pingguan-Murphy, Lu, & Xu, 2014; Jokerst, Adkins, Bisha, Mentele, Goodridge, & Henry, 2012; Lazcka, Del Campo, & Muñoz, 2007; Martinez, Phillips, Butte, & Whitesides, 2007; Nie *et al*., 2010; Rompré, Servais, Baudart, de-Roubin, Laurent, 2002; Vella *et al*., 2012; Yetisen, Akram, & Lowe, 2013). These assays focus on identifying specific bacteria in a matrix, by using reactive dyes interacting with enzymes present in only those bacteria (Adkins, Boehle, Friend, Chamberlain, Bisha, & Henry, 2017).

In this study, we used tetrazolium salts and nitrophenyl-phosphate to determine the metabolic state of *E. coli* and *Salmonella* Typhimurium on paper. Since the reactive dyes were not specific for the bacteria, we combined the assays with bacteriophages adsorbed on the paper to

capture only bacteria of interest. Bacteriophages adsorbed on materials or chemically bound to them have been used to capture *E. coli* and *Salmonella* before detection (Anany *et al*., 2011; Cademartiri, Anany, Gross, Bhayani, Griffiths, & Brook, 2010; Lakshmanan, Guntupalli, Hu, Kim, Petrenko, Barbaree, & Chin, 2007; Li, Chen, Horikawa, Shen, Simonian, & Chin, 2010; Wang, Sauvageau, & Elias, 2016). We used T4 bacteriophages for *E. coli* and P22 bacteriophages for *Salmonella*. Bacteria adsorbed on the paper or captured by immobilized bacteriophages were detected with iodophenyl-nitrophenyl-phenyl tetrazolium salt (INT) mixed with an electron transporter at neutral pH or nitrophenyl phosphate at alkaline pH. The assay at neutral pH was similar to those used for metabolically active bacteria in solution and showed a stronger purple color for metabolically active bacteria on paper (Berridge *et al*., 2005). The assay at alkaline pH, on the other hand, exhibited a stronger purple color for dormant bacteria. The colors were analyzed visually and quantified by image analysis allowing us to relate the color intensity to the metabolic state and concentration of *E. coli* and *Salmonella* on the paper.

2.2 Materials and Methods

2.2.1 Bacteria and Phage Cultures: *Salmonella enterica* subspecies *enterica* ser. Typhimurium (ATCC 19585) and *Escherichia coli* K12 (ATCC 25404) were purchased from the American Type Culture Collection (Manassas, VA), while environmental *E. coli* (*E. coli* LLV) were isolated from lake water (Lake LaVerne, Ames, IA) by plating the water on modified mTEC agar. Bacteria were propagated and enumerated (plate counts) using standard techniques with Luria Bertani (LB) broth and agar. Dormant bacteria were prepared by inoculating filtered autoclaved lake water (FALW) with 10^9 CFU/ml and storing the samples for 30 to 40 days at 4 ºC (Ozkanca & Flint, 1997). Dormant bacteria were enumerated using plate counts, before and after preparation in FALW. About 10% of these bacteria survived storage, which were classified as dormant. T4 (ATCC

11303-B4) and P22 (ATCC 19585-B1) bacteriophages were propagated and enumerated on overlays and stored in lambda buffer (100 mM NaCl, 16.6 mM $MgSO_4$, 50 mM Tris-HCl buffer pH 7.5 and 0.01% w/v gelatin) (Clokie & Kropinski, 2009).

2.2.2 Colorimetric Assays for Bacterial Enzymes on Paper: Colorimetric assays on Whatman® grade 3 filter papers were based on the reduction of tetrazolium salts or the hydrolysis of para-nitro-phenyl-phosphate. To create reaction zones the paper was cut into circles with 38.5 mm^2 surface area, or wax rings with 5 mm diameter were printed with Xerox Color Qube 8570DN™ (Norwalk, CT) and melted through the paper (5-8 minutes at 85 °C) (Carrilho, Martinez, & Whitesides, 2009). The assays were performed by first adding 10 µl of the bacteria in phosphate buffered saline (PBS) at pH 7and 5 µl of assay solution to the paper. The assay solution for metabolically active bacteria was a freshly prepared solution with 0 to 2.5 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), and 0 to 1 mM 5-methylphenazin-5-ium methyl sulfate (PMS) in PBS at pH 7 (see Table *S*1 for detailed ratios). For dormant bacteria, we used a freshly prepared solution of 0 to 3 mM INT and 1 to 45 mM nitrophenyl phosphate (PNPP) in 0.5 mM Tris buffer at pH 10 (details in Table *S*2). Using 2.5 mM INT and 1 mM PMS, the above was repeated using combined mixtures of metabolically active and dormant *Salmonella* at 10^8 CFU/mL (100% active to 100% dormant bacteria). For all samples, the bacteria and assay reacted in minutes and were dried in the dark (one to four hours) before scanning (Canon LiDE 110), while measurement of the color intensity occurred using ImageJ™ (Vella *et al*., 2012). The color for INT/PMS mixtures was measured in the green channel while INT/PNPP mixtures were analyzed in the blue channel, since these led to the strongest differences at difference concentrations of bacteria. At least five replicates of each test were performed.

2.2.3 Immobilization of Bacteriophages and Capture of Bound Bacteria: Bacteriophages were immobilized on paper by adsorption. Single circles of Whatman® grade 3 filter papers were placed vertically inside a 96-well plate, and 200 µl of bacteriophage (10^{10} PFU ml^{-1}) were added, completely covering the paper on both sides. After 48 hours at room temperature in the dark, the bacteriophage solution was removed and unreacted sites on the paper were blocked by immersing the papers in 200 µl of 1 w/v% bovine serum albumin (BSA) for one hour. The blocked papers were washed with 100 µl of PBS buffer before use. *E. coli* (10^3 – 10^7 CFU) or *Salmonella* (10^2 – 10^6 CFU) were added and allowed to interact for five minutes at 37 ºC and shaking at 300 RPM. Unbound bacteria were removed by four washes with 100 µl of PBS buffer. The captured bacteria were analyzed as described above. Eight replicates were performed for each test.

2.3 Results and Discussion

2.3.1 Development of Colorimetric Assays: Each metabolic state required its own assay on paper. Oxidoreductases in metabolically active bacteria reacted with a tetrazolium salt at neutral pH, while in dormant bacteria, oxidoreductases and alkaline phosphatases reacted with a mixture of a tetrazolium salt and nitrophenyl phosphate at alkaline pH.

2.3.2 Iodonitrotetrazolium Chloride for Metabolically Active *E. coli* on Paper: In solution, several tetrazolium salts change color in the presence of bacteria (Berridge *et al*., 2005). On paper, only iodophenyl-nitrophenyl-phenyl tetrazolium chloride (INT) showed a purple color in the presence of *E. coli*, while methyl-thiazolyl-diphenyl-tetrazolium bromide, and neotetrazolium chloride did not change color. INT developed color faster when combined methylphenazinium methyl sulfate (PMS), an electron transporter. Changing the concentrations and ratios of INT and PMS at constant *E. coli* ($5*10^3$ CFU/mm^2) did not lead obvious trends in the developed color (Fig. 1, Table *S*1). The darkest purple developed for the highest concentration of INT (2.5 mM) and

PMS (1 mM), while the second darkest purple was present at the lowest concentrations of INT (0.5 mM) and PMS (0.25 mM). Two other concentrations and ratios of INT and PMS (1.5 mM INT/0.5 mM PMS, and 2 mM INT/1 mM PMS) developed a weaker, but visible color. When images were analyzed digitally, the green component of the purple color exhibited the largest changes with concentrations of *E. coli*, and was used for quantitative analysis. At the highest concentration of INT and PMS, the green component of the color increased linearly with increasing concentrations of *E. coli* on the paper (Fig. 1), with a color change significant from background at $5*10^3$ CFU/mm^2.

Metabolically active bacteria developed a purple color while reacting with INT and PMS due to the reduction of the tetrazolium salt by oxidoreductases. While in solution, INT and PMS concentrations below the toxic level—1 mM for INT reacting in *E. coli*—are generally sufficient to detect metabolically active bacteria, on paper millimolar quantities five to forty times higher were needed (Hatzinger, Palmer, Smith, Peñarrieta, & Yoshinari, 2003; Ke *et al*., 2014; Smith & McFeters, 1997). The need for a higher concentration likely came from an interaction of INT and PMS with the paper. Both molecules are positively charged, while the paper is negatively charged. This charge difference could attract INT and PMS to the paper fibers preventing them from interacting with the bacteria. The higher concentration of INT in our assay likely determined which oxidoreductases were present in the bacteria. Only the lowest concentration of INT tested (0.5 mM) was below the toxic level suggesting that oxidoreductases involved in normal cellular processes were mainly responsible for the color change. At higher concentrations of INT, oxidoreductases expressed in response to the toxic levels of dye could also be present, such as cytochrome oxidases (Ullrich, Karrasch, Hoppe, Jeskulke, & Mehrens, 1996). The amount of these

oxidoreductases depended on the concentration of INT as observed by an increase in the color with increasing concentration of INT above the toxic level (Fig 1).

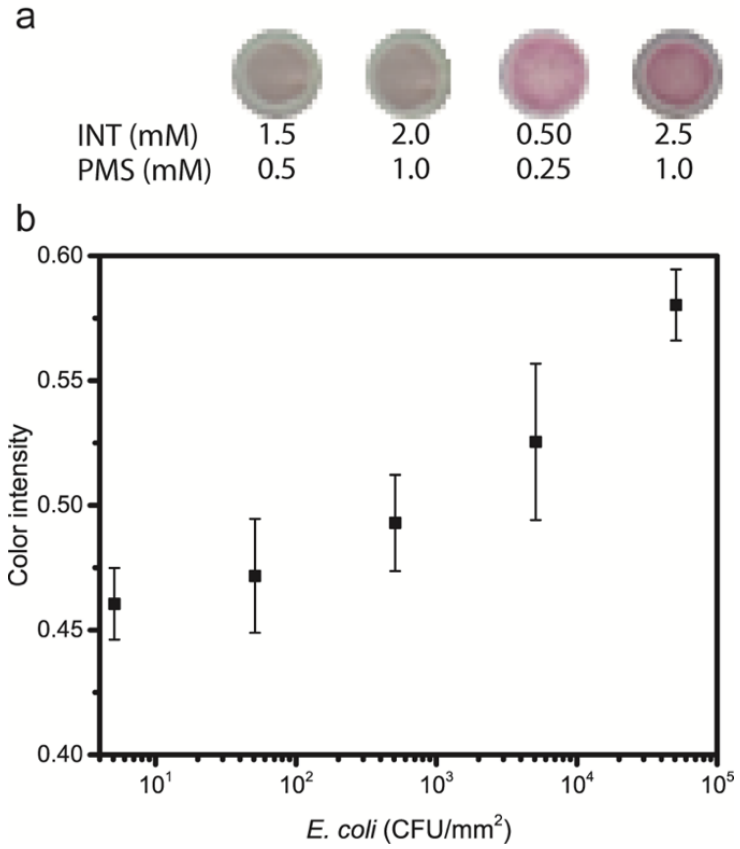

Figure 1. INT/PMS assay on paper detected metabolically active *E. coli*. (**Panel a**) Distinct ratios of INT and PMS produced different color intensity values on paper at the same concentration of *E. coli*. (**Panel b**) Different concentration of *E. coli* on paper detected by the assay with highest color intensity 2.5 mM INT and 1 mM PMS. The green component of the purple color was used for analysis. Error bars correspond to two standard errors (n=6).

2.3.3 Para-nitrophenylphosphate and INT for Dormant *E. coli* on Paper: The detection of dormant bacteria was based on two classes of enzymes: oxidoreductases and alkaline phosphatases (ALP). These enzymes have been observed to be active in dormant bacteria. We used INT and para-nitrophenyl phosphate (PNPP) as substrates for these enzymes. PNPP is cleaved by ALP to produce yellow para-nitrophenol while INT is transformed to purple formazan by action of

oxidoreductases. Considering that para-nitrophenol was difficult to observe on the white paper, and taking advantage of the presence of both type of enzymes in dormant bacteria, we combined PNPP and INT substrates to detect simultaneously ALP and oxidoreductases activities. The combined substrate INT/PNPP led to a stronger purple color that was easier to distinguish on paper compared to these substrates alone.

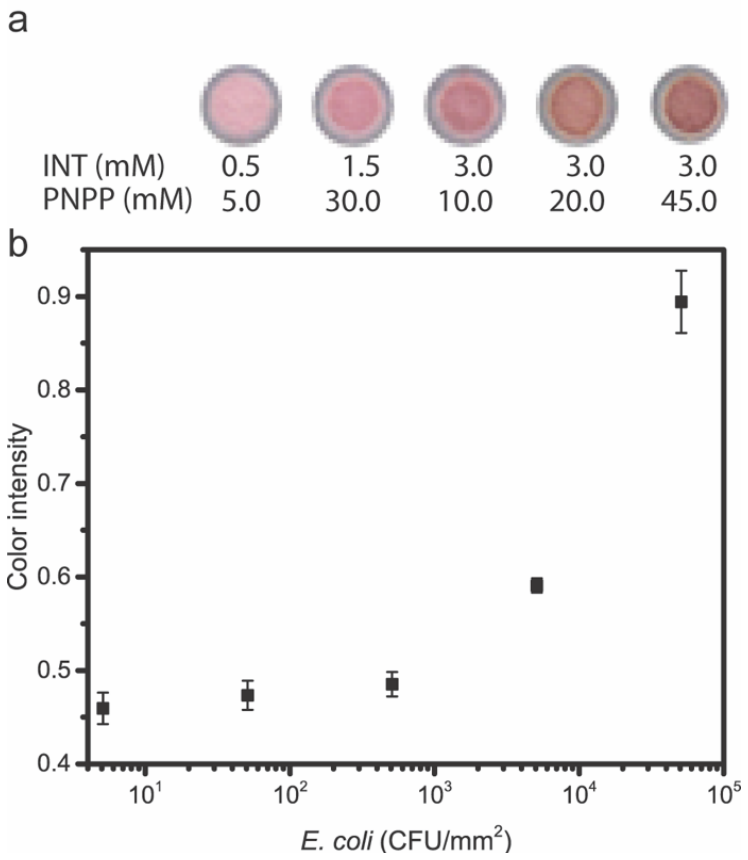

Figure 2. INT/PNPP assay on paper detected dormant *E. coli*. (**Panel a**) Distinct ratios of INT and PNPP produced different color intensity values on paper at the same concentration of *E. coli*. (**Panel b**) Different concentration of *E. coli* on paper detected by the assay with highest color intensity 3 mM INT and 45mM PNPP. The blue component of the purple color was used for analysis. Error bars correspond to two standard errors (n=6).

For dormant *E. coli* at pH 10, the color of the assay increased with increasing concentration of INT (Fig. 2, Table *S*2), over a wider range of INT concentrations than at pH 7 due to an increased solubility of INT (3 mM versus 2.5 mM). Adding PNPP (10 to 45 mM) to a constant concentration

of INT (3 mM) led to an increase in color with increasing PNPP concentration as long as 5 mM of PNPP was present (Fig. 2a). The strongest color in the blue channel developed for the highest concentrations of INT/PNPP (3 mM/45 mM), which was used to measure to color intensity at different concentrations of dormant *E. coli* (Fig. 2b). There was a visible difference in color between 10^3 CFU/mm^2 and 10^4 CFU/mm^2 (Fig. S1), which led to a significant increase in the color intensity in the blue channel. PNPP likely reacted with alkaline phosphates in the periplasm, while INT reacted with oxidoreductases in cytosol and periplasm (Clegg *et al*., 2006; Ozkanca & Flint, 1996; Yang & Metcalf, 2004). As with the assay for metabolically active bacteria, millimolar concentrations of INT were required for the darkest color changes. These higher concentrations suggest that some dye adsorbed on the paper and oxidoreductases present in dormant bacteria and those for detoxification reacted with the INT.

2.3.4 Identifying the Metabolic States of Dormant *E. coli* and *Salmonella* Typhimurium on Paper: After developing assays for metabolically active and dormant *E. coli*, we tested both assays on metabolically active and dormant *E. coli* and *Salmonella* at different concentrations. At each concentration and metabolic state, the bacteria were analyzed on separate papers with mixtures of INT (2.5 mM) and PMS (1 mM) at a neutral pH (INT/PMS), or INT (3 mM) and PNPP (45 mM) at alkaline pH (INT/PNPP). Papers were dried completely before analysis (one to four hours), although a color change for the higher bacterial concentrations ($5*10^5$ CFU/mm^2) occurred within five minutes.

As expected, INT/PMS showed stronger color for metabolically active bacteria, while INT/PNPP exhibited stronger color for dormant bacteria (Fig. 3). The extent of the color difference depended on the bacterium and its concentration. The INT/PMS assay for metabolically active

Salmonella showed a color different to the background at $5*10^5$ CFU/mm^2, while *E. coli* demonstrated a difference at $5*10^2$ CFU/mm^2.

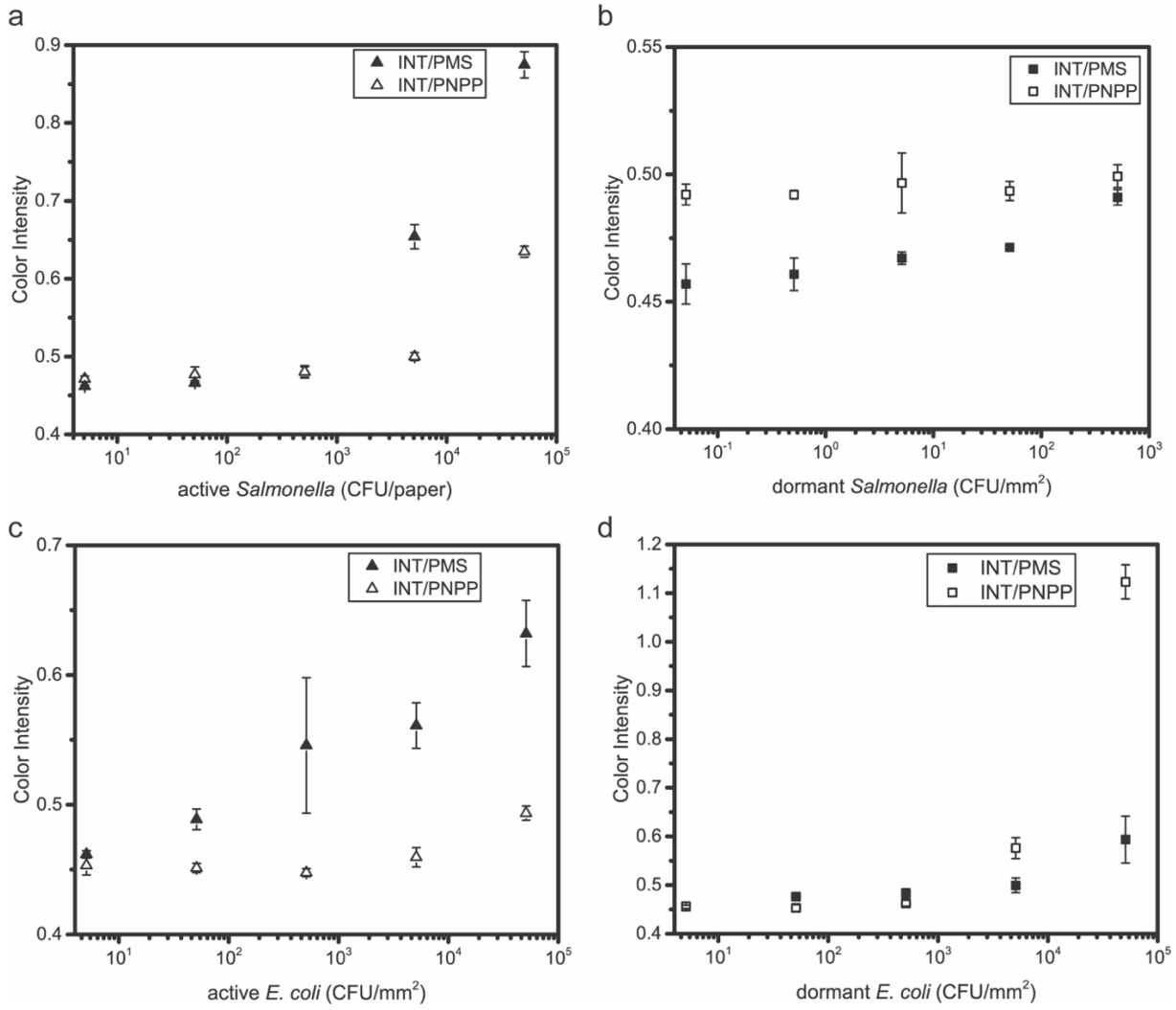

Figure 3. Identification of bacterial metabolisms by colorimetric assays on paper. (**Panel a,c**) Metabolically active cultures *Salmonella* Typhimurium (**Panel a**) and *E. coli* (**Panel c**) reacted with INT/PMS (closed triangles) and INT/PNPP (open triangles) solutions on paper. (**Panel b,d**) Dormant *Salmonella* Typhimurium (**Panel b**) and *E. coli* (**Panel d**) reacted with the INT/PMS (closed squares) and INT/PNPP (open squares) on paper. Reported concentrations are 38.5 times higher if expressed per paper. INT/PMS values: green component of the color, INT/PNPP values: blue component of the color. Error bars correspond to two standard errors (n=5).

On the other hand, for dormant bacteria and the INT/PNPP assay, *Salmonella* changed color at 50 CFU/mm^2, compared to $5*10^4$ CFU/mm^2 *E. coli*. The difference in color between the

assays for the same metabolic state was generally more distinct at higher concentrations of bacteria (Fig. 3), except for dormant *Salmonella*. For example, for $5*10^2$ CFU/mm^2 of metabolically active *E. coli*, the color for INT/PMS was 8% higher than for INT/PNPP, while at $5*10^5$ CFU/mm^2 it was 30% higher. The largest difference in color between INT/PMS and INT/PNPP was seen for dormant *E. coli* at $5*10^5$ CFU/mm^2, when INT/PNPP showed a 50% stronger color.

Aside from the concentration of the dyes, the concentration and species of the bacteria influenced the color. In general, higher concentrations of bacteria led to stronger colors allowing a semi-quantitative measure for the concentration of *E. coli* and *Salmonella* at each metabolic state. For example, metabolically active *E. coli* reacting with INT/PMS developed a color at lower concentrations compared to dormant *E. coli* reacting with INT/PNPP ($5*10^2$ CFU/mm^2 in Fig. 3c compared to $5*10^4$ CFU/mm^2 in Fig. 3d). The weaker color in dormant *E. coli* was likely due to fewer expressed oxidoreductases. When comparing dormant *E. coli* to dormant *Salmonella* the latter exhibited color change at lower concentrations (50 CFU/mm^2 in Fig. 3b), suggesting more oxidoreductases in dormant *Salmonella*. At higher concentrations, the difference in color between the assays was generally more pronounced, making it easier to distinguish between metabolically active and dormant bacteria. High concentrations of bacteria switching from a dormant to a metabolically active state, for example, are associated with cyanobacterial blooms in environmental waters that can lead to the death of wildlife or are linked to failed bioethanol productions when dormant lactic acid bacteria revert to an active metabolic state during fermentation (Hallegraeff, 1993; Murphree *et al*., 2014).

After establishing a relation between the color intensity of each assay, and the metabolic state and concentration of bacteria, we measured metabolically active *Salmonella* in a mixture with dormant *Salmonella* (100% active to 100% dormant) at constant bacteria concentration (10^8

CFU/mL). The mixtures on the papers were analyzed with INT/PMS (2.5 mM/1 mM). As expected, INT/PMS exhibited a darker color for mixtures with higher concentrations of metabolically active *Salmonella*. The color decreased linearly (R^2 = 0.9997) with decreasing number of metabolically active until 25% of active *Salmonella*, which exhibited the same color as 100% dormant *Salmonella* (Fig. 4). This linear decrease led to 8%, 14%, and 19% weaker color in the green channel from 100% active *Salmonella* to 25% active *Salmonella*. This difference in color was visible by eye (Fig. 4). This linear decrease in the color intensity allows the determination of the ratio of active and dormant bacteria in a mixture as long as the overall concentration is constant. For mixtures with different overall concentrations of bacteria, the color intensity, which also depends on the concentration of bacteria, will have to be normalized.

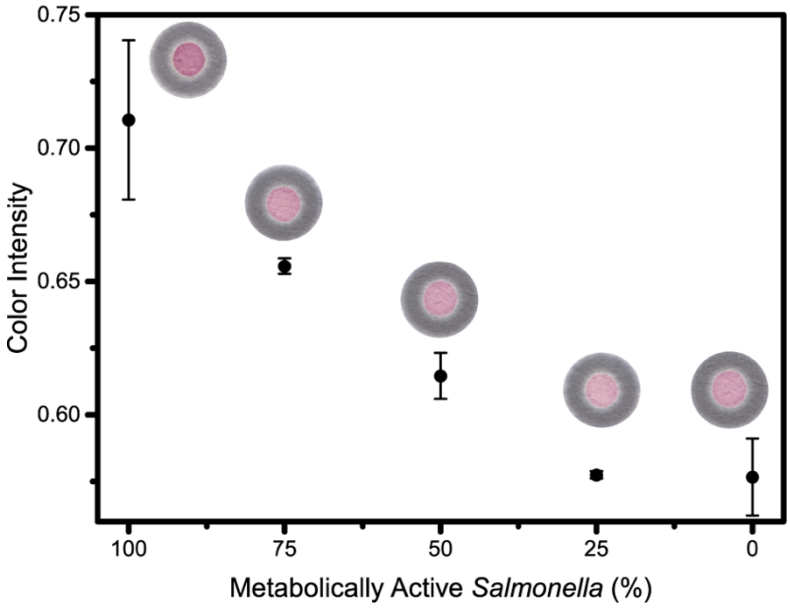

Figure 4. INT/PMS assay on paper detected mixtures of metabolically active and dormant *Salmonella* at 10^8 CFU/mL. The green component of the purple color was used for analysis. Error bars correspond to two standard errors (n=5). Inserts: representative images of the color on paper. Diameter of the circles is 5 mm.

2.3.5 Detection of Dormant *E. coli* and Metabolically Active *Salmonella* After Capture by Bacteriophages on Paper: Detection of specific bacteria with the non-specific INT/PMS and INT/PNPP assays required specific capture of the bacteria to defined areas of the paper. We used bacteriophages adsorbed on the paper to achieve specificity. T4 bacteriophages were used for *E. coli*, while P22 bacteriophages were used for *Salmonella*. T4 bacteriophages were stable to washing losing about 20% of active bacteriophages. T4 bacteriophage adsorbed on filter paper bound dormant *E. coli* and changed color in the presence of INT/PNPP. Papers immersed for 48 hours in the bacteriophage solution, showed the darkest purple with INT/PNPP after *E. coli* capture. A shorter time of adsorption reduced the color intensity, suggesting a lower number of captured bacteria. Washing the papers with the adsorbed T4 bacteriophages up to eight times before capturing the bacteria, did not reduce the color intensity of bound *E. coli.* The color only changed with the number of *E. coli* captured on the paper, with stronger colors for higher numbers of *E. coli* (Fig. 5). P22 bacteriophages adsorbed on paper captured metabolically active *Salmonella* that changed color with INT/PMS as low as 3 CFU/mm^2.

The developed assays were not specific for a given bacterium, but capturing specific bacteria with bacteriophages adsorbed on the paper allowed us to determine the metabolic state solely of the bacteria of interest. Even on unmodified paper a sufficient number of bacteriophage tails were available to capture their host bacteria. The unmodified paper, however, required a longer adsorption time compared to cellulose membranes with cationic charge (Anany *et al*., 2011). This longer time was likely due to weaker attractive forces between the bacteriophages and the paper, which led to slower adsorption on the paper fibers. Once bacteriophages were adsorbed, they were stable to washing and able to capture their host bacteria.

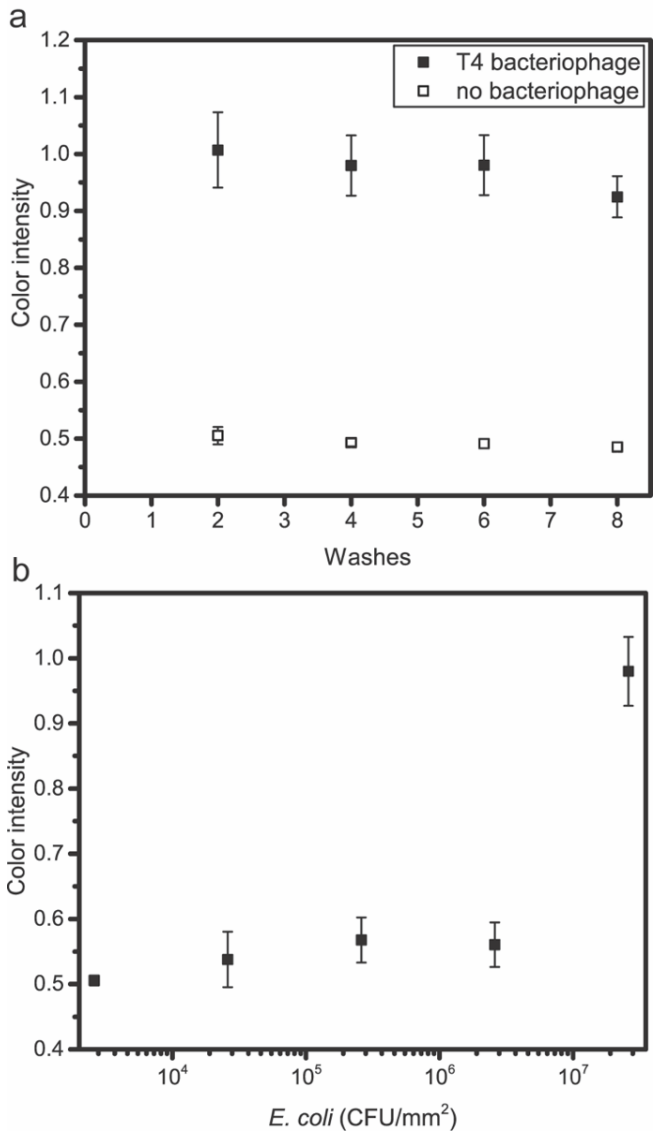

Figure 5. Colorimetric detection of dormant *E. coli* bound to T4 bacteriophage on filter paper. (**Panel a**) Change in the blue component of the color depending on the number of washes with PBS after capture of dormant *E. coli* and before analysis with INT/PNPP (3.28/45 mM). Error bars correspond to two standard errors (n=8). (**Panel b**) Change in the blue component of the color depending on the concentration of dormant *E. coli*. *E. coli* were bound to bacteriophages and unbound bacteria were removed by four washes with PBS buffer before INT/PNPP (3.28/45 mM) solution was added. Error bars correspond to two standard errors (n=8).

Dormant *E. coli* captured with T4 bacteriophages exhibited a higher limit of detection with the INT/PNPP assay compared to adsorbed *E. coli* (10^4 CFU/mm^2 in Fig. 3d versus 10^6 CFU/mm^2 in Fig. 5b), while metabolically active *Salmonella* captured with P22 bacteriophages had a lower

limit of detection with the INT/PMS assay compared to adsorbed *Salmonella*. These differences in the limit of detection for papers with and without bacteriophages and for *E. coli* and *Salmonella* were likely due to differences in experimental protocols, the nature of the bacteriophages and their interactions with the bacteria. We did not wash papers with adsorbed bacteria to control the number of bacteria per paper for calibration and control, while we removed unbound bacteria, lowering their numbers before performing the assays. T4 and P22 bacteriophages belong to two different families, while T4 bacteriophages are a member of the myoviridae family and have a long contractile tail, P22 bacteriophages are a member of the podoviridae family with no tail. The long tail in the T4 bacteriophages was more sensitive to environmental factors and potentially denatured during the adsorption on the paper fibers. Bacteriophages also likely preferentially bound metabolically active bacteria as shown for *Salmonella* bacteriophages on gold surfaces (Fernandes *et al*., 2014).

2.4 Conclusions

Both beneficial and pathogenic bacteria can switch their metabolic states depending on their environment. Since these changes can alter their behavior and influence environmental and industrial processes, it is important to determine their metabolic state in a short time. In this study, we developed two paper-based assays that distinguished between metabolically active and dormant *E. coli* and *Salmonella* in water, and have the potential to be used with other bacteria, as they target classes of enzymes common in most bacteria. Compared to other paper-based assays for bacteria, our assays did not require bacterial lysis to release the oxidoreductases and alkaline phosphatases before reaction with the dyes, reducing the steps involved in the determination of the metabolic state of the bacteria (Jokerst *et al*., 2012). A drawback to not lysing the bacteria was the higher concentrations of *E. coli* and *Salmonella* required for the color change, as the dyes had to cross the

membrane of the bacteria before reacting with the enzymes. If the determination of the metabolic state of lower concentrations of bacteria is needed, our assay could be combined with chemical or biological lysis once the bacteria are immobilized on the paper.

In summary, we developed two paper-based colorimetric assays to distinguish between metabolic states in *E. coli* and *Salmonella*. These assays are not specific to a certain bacterial strain, and adaptable to other bacterial species or cells containing oxidoreductases and alkaline phosphatases. Combining these non-specific assays with bacteriophages allowed us to determine the metabolic state of specific bacteria. Independent of the specificity, the assays were fast (< 30 minutes), simple and low-cost, allowing a semi-quantitative determination of the concentration of bacteria at each metabolic state. The assays have the potential of being portable, which will allow the rapid determination of the metabolic state of bacteria, and with that can prevent negative environmental and industrial effects from rapid bacterial growth.

Author Contributions

Stephanie A. Hice and Miguel C. Santoscoy contributed equally to this work.

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Supporting Information

Table S1-S2, and Figure S1 can be found in Appendix A.

References

Achbergerová, L., Nahálka, J., Lartigue, C., Noskov, V., Chuang, R., Algire, M., … Venter, J. (2011). Polyphosphate - an ancient energy source and active metabolic regulator. *Microbial Cell Factories, 10*(63). doi:10.1186/1475-2859-10-63

Adkins, J. A., Boehle, K., Friend, C., Chamberlain, B., Bisha, B., & Henry, C.S. (2017). Colorimetric and electrochemical bacteria detection using printed paper- and transparency-based analytic devices. *Analytical Chemistry, 89*(6), 3613-3621. doi:10.1021/acs.analchem.6b05009

Almaas, E., Kovács, B., Vicsek, T., Oltvai, Z. N., & Barabási, A. L. (2004). Global organization of metabolic fluxes in the bacterium *Escherichia coli*. *Nature, 427*(6977), 839-843. doi:10.1038/nature02289

Anany, H., Chen, W., Pelton, R., & Griffiths, M. W. (2011). Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat by using phages immobilized on modified cellulose membranes. *Applied and Environmental Microbiology, 77*(18), 6379-87. doi:10.1128/AEM.05493-11

Ayyash, S., Wu, W. I., & Ravi Selvaganapathy, P. (2014). Fast and inexpensive detection of bacterial viability and drug resistance through metabolic monitoring. In: 2014 IEEE Healthcare Innovation Conference, HIC 2014. (pp. 22-25)

Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T. F., … Bumann, D. (2006). Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature, 440*(7082), 303-307. doi:10.1038/nature04616

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review, 11*, 127-152. doi:10.1016/S1387-2656(05)11004-7

Bessey, O. A., Lowry, O. H., & Brock, M. J. (1946). A method for the rapid determination of alkaline phosphates with five cubic millimeters of serum. *Journal of Biological Chemistry, 164*, 321-329.

Blake, M. S., Johnston, K. H., Russell-Jones, G. J., & Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal Biochem, 136*, 175-179. doi:10.1016/0003-2697(84)90320-8

Cademartiri, R., Anany, H., Gross, I., Bhayani, R., Griffiths, M., & Brook, M. A. (2010). Immobilization of bacteriophages on modified silica particles. *Biomaterials, 31*(7), 1904-1910. doi:10.1016/j.biomaterials.2009.11.029

Carrilho, E., Martinez, A. W., & Whitesides, G. M. (2009). Understanding wax printing: A simple micropatterning process for paper-based microfluidics. *Analytical Chemistry, 81*(16), 7091-7095. doi:10.1021/ac901071p

Clegg, S. J., Jia, W., & Cole, J. A. (2006). Role of the *Escherichia coli* nitrate transport protein, NarU, in survival during severe nutrient starvation and slow growth. *Microbiology, 152*(7), 2091-2100. doi:10.1099/mic.0.28688-0

Clokie, M. R. J., & Kropinski, A. M. (Eds.) (2009). Bacteriophages: Methods and protocols. (Vols. 1-2). New York, NY: Springer.

Coates, A. R. M. (2003). Dormancy and low-growth states in microbial disease. Cambridge, UK: Cambridge University Press.

Fernandes, E., Martins, V. C., Nóbrega, C., Carvalho, C. M., Cardoso, F. A., Cardoso, S., … Azeredo, J. (2014). A bacteriophage detection tool for viability assessment of *Salmonella* cells. *Biosensors and Bioelectronics, 52*(15), 239-246. doi:10.1016/j.bios.2013.08.053

Fiksdal, L., Pommepuy, M., Caprais, M. P., & Midttun, I. (1994). Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. *Applied and Environmental Microbiology, 60*(5), 1581-1584.

Gómez, R., Bashir, R., & Bhunia, A. K. (2002). Microscale electronic detection of bacterial metabolism. *Sensors Actuators B: Chemical, 86*(2-3), 198-208. doi:10.1016/S0925-4005(02)00175-2

Hallegraeff, G. M. (1993). On the global increase of harmful algal bloom. *Wetlands Australia, 12*, 2-15.

Hatzinger, P. B., Palmer, P., Smith, R. L., Peñarrieta, C. T., & Yoshinari, T. (2003). Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *Journal of Microbiological Methods, 52(1)*, 47-58. doi:10/1016/S0167-7012(02)00132-X

Hu, J., Wang, S., Wang, L., Li, F., Pingguan-Murphy, B., Lu, T. J., & Xu, F. (2014). Advances in paper-based point-of-care diagnostics. *Biosensors and Bioelectronics, 54*, 585-597. doi:10.1016/j.bios.2013.10.075

Jokerst, J. C., Adkins, J. A., Bisha, B., Mentele, M. M., Goodridge, L. D., & Henry, C. S. (2012). Development of a paper-based analytical device for colorimetric detection of select foodborne pathogens. *Analytical Chemistry, 84*(6), 2900-2907. doi:10.1021/ac203466y

Jones, K. (2001). Campylobacters in water, sewage and the environment. *Journal of Applied Microbiology, 90*(S6), 68S-79S. doi:10.1046/j.1365-2672.2001.01355.x

Ke, C., He, Y., He, H., Yang, X., Li, R., Yuan, J., … van den Heuvel, L. P. (2014) A new spectrophotometric assay for measuring pyruvate dehydrogenase complex activity: A comparative evaluation. *Analytical Methods, 6*, 6381-6388. doi:10.1039/C4AY00804A

Lakshmanan, R. S., Guntupalli, R., Hu, J., Kim, D., Petrenko, V. A., Barbaree, J. M., & Chin, B. A. (2007). Phage immobilized magnetoelastic sensor for the detection of *Salmonella* Typhimurium. *Journal of Microbiological Methods, 71*(1), 55-60. doi:10.1016/j.mimet.2007.07.012

Lazcka, O., Del Campo, F. J., & Muñoz, F. X. (2007). Pathogen detection: A perspective of traditional methods and biosensors. *Biosensors and Bioelectronics, 22*(7), 1205-1217. doi:10.1016/j.bios.2006.06.036

Lennon, J. T., & Jones, S. E. (2011). Microbial seed banks: The ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology, 9*, 119-130. doi:10.1038/nrmicro2504

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology, 5*, 48-56. doi:10.1038/nrmicro1557

Li, L., Mendis, N., Trigui, H., Oliver, J. D., & Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in Microbiology, 5*, 258. doi:10.3389/fmicb.2014.00258

Li, S., Li, Y., Chen, H., Horikawa, S., Shen, W., Simonian, A., & Chin, B. A. (2010). Direct detection of *Salmonella* Typhimurium on fresh produce using phage-based magnetoelastic biosensors. *Biosensors and Bioelectronics, 26*(4), 1313-1319. doi:10.1016/j.bios.2010.07.029

Martinez, A. W., Phillips, S. T., Butte, M. J., & Whitesides, G. M. (2007). Patterned paper as a platform for inexpensive, low-volume, portable bioassays. *Angewandte Chemie - International Edition,* 46, 1318-1320. doi:10.1002/anie.200603817

Murphree, C. A., Heist, E. P., & Moe, L. A. (2014). Antibiotic resistance among cultured bacterial isolates from bioethanol fermentation facilities across the United States. *Current Microbiology, 69*(3), 277-285. doi:10.1007/s00284-014-0583-y

Nie, Z., Nijhuis, C. A., Gong, J., Chen, X., Kumachev, A., Martinez, A. W., … Whitesides, G. M. (2010). Electrochemical sensing in paper-based microfluidic devices. *Lab Chip, 10*(4), 477-483. doi:10.1039/b917150a

Ozkanca, R., & Flint, K. P. (1996). Alkaline phosphatase activity of *Escherichia coli* starved in sterile lake water microcosms. *Journal of Applied Bacteriology, 80*, 252-258.

Ozkanca, R., & Flint, K. P. (1997). Relationship between respiratory enzymes and survival of *Escherichia coli* under starvation stress in lake water. *Journal of Applied Microbiology, 82*(3), 301-309.

Pelton, R. (2009). Bioactive paper provides a low-cost platform for diagnostics. *Trends in Analytical Chemistry, 28*(8), 925-942. doi:10.1016/j.trac.2009.05.005

Poncet, S., Milohanic, E., Mazé, A., Nait Abdallah, J., Aké, F., Larribe, M., … Deutscher, J. (2009). Correlations between carbon metabolism and virulence in bacteria. *Contributions to Microbiology, 16*, 88-102. doi:10.1159/000219374

Rodriguez, G. G., Phipps, D., Ishiguro, K., & Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied and Environmental Microbiology, 58*(6), 1801-1808.

Rompré, A., Servais, P., Baudart, J., de-Roubin, M. R., & Laurent, P. (2002). Detection and enumeration of coliforms in drinking water: Current methods and emerging approaches. *Journal of Microbiological Methods, 49*(1), 31-54. doi:10.1016/S0167-7012(01)00351-7

Smith, J. J., & McFeters, G. A. (1997). Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12. *Journal of Microbiological Methods, 29*(3), 161-175. doi:10.1016/S0167-7012(97)00036-5

Ullrich, S., Karrasch, B., Hoppe, H., Jeskulke, K., & Mehrens, M. (1996). Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Applied and Environmental Microbiology, 62*(12), 4587-4593.

Vella, S. J., Beattie, P., Cademartiri, R., Laromaine, A., Martinez, A. W., Phillips, S. T., … Whitesides, G. M. (2012). Measuring markers of liver function using a micropatterned paper device designed for blood from a fingerstick. *Analytical Chemistry, 84*(6), 2883-2891. doi:10.1021/ac203434x

Wang, C., Sauvageau, D., & Elias, A. (2016). Immobilization of active bacteriophages on polyhydroxyalkanoate surfaces. *ACS Applied Materials and Interfaces, 8*(2), 1128-1138. doi:10.1021/acsami.5b08664

Yang, K., & Metcalf, W. W. (2004). A new activity for an old enzyme: *Escherichia coli* bacterial alkaline phosphatase is a phosphite-dependent hydrogenase. *Proceedings of the National Academy of Sciences of the United States of America, 101*(21), 7919-7924. doi:10.1073/pnas.0400664101

Yetisen, A. K., & Akram, M. S., Lowe, C. R. (2013). Paper-based microfluidic point-of-care diagnostic devices. *Lab Chip, 13*(12), 2210-2251. doi:10.1039/c3lc50169h

CHAPTER 3

CAPTURE, CONCENTRATION AND DETECTION OF *SALMONELLA* IN FOODS USING MAGNETIC IONIC LIQUIDS AND RECOMBINASE POLYMERASE AMPLIFICATION

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Abstract

We have previously investigated the extraction and concentration of bacteria from model systems using magnetic ionic liquid (MIL) solvents, while retaining their viability. Here, we combine MIL-based sample preparation with isothermal amplification and detection of *Salmonella*-specific DNA using Recombinase Polymerase Amplification (RPA). After initial developmental work with *Serratia marcescens* in water, *Salmonella* Typhimurium ATCC 14028 was inoculated in water, 2% milk, almond milk or liquid egg samples and extracted using one of two MILs, including: trihexyl(tetradecyl)phosphonium cobalt(II) hexafluoroacetylacetonate ($[P_{66614}^+][Co(hfacac)_3^-]$) and trihexyl(tetradecyl)phosphonium nickel(II) hexafluoroacetylacetonate ($[P_{66614}^+][Ni(hfacac)_3^-]$). Viable cells were recovered from the MIL extraction phase after the addition of modified LB broth, followed by a 20 min isothermal RPA assay. Amplification was carried out using supersaturated sodium acetate heat packs and results compared to those using a conventional laboratory thermocycler set to a single temperature. Results were visualized using either gel electrophoresis or nucleic acid lateral flow immunoassay (NALFIA). The combined MIL-RPA approach enabled detection of *Salmonella* at levels as low as 10^3 CFU mL^{-1}. MIL-based sample preparation required less than 5 min to capture and concentrate sufficient cells for detection using RPA, which (including NALFIA or gel-based

analysis) required approximately 30 - 45 min. Our results suggest the utility of MILs for the rapid extraction and concentration of pathogenic microorganisms in food samples, providing a means for physical enrichment that is compatible with downstream analysis using RPA.

3.1 Introduction

Salmonella is a ubiquitous, Gram-negative bacterium that is widespread in the environment and is a contaminant in various foods, food ingredients, and in industrial food processing environments (Besser, 2018). Infection typically results from ingestion of tainted food products, including consumption of contaminated poultry, eggs and dairy products. The Centers for Disease Control and Prevention (CDC) estimates that nontyphoidal *Salmonella* spp. are responsible for 1.2 million cases of illness, 19,000 hospitalizations and nearly 380 deaths in the United States annually, resulting in an economic burden greater than 3.4 billion USD (Hoffmann, Maculloch, & Batz, 2015). Therefore, rapid, streamlined and field-deployable methods for detection of *Salmonella* spp. and other foodborne pathogens are crucial for ensuring the safety and quality of the foodstream.

Current detection techniques used by the food industry include standard culture methods and polymerase chain reaction (PCR), due to the selectivity, reliability and regulatory acceptance of these techniques. However, culture-based methods for *Salmonella* may require between several days to more than a week, depending on the sample (Brehm-Stecher, Young, Jaykus, & Tortorello, 2009; US Department of Agriculture [USDA], 2009;). To address these time-to-result issues, the food industry relies PCR as a means for rapid screening of food samples for contamination. Although PCR has gained wide acceptance in the food industry over the past 20 years, the thermal cyclers needed for this approach can be expensive and most systems remain bench-bound or have limited portability (Brehm-Stecher & Johnson, 2007).

Recently, recombinase polymerase amplification (RPA) has demonstrated promise as an alternative means for the rapid detection of microbial pathogens and viruses (Murinda *et al*., 2014). Unlike PCR, which relies on high heat to denature and separate duplex DNA (dsDNA), RPA accomplishes this at lower temperatures using an enzyme, recombinase (Li & Macdonald, 2015). While RPA maintains several similarities to PCR, including exponential amplification of target sequences, a major difference between the two detection methods is the temperature profile used for amplification. RPA is an isothermal process, which obviates the need for a thermal cycler, allowing use of simple, small and inexpensive heating devices. The optimal temperature of RPA has been reported to be 37 – 42 °C, but amplification of specific products has been demonstrated at temperatures ranging from 25 – 45 °C (TwistDx, 2016). Suitable product amplification can be achieved in less than 20 min with RPA, as time-consuming ramping between separate denaturation, annealing and extension temperatures is not required (Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014). Together, these attributes make RPA advantageous for use in resource-poor environments.

Although RPA products can be visualized using gel electrophoresis, gels require specialized equipment and are time-consuming, taking upwards of 35 min to get results. As an alternative, disposable, colorimetric lateral flow devices can be used for rapid (<10 min) amplicon detection. These paper- or nitrocellulose-based devices rely on capillary action and therefore have no requirement for power. Nucleic acid lateral flow immunoassays (NALFIA) like those used in this work with RPA are typically based on a sandwich-type assay (Posthuma-Trumpie, Korf, & van Amerongen, 2009). Like RPA itself, these NALFIA devices are portable, economical and simple to use outside of laboratory settings – characteristics that make them ideal for use in the field.

While nucleic acid amplification techniques are highly specific, their successful use in foods may be limited by low pathogen levels (Soo, 2013). Without a suitable means for capture, concentration and purification of bacteria prior to downstream analysis ("extraction" in purely chemical terms), the detection of pathogens in contaminated food samples may suffer from lack of reproducibility or from poor sensitivity due to inhibitory substances carried over from the sample matrix. Cultural enrichment is commonly used prior to detection, allowing dilution of food matrix-associated assay inhibitors, recovery of stressed or injured cells and generation of a detectable threshold of cells. However, key drawbacks include increased assay time, outgrowth of target organisms by competitive microflora and loss of information on initial pathogen load (Brehm-Stecher *et al*., 2009).

While filtration- and centrifugation-based sample preparation techniques can enable physical enrichment of cells, clogging of filters or co-isolation of particles or debris that may interfere with assay performance can be problematic (Soo, 2013). Magnetic techniques can also be used for capture, concentration and purification of cells from complex food matrices, including the use of magnetic microbeads or nanoparticle substrates functionalized with pathogen-specific antibodies. Other means for functionalizing substrates for cell capture include the use of cationic charge, which is non-selective, or those that depend on semi-selective cell-ligand interactions, such as lectins, antimicrobial peptides or antibiotics (Dao *et al*., 2018). In these approaches, functionalized magnetic beads or nanoparticles are dispersed throughout a sample slurry where they encounter and bind to bacteria. A magnetic field is then applied for physical isolation of the cell-enriched sorbent (Soo, 2013). Drawbacks to particulate magnetic sorbents such as microbeads may include aggregation, diffusion- or suspension-based limitations or poor access to microscopic physical niches where bacteria may be present. These issues may result in lower extraction

efficiencies and/or clogging of microfluidic systems. These problems can be addressed, but not completely avoided, through the use of functionalized nanoparticles (Soo, 2013).

Magnetic ionic liquids (MILs) are paramagnetic molten salts comprised of organic/inorganic cations and anions that exhibit melting points at or below 100 °C. Similar to conventional ionic liquids (ILs), MILs possess negligible vapor pressures at ambient temperatures and tunable physicochemical properties including viscosity, solvent miscibility, and solvation capabilities (Clark, Nacham, Purslow, Pierson, & Anderson, 2016; Pierson *et al*., 2017; Santos, Albo, & Irabien, 2014). Owing to their tunable chemical structures and susceptibility to magnetic fields, MILs have been applied for the analysis of hormones in biological fluids acidic pharmaceuticals and endocrine disrupters, and the extraction and preservation of nucleic acids (Chatzimitakos, Binellas, Maidatsi, & Stalikas, 2016; Clark *et al*., 2015; Clark, Sorensen, Nacham, & Anderson, 2016; Merib, Spudeit, Corazza, Carasek, & Anderson, 2018). Very recently, MILs were also investigated as solvents for the preconcentration of viable bacteria for culture and PCR-based detection (Clark, Purslow, Pierson, Nacham, & Anderson, 2017). By dispersing the hydrophobic MIL in an aqueous suspension of *Escherichia coli* K12, viable cells were rapidly extracted and concentrated for downstream analysis using qPCR. However, the ability of MIL solvents to extract industrially-relevant foodborne pathogens, such as *Salmonella* Typhimurium, in complex food matrices was not tested. In this study, we report development of a method for the preconcentration and detection of *S.* Typhimurium that capitalizes on the rapid and cell-compatible extraction capabilities of MIL solvents and the portability, simplicity and rapidity of a RPA detection platform.

3.2 Materials and Methods

3.2.1 Magnetic Ionic Liquids: The structures of the three MIL solvents examined in this work are shown in Fig. 1, panel a. Synthesis and characterization of the MILs was performed as previously described (Pierson *et al*., 2017). MIL solvents were purified by liquid-liquid extraction with acetonitrile/hexane and dried *in vacuo*. MILs were kept for long-term storage in capped glass vials and MILs were stored in a dessicator for at least 24 h prior to use.

3.2.2 Bacteria and Culture Conditions: *Serratia marcescens* (originally from Carolina Biological Supply Company, Burlington, NC, USA) was sourced from a teaching lab at Iowa State. *Salmonella enterica* subspecies *enterica* ser. Typhimurium ATCC 14028 and *Escherichia coli* ATCC 25922 were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Overnight cultures (10 mL) of *S. marcescens* were grown at 25 °C in 250 mL glass Erlenmeyer flasks containing Luria Bertani (LB) broth (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) supplemented with 1% (wt/vol) glycerol to accelerate production of the red pigment prodigiosion (Haddix *et al*., 2008). Flasks were incubated with shaking at 190 rpm in a Shel Lab Shaking Incubator (Sheldon Manufacturing, Inc., Cornelius, OR, USA). *S.* Typhimurium and *E. coli* were grown in 14 mL polystyrene round-bottom tubes (Corning Inc., Corning, NY, USA) containing 10 mL Tryptic Soy Broth (TSB) (BD), and incubated at 37 °C. Organisms were enumerated using Tryptic Soy Agar (TSA) plates (BD).

3.2.3 Pasteurized Liquid Food Products: Two-percent milk (Hy-Vee Reduced Fat Milk), almond milk (Hy-Vee All Natural Original) and a liquid egg product (Hy-Vee 99% Real Egg) were purchased from a local grocery store (Hy-Vee, Ames, IA) for evaluation of MIL-based capture in liquid food products. All foods were evaluated before the “Sell by”, “Best if used by” or “Use by” dates listed on their packaging.

3.2.4 MIL-Based Extraction of Viable Bacteria: A representative schematic for the MIL-based extraction of bacteria is depicted in Fig. 1, panel b. A 1 mL aliquot of diluted cell suspension, artificially spiked milk, almond milk or liquid egg product was added to a 2 mL or 4 mL screw cap glass vial. A small volume of MIL (e.g., 15 μL) was added and vortexed vigorously for 30 s to create a cell-capturing microdroplet dispersion. With some samples (e.g. the egg product, due to viscosity and foaming), a magnet was applied externally to concentrate the cell-enriched MIL, although this step was not necessary with some samples, as the hydrophobic, denser-than-water MIL droplets were able to sink to the bottom of the extraction vial. After gravity-based deposition or magnetic extraction, the aqueous phase was then discarded and the MIL was subjected to a brief wash step using 1 mL of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA), to ensure adequate removal of residual bacteria that were not captured by the MIL microdroplets. Recovery of viable cells from the MIL extraction phase was achieved through a "back-extraction" step that involved vortexing the cell-enriched MIL in 1 mL or 200 μL of a nutritive medium comprised of tryptone (20 g/L; "2x tryptone") and NaCl (10 g/L; "1x NaCl") for 2 min. After back-extraction, captured bacteria were detected using microbiological culture or RPA. Prior to RPA, the cell-enriched back-extraction media was heated at 100 °C for 10 min for cell lysis and release of target nucleic acids. The MIL-RPA method was compared to a commercial nucleic acid sample preparation approach using the PrepMan Ultra Sample Preparation Reagent (PMU; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

3.2.5 Plating and Enumeration: Following back-extraction, aliquots of the cell-enriched modified LB media were serially diluted in 0.1% peptone water. A 10 μL aliquot of each dilution was applied to the appropriate lane on square, gridded TSA plates as described previously (Jett, Hatter, Huycke, & Gilmore, 1997). The track plates were then tilted to an angle of~80° for 15 min

to allow the deposited liquid to travel toward the opposite end of the plate. Plates were incubated for 48 h at 25 °C (*S. marcescens*) or for 24 h at 37 °C (*S.* Typhimurium). Colonies were manually counted for determination of the number of CFUs in each sample. The enrichment factor (E_F) for the MIL-based method was calculated using Equation 1, where C_{MIL} represents the concentration of bacteria in suspension following MIL-based extraction and C_S is the concentration of bacteria in the initial sample.

$$E_F = \frac{C_{MIL}}{C_S} \quad (1)$$

3.2.6 Recombinase Polymerase Amplification: RPA TwistAmp Basic and TwistFlow *Salmonella* were purchased from TwistDx (Cambridge, UK). RPA was carried out according to the manufacturer's instructions and results were visualized using either gel electrophoresis (TwistAmp Basic kit) or a chromatographic lateral flow assay (TwistFlow *Salmonella* kit). Using the TwistAmp Basic kit, a 340 bp region of the putative dienelactone hydrolase gene (DLH) was amplified using the following primers: (Forward primer) 5'-GCC GGG CAG CRA TTA TTC TGC ATG AA-3'and (Reverse primer) 5'-TGG CGT ATA CGG GAA CCG TAA TAG CA-3'. An *in silico* analysis of this primer set using the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) indicated that within the *Salmonella enterica* I database (NCBI taxonomy ID: 59201), the primer set matched several subspecies I serotypes, including the top three disease-causing serotypes identified in the most recent Centers for Disease Control and Prevention (CDC) *Salmonella* Surveillance Report (CDC, 2018b; Ye *et al*., 2012). These include *S.* Enteritidis (225 hits within the *S. enterica* I database), *S.* Typhimurium (57 hits) and *S.* Newport (21 hits). Because the Primer-Blast software does not accommodate degenerate bases (the DLH primer set contains an "R", which indicates either an "A" or a "G" in this position), it is expected that additional *Salmonella* serovars will be detected with this primer

set. An *invA* target, using primers described by Liu *et al*. was also investigated for use in gel electrophoresis-based experiments (Liu, Zang, Du, Li, & Wang, 2017). For both assays, primers were diluted with nuclease free water from a 100 µM stock of mixed primers to a working concentration of 10 µM. Because the DLH primer set resulted in higher amplicon production, it was used in subsequent experiments.

For the TwistFlow *Salmonella* kit (also targeting the *invA* gene), a master mix containing primer in rehydration buffer and nuclease-free water was prepared. Sample DNA was obtained from the MIL back-extraction, or using the PMU approach as per manufacturer's instructions. For each kit, master mix, plus 1 µL of sample DNA was added to the lyophilized RPA reagents contained in a PCR tube, where the entire volume was mixed using a pipette. Following this, 2.5 µL of 280 mM magnesium acetate was added to initiate amplification. Sample tubes were inverted vigorously 10 times, vortexed for 10 s, followed by centrifugation for 5 s to draw the sample to the base of the tube. This mixing process was repeated after 4 min of incubation, and after completion of incubation. For both the TwistAmp Basic and TwistFlow *Salmonella* kits, reactions were incubated using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 40 °C for 20 min. *S.* Typhimurium was tested for each RPA kit, with *E. coli* serving as a negative control.

3.2.7 Gel Electrophoresis: After heating, RPA products generated using the TwistAmp Basic kit were mixed with 10 µL of 6X bromophenol blue/xylene cyanol FF loading dye and loaded on a 1% agarose gel stained with either SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) or GelRed (Biotium, Fremont, CA, USA). Using 1X TBE as the running buffer, samples were electrophoresed using a Mupid-2Plus Submarine Electrophoresis System (Mupid Co., Ltd., Tokyo, JP), for 35 min at 100 V. Bands were visualized using either a Safe Imager 2.0 Blue Light

Transilluminator (Thermo Fisher Scientific), or an Azure Biosystems c300 imaging system (Azure Biosystems, Dublin, CA, USA) at 302 nm with a 20 s exposure time.

3.2.8 Lateral Flow Assay: Single-tube amplification products generated using the TwistFlow *Salmonella* kit were added directly to a nucleic acid lateral flow immunoassay (NALFIA) disposable cartridge (Ustar Biotechnologies (Hangzhou) Ltd., Hangzhou, CN). The NALFIA relies on visual detection of a test band facilitated by the extension of biotin and 6-carboxyfluorescein (6-FAM) labeled primers during RPA. The amplification product is visible by eye as a result of aggregation of streptavidin-conjugated gold nanoparticles, which bind to the biotin-labeled 5' end of the double-stranded amplicon. The terminal 6-FAM group of the amplicon is also selectively captured by the anti-FAM antibody, which is embedded in the test line on the lateral flow strip. A control line consisting of biotin-conjugated BSA exhibits strong affinity for any remaining streptavidin-conjugated gold nanoparticles and can be visualized for a valid assay. Generation of a red band at the test position indicates successful amplification of the double-stranded product, sandwiched between the bound anti-FAM antibody and the streptavidin-conjugated gold nanoparticles. A positive result was recorded if both the control and test bands were identified within 10 min, whereas detection of only the control band indicated a negative result.

3.3 Results and Discussion

3.3.1 Improved MIL Extraction Conditions for Gram-Negative Bacteria: To begin our investigation of the MIL capture process for bacteria other than the previously-reported *E. coli* K-12, we selected the non-pathogenic *Serratia marcescens*. *S. marcescens*, a Gram-negative bacterium in the same family as *Salmonella*, produces the reddish-orange pigment prodigiosin, allowing its unambiguous visual detection when concentrated and facilitating its use as a model Gram-negative bacterium in development of pre-analytical sample preparation methods

(Rossmanith, Frühwirth, Süss, Schopf, & Wagner, 2010). Since the chemical structure of the MIL has profound implications on its extraction behavior, three MILs were studied for bacterial extraction (Fig. 1a). By vigorously dispersing a small volume of MIL (e.g., 15 µL) in an aqueous suspension of *S. marcescens* (1×10^3 CFU mL^{-1}), cells were extracted into the resulting MIL microdroplets and cell-enriched microdroplets were harvested based on either MIL density (~1.3 g mL^{-1} for the Ni(II) MIL) or through manipulation with an external magnetic field (**Fig. 1b**) (Clark *et al*., 2015; Trujillo-Rodríguez *et al*., 2016).

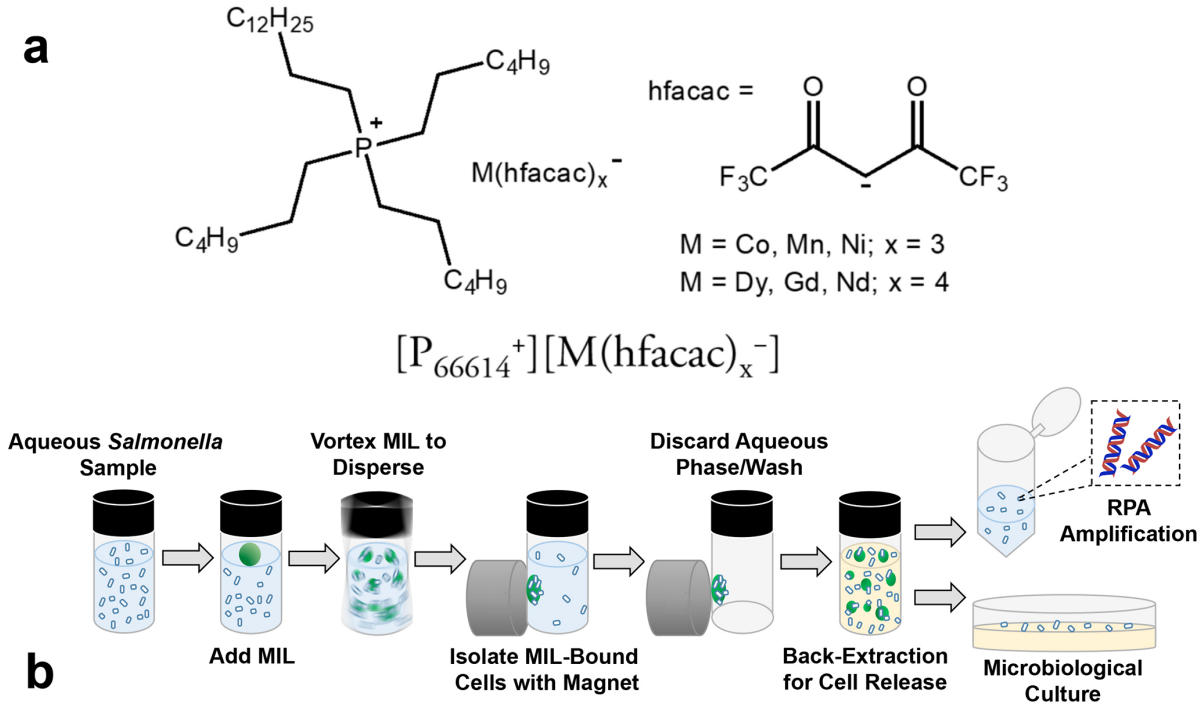

Figure 1. (**Panel a**) Structures of MILs evaluated in this study; (**Panel b**) Schematic for the extraction and preconcentration of *Salmonella* Typhimurium from aqueous samples, followed by downstream analysis using RPA amplification and microbiological culture detection methods. Panel b adapted from Clark *et al*., 2017.

After the cell-enriched MIL was rinsed with deionized water, bacteria were recovered from the extraction phase using Luria Bertani nutrient broth (LB, per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl), plated, and incubated at 25 °C for 48 h prior to enumeration. Of the three MILs studied,

the Ni(II) and Co(II) MILs yielded comparable colony forming units (CFUs), whereas no growth was detected after extraction using the Dy(III) MIL (Fig. 2, inset). Despite possessing identical cation moieties ($[P_{66614}^{+}]$) and ligands (hfacac), the extraction of *S. marcescens* by MILs strongly depended on the identity of the metal component. This phenomenon has also been observed when using similar MIL solvents for the preconcentration of nucleic acids from aqueous solution (Emaus, Clark, Hinners, & Anderson, 2018).

To maximize the recovery of viable bacteria from the MIL extraction phase, several LB-based back-extraction media varying in ionic strength and nutrient composition were investigated (Fig. 2). For these experiments, suspensions of *S. marcescens* (1×10^3 CFU mL^{-1}, in 1 mL 0.1% peptone) were vortexed for 30 s with 15 µL of the Co(II) MIL, then resuspended for a 2 min back-extraction into 1 mL of either water (control), LB medium or 7 variations of the basic LB medium recipe. Back-extraction using deionized water resulted in the lowest quantity of bacteria recovered from the MIL while the greatest quantity of cells was obtained with a nutrient-rich tryptone medium supplemented with NaCl (2x T, 1x NaCl, Fig. 2). Back-extracted samples were diluted 100-fold prior to plating to ensure that countable dilutions within the statistically valid range of 25 – 250 CFU were obtained. Our results show that *S. marcescens* cells were physically enriched by the MIL to levels between 5 and 6 times higher than their initial concentration. Apart from the higher ionic strength of the best back-extraction media, which has previously been shown to assist in the recovery of Gram-negative bacteria, it is conceivable that the hydrophobic MIL solvent imposes stress on the cell in a process that is attenuated by transferring the bacteria to a supportive nutrient media (Bhaganna *et al*., 2010; Clark *et al.*, 2017). Because the 2x T, 1x NaCl back-extraction medium provided the highest recoveries for *S. marcescens*, it was selected for use in subsequent experiments.

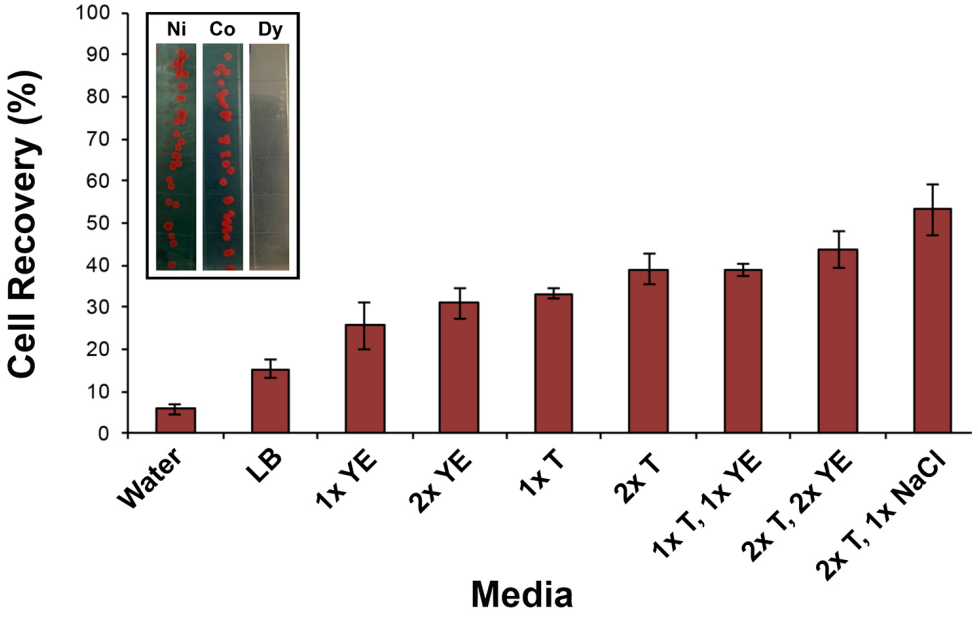

Figure 2. Recovery of *S. marcescens* extracted with Co(II) MIL as a function of back-extraction medium composition. Percentage of initial cell load recovered from the Co(II) MIL extractant using aqueous back-extraction media of different ionic composition is shown. A suspension of *S. marcescens* was prepared and captured with the Co(II) MIL as described in the text, then back-extracted into water (control), LB medium or 7 variations of the basic LB medium recipe. Back-extraction media used: **1X YE** (5 g L^{-1} yeast extract); **2x YE** (10 g L^{-1} yeast extract); **1x T** (10 g L^{-1} tryptone); **2x T** (20 g L^{-1} tryptone); **1x T, 1x YE** (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract); **2x T, 2x YE** (20 g L^{-1} tryptone, 10 g L^{-1} yeast extract); **2x T, 1x NaCl** (20 g L^{-1} tryptone, 10 g L^{-1} NaCl). Average cell recoveries for three separate experiments are shown. **Inset:** Representative cell growth on track plates obtained following the extraction of *S. marcescens* using Ni(II), Co(II) and Dy(III) MILs.

Next, we investigated our MIL-based method for the preconcentration of *S.* Typhimurium, using the 2x T, 1x NaCl back-extraction medium. As with *S. marcescens*, similar recoveries of viable *Salmonella* were observed for the Ni(II) and Co(II) MILs, resulting in enrichment factors of approximately 12, which is comparable to previous enrichment factors for the MIL-based extraction of *E. coli* (Clark *et al*., 2017). The work reported here represents the first use of MILs as solvents for the preconcentration of viable pathogenic bacteria from aqueous suspensions. Because Ni(II) and Co(II) results were similar, subsequent experiments toward coupling MIL-

based bacterial extraction with molecular detection using RPA were performed using the [P_{66614}^+] [$Ni(hfacac)_3^-$] MIL.

3.3.2 Combining MIL-based Extraction with *Salmonella*-Targeted RPA: In an effort to identify improved approaches for food analysis, we investigated the feasibility of coupling our MIL-based method for capture and concentration of *S.* Typhimurium from aqueous media with the speed and simplicity of RPA analysis. Briefly, after preconcentration and recovery of *S.* Typhimurium from an aqueous sample using the Ni(II) MIL, the back-extraction suspension was heated for 10 min at 100 °C to lyse the bacteria and release their nucleic acids for downstream RPA analysis. Initially, we compared two primer sets for the amplification of nucleic acids from *S.* Typhimurium: primers described by Liu *et al*. targeting the *invA* gene, which codes for a protein involved in invasion of host intestinal epithelia by *Salmonella*, and primers identified *via* comparative genomic analysis and amplifying a 340 bp region of a putative dienelactone hydrolase gene (DLH, this study) (Liu *et al*., 2017). Although both primer sets successfully amplified DNA from the bacteria recovered from the MIL phase, gel electrophoresis results demonstrated that the DLH-targeted primers yielded bands with higher fluorescence intensities (Fig. S1). As a control, *E. coli* ATCC 25922 (10^5 CFU mL^{-1}) was extracted using the Ni(II) MIL, lysed and examined *via* DLH-based RPA. No amplicon was detected, indicating good selectivity of the DLH primers for *Salmonella* (Fig. S1).

3.3.3 Evaluating Use of a Power-free Heat Source for *Salmonella* -Targeted RPA: A major limitation of many nucleic acid amplification methodologies is their reliance on electricity to power a heat source such as a thermal cycler in PCR or a heat block for isothermal methods. In an effort to circumvent this limitation, we examined supersaturated sodium acetate heat packs - a

small, portable consumer-grade novelty product used in handwarmers and earmuffs (Cristalheat, www.xUmp.com) - as a power-free means for driving RPA.

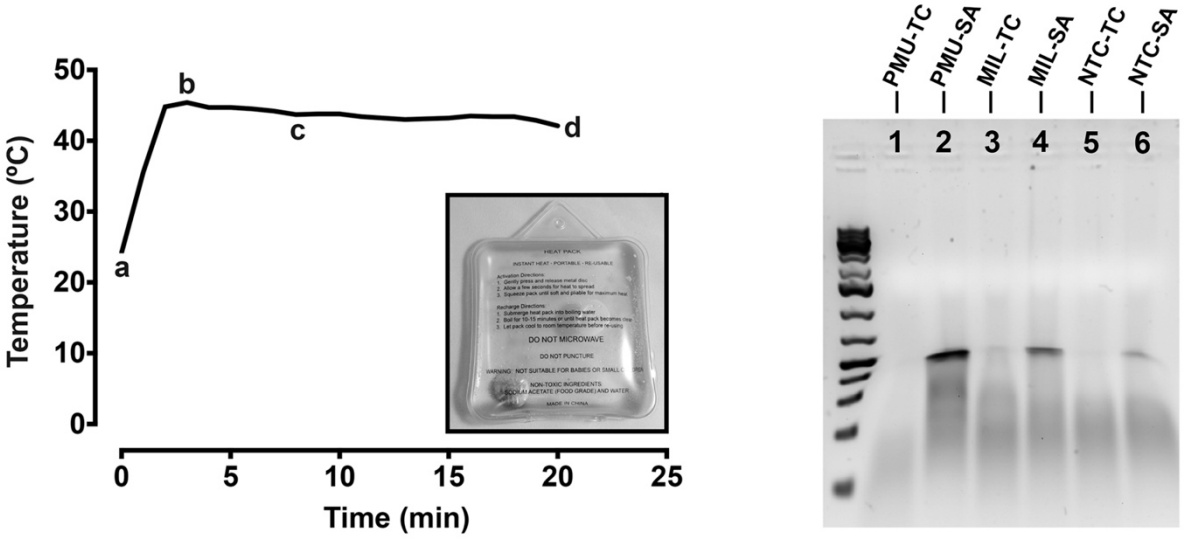

Figure 3. Evaluation of sodium acetate heat pack for power-free incubation of RPA reactions. In order to assess the utility of sodium acetate heat packs (inset) for portable and power-free incubation of RPA reactions, we measured the internal temperature of a template-free RPA reaction tube sandwiched between two activated heat packs using a fiber optic temperature monitoring system as described in the text. **Lefthand panel:** Sodium acetate heat packs were able to provide near-optimal RPA reaction temperatures (reported range, 37 °C – 42 °C) over typical amplification times. Temperatures at various points along the time-temperature curve are 25.2 °C (initial temperature, point a), 45.4 °C (point b), 43.7 °C (point c) and 42.1 °C (point d). **Righthand panel:** Comparison of DLH-RPA reactions driven with a thermal cycler (TC) or with sodium acetate heat packs (SA), using bacterial DNA obtained using the PrepMan Ultra Sample Preparation Reagent (PMU) or *via* MIL-based extraction (MIL).

In initial work, we measured the internal temperature of a template-free RPA reaction tube sandwiched between two activated heat packs using an OPTOCON FOTEMP1-4 fiber optic temperature monitoring system (Optocon AG, Dresden, Germany). Data were collected using the FOTEMP Assistant software, exported to Microsoft Excel and plotted in Prism graphing software (Prism 7 for Mac OS X, v. 7.0d, GraphPad Software, La Jolla, CA) (Fig. 3). The temperature of the RPA mixture increased rapidly after heat pack activation, reached equilibrium between 42 °C

and 44 °C (Fig. 3a) and remained within optimal RPA temperature range for up to 40 min. We then tested the performance of heat pack-driven RPA using the DLH primer set and MIL-extracted *S.* Typhimurium. Intense amplicon bands were seen for sodium acetate-driven DLH-RPA with DNA isolated using either the PrepMan Ultra Sample Preparation Reagent (PMU-SA) or with MIL-extracted cells (MIL-SA) (Fig. 3b). Although a band was seen with the sodium acetate-heated no-template control (NTC-SA), it is expected that use of lateral flow-based detection would enable differentiation of legitimate amplicons from spurious NTC bands sometimes seen on agarose gels with RPA (Bentahir, Ambroise, Delcorps, Pilo, & Gala, 2018; Rohrman & Richards-Kortum, 2012). These results highlight the utility of sodium acetate heat packs as a viable, power-free means for amplifying nucleic acids from microbial pathogens using RPA.

3.3.4 Comparison of Methods for Amplicon Detection, Further Improvement of MIL Approach: All elements of a detection assay (cell capture, release of nucleic acids, amplification of target DNA and product detection) may impact the quality of the final result. With this in mind, we evaluated different methods for nucleic acid release in conjunction with further improvement of the MIL-based workflow (larger sample vial size, smaller back-extraction volume) and two approaches for amplicon detection (gel electrophoresis, nucleic acid lateral flow immunoassay [NALFIA]). For direct comparison of methods for release of nucleic acids prior to RPA, we compared heating of the cell-enriched MIL to 100°C (with modifications, as described below) with use the commercial reagent PMU. For both approaches, aqueous samples were inoculated with *S.* Typhimurium at concentrations ranging from 10^3 to 10^6 CFU mL^{-1}, followed by DLH-targeted RPA.

The PMU method was used according to the manufacturer's instructions. Briefly, cells from a liquid suspension were lysed in 200 µL of PMU reagent, followed by heating (15 min,

100°C) and centrifugation to separate cellular debris from the DNA-containing supernatant. The PMU method facilitated consistent detection down to 10^4 CFU mL^{-1}, while detection at lower levels was inconsistent (Fig. 4 gel image, lane 1).

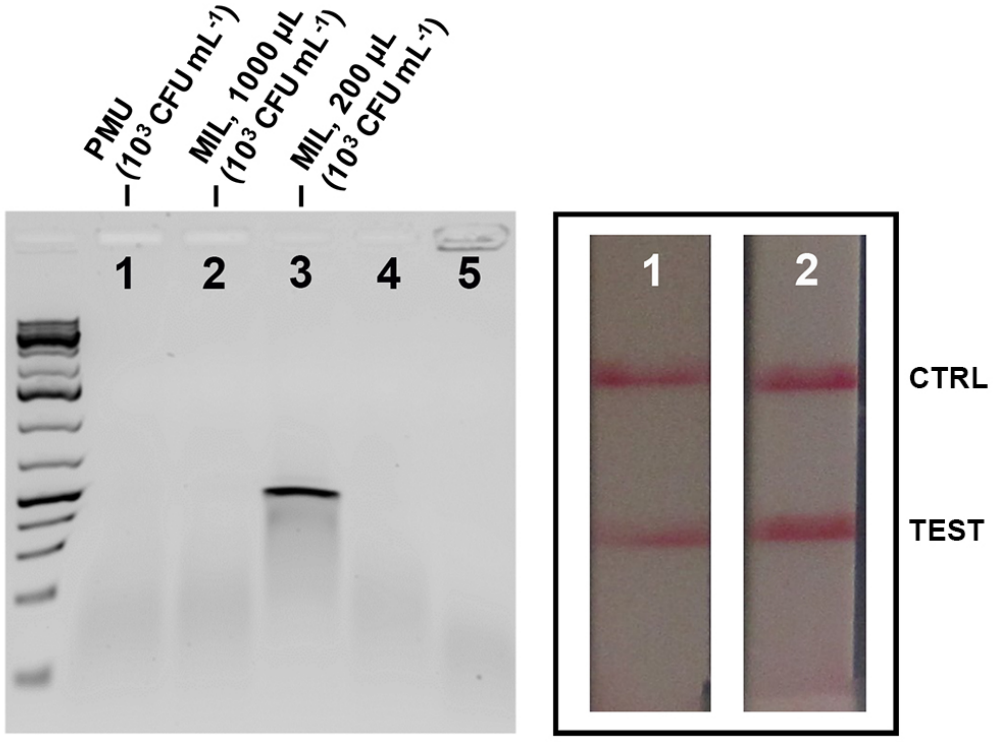

Figure 4. MIL-RPA with Ni(II) MIL. The combined MIL and RPA approach was visualized using gel electrophoresis (35 min) or lateral flow (10 min). MIL-based extraction was compared with concurrent extraction using PrepMan Ultra Sample Preparation Reagent (PMU). For gel electrophoresis (**lefthand panel**), PMU LOD was consistently identified as 10^4 CFU mL^{-1}, however, bands were inconsistently present at lower levels (lane 1), or were less defined than those for the Ni(II) MIL at the same level (lane 3). Lanes 4, 5 represent no template controls (NTC). With lateral flow (**right-hand panel**) detection was achieved as low as 10^4 CFU mL^{-1} for both PMU (strip 1) and the MIL (strip 2).

PMU was not evaluated for lysis of MIL-captured cells, as this would have introduced additional assay elements, namely use of a chemical lysis reagent and a centrifugation step. Based on observations of liquid behavior during vortexing, we hypothesized that slight modifications to our established MIL-RPA approach might result in process improvements. By decreasing the sample

vial size from 4 mL to 2 mL and decreasing the back-extraction volume from 1 mL to 200 µL, we found we could lower our detection limit to 10^3 CFU mL^{-1}, presumably due to enhanced contact between the MIL and back extraction solution when a smaller sample vial was used and the increased concentration of bacteria in the smaller back-extraction volume (Fig. 4 gel image, lane 3). These slight modifications enabled us to improve the sensitivity of our streamlined "capture, concentrate, heat and amplify" MIL-RPA process, without the addition of further assay elements. Without these process modifications, the limit of detection for our MIL-RPA approach was 10^4 CFU mL^{-1} for gel electrophoresis-based visualization of *Salmonella* DLH amplicons.

Because conventional gel electrophoresis is inherently laboratory-bound and time consuming, we sought to evaluate the use of a rapid, portable alternative for amplicon detection. Due to its simplicity and portability, NALFIA is often used in resource-limited environments, or in non-laboratory settings, as it does not require electricity or laboratory equipment. Using the Ni(II) MIL for preconcentration and extraction of aqueous samples of *S.* Typhimurium at concentrations ranging from 10^3 to 10^6 CFU mL^{-1}, RPA was carried out using the TwistFlow *Salmonella* kit from TwistDx (Posthuma-Trumpie *et al*., 2009). Test results were determined using a 5 min NALFIA step. Initially, the combined MIL-RPA-NALFIA approach facilitated detection at levels as low as 10^5 CFU mL^{-1}. Since the detection limits using NALFIA were 2 log higher than with gel electrophoresis, we investigated whether metal ions released from the hydrophobic MIL phase (e.g., Ni^{2+}) during back-extraction had any influence on the outcome of the NALFIA step (Emaus *et al*., 2018). For these experiments, PMU samples were spiked with levels of $NiCl_2$ ranging from 0.2 mM to 2 mM. Visible control and test bands were observed for all $NiCl_2$ samples, suggesting that Ni^{2+} potentially released from the MIL did not inhibit the NALFIA. It is important to note that the NALFIA targets the *invA* gene, using primers and conditions developed by the

manufacturer. Our choice of the dienelactone hydrolase gene target for the gel electrophoresis experiments may also have contributed to differences in observed detection limits due to differences in amplification efficiency between the two primer sets. In order to improve detection limits, the back-extraction volume was decreased to 200 μL, resulting in detection limits for *S.* Typhimurium as low as 10^3 CFU mL^{-1} with the Ni(II) MIL (Fig. 4). The PMU method provided detection of *Salmonella* at levels as low as 10^4 CFU mL^{-1} (Fig. 4), but once again requires the use of a benchtop centrifuge that is incompatible with pathogen analysis in the field or in resource limited settings.

3.3.5 MIL-RPA for detection of *Salmonella* in Liquid Food Samples: In order to examine the application of the combined MIL-RPA method in a practical setting, it was applied for the detection of *S.* Typhimurium in food samples including milk (2% milk fat) and almond milk. *S.* Typhimurium cells were inoculated into 1 mL samples at 10^5 CFU mL^{-1} and extracted using the Ni(II) MIL under improved conditions. The combined approach enabled detection of S. Typhimurium at 10^5 CFU mL^{-1} (Fig. 5a). The PMU method did not consistently detect *S.* Typhimurium in spiked 2% milk samples. To explore whether dilution of samples (and of potentially interfering substances such as fats and proteins) might lead to improvements in both PMU and MIL samples, samples were diluted with 0.1% peptone water (PW) in some experiments. Interestingly, and for unknown reasons, PW appeared to reduce the efficacy of both PMU extraction of cell DNA and MIL-based cell capture, with the diluted MIL sample not yielding a detectable band (Fig 5a, lane 6). These results suggest examination of alternate diluents (i.e. molecular-grade water) in future experiments to explore whether such inhibition may be avoided and if a more appropriate dilution medium may represent a viable approach for reducing sample complexity, if needed. Because *Salmonella* spp. have been especially problematic in eggs, with large outbreaks occurring in 2010 (almost a half a

billion eggs recalled) and 2018 (almost 207 million eggs recalled), the MIL-based preconcentration method was applied to liquid egg samples spiked with concentrations of *S.* Typhimurium ranging from 10^3 to 10^5 CFU mL^{-1} (Allard *et al*., 2013; CDC, 2018a).

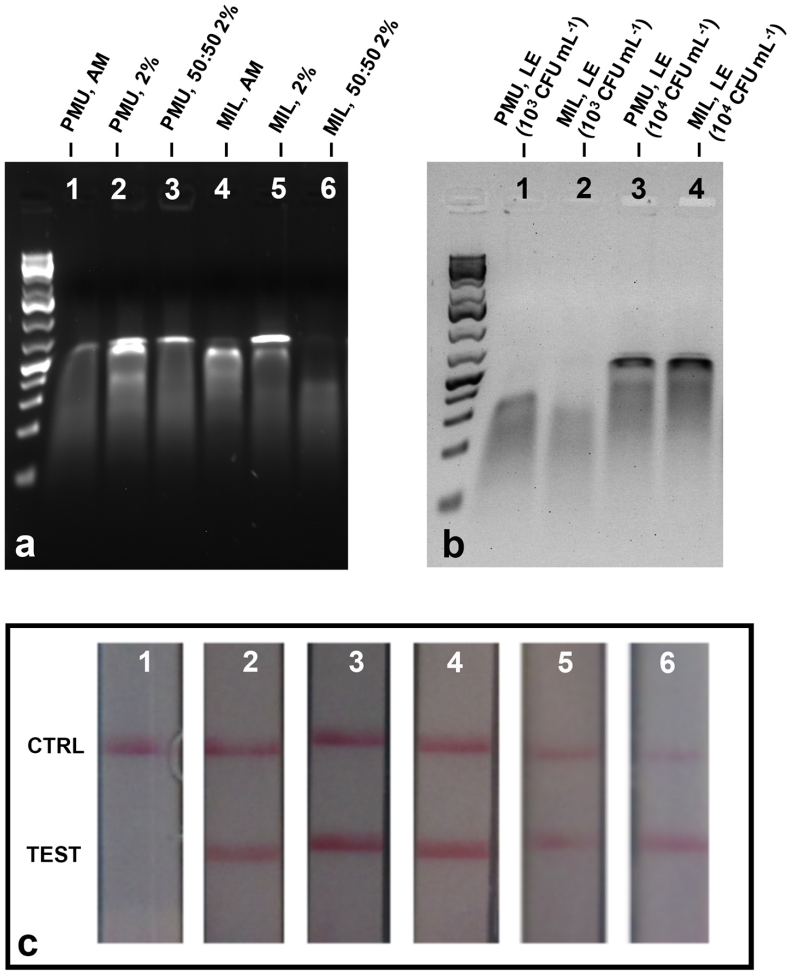

Figure 5. RPA-based detection of *Salmonella* Typhimurium in liquid food products. The combined MIL and RPA approach was applied to almond milk, milk (2% milk fat), 50:50 dilution of milk (2% milk fat: 0.1% peptone water) and liquid egg product. Results were visualized using gel electrophoresis (35 min) or lateral flow (10 min). MIL-based extraction was compared with concurrent extraction using PrepMan Ultra Sample Preparation Reagent (PMU). For *Salmonella*-spiked almond milk (**Panel a, lanes 1, 4**), milk (2% milk fat) (**Panel a, lanes 2, 5**) and 50:50 milk dilution samples (**Panel a, lanes 3, 6**) using gel electrophoresis and either extraction method (PMU or MIL), detection was achieved at 10^5 CFU mL^{-1}. For liquid egg samples spiked with *S.* Typhimurium (**Panel b**), PMU and MIL LOD was 10^4 CFU mL^{-1} (**Panel b, lanes 3, 4**). Using lateral flow (**Panel c**), for *Salmonella*-spiked liquid egg samples, LOD was identified 10^4 CFU mL^{-1} for both PMU (**Panel c, 3, 4**) and MIL-extracted samples (**Panel c, 5, 6**). Strips 1 and 2 are the no template control (NTC) and internal control, respectively.

The foamy nature of the liquid egg sample initially caused challenges in recovering a sufficient volume of MIL for downstream detection bacteria. However, ~1 min exposure of the sample to a 0.66 T rod magnet to the base of the 2 mL glass vial facilitated collection of the cell-enriched MIL solvent, enabling detection limits as low as 10^4 CFU mL^{-1} (Fig. 5b). The commercial PMU method was concurrently compared to the MIL-based approach and exhibited an identical detection limit using RPA and gel electrophoresis. However, the PMU method resulted in less intense bands than those from the MIL-based extraction method (Fig. 5b). The Ni(II) MIL was also applied for preconcentration of *S.* Typhimurium in liquid egg samples coupled to RPA and NALFIA readout. The combined MIL-RPA-NALFIA approach facilitated detection at levels as low as 10^4 CFU mL^{-1} in inoculated liquid egg samples. Once again, this method was concurrently compared with the use of PMU, which maintained similar detection levels (Fig. 5c) but required a centrifugation step that is not compatible with field sampling or on-site analysis.

3.4 Conclusions

We report further improvement of our previously described method for capture of Gram-negative bacteria and the first use of this approach for capture and concentration of a human pathogen from aqueous suspensions, including at-risk foods. Post-capture growth on non-selective media suggested a lack of cytotoxicity with this approach. We coupled capture and concentration of *S.* Typhimurium to RPA, a rapid isothermal method for DNA amplification, and found that we could drive RPA reactions using inexpensive and regenerable sodium acetate heat packs, eliminating the need for an external power source. *Salmonella* RPA amplicons could be detected in <10 min using a simple chromatographic readout. Our approach is simple, streamlined and amenable to analyses in the field or in other resource-limited environments.

Apart from their magnetic properties, the MILs used here have other advantageous characteristics useful for the analysis of aqueous foods or food suspensions. Unlike other recently reported MILs, which have reported room temperature densities that are on par with that of water, the Ni(II) and Co(II) MILs used here had densities of ~1.3 g mL^{-1}, which lie between the densities of glycerol (1.26 g mL^{-1}) and corn syrup (~1.4 g mL^{-1}) (Santos, Albo, Rosatella, Afonso, & Irabien, 2014). Because the MILs used in this study are both hydrophobic and denser than water, they are well-suited for analysis of aqueous solutions such as liquid foods or food suspensions, and can be collected through either simple density-based sedimentation or with application of an external magnet. For automated and high-throughput applications in the food industry, it is possible that use of a strong electromagnet for post-extraction collection of MILs could result in fast and uniform capture of cell-enriched MILs. It may also be possible to minimize the costs and environmental impacts of high-throughput use of MILs in food testing by developing methods capable of recycling MILs for multiple rounds of cell capture.

The approach described here bridges the disciplines of food science, materials science and chemistry, providing new tools for rapid and efficient extraction of viable cells in support of pathogen detection efforts. Future work will focus on extending MIL-RPA to additional foodborne pathogens, including the Gram-positive pathogen *Listeria monocytogenes* and assessment of any negative impacts that MIL-based capture may have on bacterial physiology, such as injury or antimicrobial activity, with the development of approaches for mitigation of these impacts, should they occur.

Author Contributions

Stephanie A. Hice and Kevin D. Clark contributed equally to this work.

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Supporting Information

Figure S1 can be found in Appendix B.

References

Besser, J. M. (2018). *Salmonella* epidemiology: A whirlwind of change. *Food Microbiology, 71*, 55-59.

Allard, M. W., Luo, Y., Strain, E., Pettengill, J., Timme, R., Wang, C., . . . Brown, E. W. (2013). On the evolutionary history, population genetics and diversity among isolates of *Salmonella* Enteritidis PFGE pattern JEGX01.0004. *PLoS One, 8*(1), e55254. doi:10.1371/journal.pone.0055254

Bentahir, M., Ambroise, J., Delcorps, C., Pilo, P., & Gala, J. L. (2018). Sensitive and Specific Recombinase Polymerase Amplification Assays for Fast Screening, Detection, and Identification of Bacillus anthracis in a Field Setting. *Applied and Environmental Microbiology, 84*(11). doi:10.1128/AEM.00506-18

Bhaganna, P., Volkers, R. J., Bell, A. N., Kluge, K., Timson, D. J., McGrath, J. W., . . . Hallsworth, J. E. (2010). Hydrophobic substances induce water stress in microbial cells. *Microbial Biotechnology, 3*(6), 701-716. doi:10.1111/j.1751-7915.2010.00203.x

Brehm-Stecher, B., Young, C., Jaykus, L. A., & Tortorello, M. L. (2009). Sample preparation: the forgotten beginning. *Journal of Food Protection, 72*(8), 1774-1789.

Brehm-Stecher, B. F., & Johnson, E. A. (2007). *Rapid methods for detection of Listeria* (E. T. Ryser & E. H. Marth Eds.). New York: CRC Press.

Centers for Disease Control and Prevention (CDC). (2018a). Multistate outbreak of S*almonella* Braenderup infections linked to Rose Acre Farms shell eggs (final update). Retrieved from https://www.cdc.gov/salmonella/braenderup-04-18/index.html

Centers for Disease Control and Prevention (CDC). (2018b). *National Salmonella Surveillance Annual Report, 2016*. Retrieved from https://www.cdc.gov/nationalsurveillance/salmonella-surveillance.html

Chatzimitakos, T., Binellas, C., Maidatsi, K., & Stalikas, C. (2016). Magnetic ionic liquid in stirring-assisted drop-breakup microextraction: Proof-of-concept extraction of phenolic endocrine disrupters and acidic pharmaceuticals. *Analytica Chimica Acta, 910*, 53-59. doi:10.1016/j.aca.2016.01.015

Clark, K. D., Nacham, O., Purslow, J. A., Pierson, S. A., & Anderson, J. L. (2016). Magnetic ionic liquids in analytical chemistry: a review. *Analytica Chimica Acta, 934*, 9-21. doi: 10.1016/j.aca.2016.06.011

Clark, K. D., Nacham, O., Yu, H., Li, T., Yamsek, M. M., Ronning, D. R., & Anderson, J. L. (2015). Extraction of DNA by magnetic ionic liquids: Tunable solvents for rapid and selective DNA analysis. *Analytical Chemistry, 87*, 1552-1559. doi:10.1021/ac504260t

Clark, K. D., Purslow, J. A., Pierson, S. A., Nacham, O., & Anderson, J. L. (2017). Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR amplification and culture-based diagnostics. *Analytical and Bioanalytical Chemistry, 409*(21), 4983-4991.

Clark, K. D., Sorensen, M., Nacham, O., & Anderson, J. L. (2016). Preservation of DNA in nuclease-rich samples using magnetic ionic liquids. *RSC Advances, 6*, 39846-39851. doi:10.1039/C6RA05932E

Dao, T. N. T., Lee, E. Y., Koo, B., Jin, C. E., Lee, T. Y., & Shin, Y. (2018). A microfluidic enrichment platform with a recombinase polymerase amplification sensor for pathogen diagnosis. *Analytical Biochemistry, 544*, 87-92. doi:10.1016/j.ab.2017.12.030

Emaus, M. N., Clark, K. D., Hinners, P., & Anderson, J. L. (2018). Preconcentration of DNA using magnetic ionic liquids that are compatible with real-time PCR for rapid nucleic acid quantification. *Analytical and Bioanalytical Chemistry, 410*(17), 4135-4144. doi:10.1007/s00216-018-1092-9.

Haddix, P. L., Jones, S., Patel, P., Burnham, S., Knights, K., Powell, J. N., & LaForm, A. (2008). Kinetic analysis of growth rate, ATP, and pigmentation suggests an energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. *Journal of Bacteriology, 190*(22), 7453-7463.

Hoffmann, S., Maculloch, B., & Batz, M. (2015). Economic burden of major foodborne illnesses acquired in the United States. *Current politics and economics of the United States, Canada and Mexico, 17*(4), 543.

Jett, B. D., Hatter, K. L., Huycke, M. M., & Gilmore, M. S. (1997). Simplified agar plate method for quantifying viable bacteria. *Biotechniques, 23*(4), 648-650.

Kersting, S., Rausch, V., Bier, F. F., & von Nickisch-Rosenegk, M. (2014). Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. *Microchimica Acta, 181*(13-14), 1715-1723.

Li, J., & Macdonald, J. (2015). Advances in isothermal amplification: novel strategies inspired by biological processes. *Biosensors and Bioelectronics, 64*, 196-211.

Liu, H. B., Zang, Y. X., Du, X. J., Li, P., & Wang, S. (2017). Development of an isothermal amplification-based assay for the rapid visual detection of *Salmonella* bacteria. *Journal of Dairy Science, 100*(9), 7016-7025. doi:10.3168/jds.2017-12566.

Merib, J., Spudeit, D. A., Corazza, G., Carasek, E., & Anderson, J. L. (2018). Magnetic ionic liquids as versatile extraction phases for the rapid determination of estrogens in human urine by dispersive liquid-liquid microextraction coupled with high-performance liquid chromatography-diode array detection. *Analytical and Bioanalytical Chemistry*, 1-11.

Murinda, S. E., Ibekwe, A. M., Zulkaffly, S., Cruz, A., Park, S., Razak, N., . . . Muthaiyah, K. (2014). Real-time isothermal detection of Shiga toxin–producing *Escherichia coli* using recombinase polymerase amplification. *Foodborne Pathogens and Disease, 11*(7), 529-536.

Pierson, S. A., Nacham, O., Clark, K. D., Nan, H., Mudryk, Y., & Anderson, J. L. (2017). Synthesis and characterization of low viscosity hexafluoroacetylacetonate-based hydrophobic magnetic ionic liquids. *New Journal of Chemistry, 41*, 5498-5505. doi:10.1039/c7nj00206h

Posthuma-Trumpie, G. A., Korf, J., & van Amerongen, A. (2009). Lateral flow (immuno) assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Analytical and Bioanalytical Chemistry, 393*(2), 569-582.

Rohrman, B. A., & Richards-Kortum, R. R. (2012). A paper and plastic device for performing recombinase polymerase amplification of HIV DNA. *Lab Chip, 12*(17), 3082-3088. doi:10.1039/c2lc40423k

Rossmanith, P., Frühwirth, K., Süss, B., Schopf, E., & Wagner, M. (2010). The use of chromogenic bacteria as coloured substitutes for pathogens: a simple strategy during design and development of a new method for sample pretreatment. *Letters in Applied Microbiology, 50*(2), 230-233. doi:10.1111/j.1472-765X.2009.02751.x

Santos, E., Albo, J., & Irabien, A. (2014). Magnetic ionic liquids: Synthesis, properties and applications. *RSC Advances, 4*(75), 40008-40018. doi:10.1039/C4RA05156D

Santos, E., Albo, J., Rosatella, A., Afonso, C. A. M., & Irabien, Á. (2014). Synthesis and characterization of magnetic ionic liquids (MILs) for CO2 separation. *Journal of Chemical Technology and Biotechnology, 89*, 866-871.

Soo, H. S., Brehm-Stecher, B.F, Jaykus, L.A. (2013). Advances in separation and concentration of microorganisms from food samples. In J. Sofos (Ed.), (pp. 173-192). Cambridge, UK: Woodhead Publishing.

Trujillo-Rodríguez, M. J., Nacham, O., Clark, K. D., Pino, V., Anderson, J. L., Ayala, J. H., & Afonso, A. M. (2016). Magnetic ionic liquids as non-conventional extraction solvents for the determination of polycyclic aromatic hydrocarbons. *Analytica Chimica Acta, 934*, 106-113. doi:10.1016/j.aca.2016.06.014

TwistDx. (2016). Evaluation of isothermal recombinase polymerase amplification incubation temperature tolerance. *BioTechniques, 61,* 328.

US Department of Agriculture (USDA) (2009). Procedures for collection, isolation, and identification of *Salmonella* from environmental samples, cloacal swabs, chick box papers, and meconium samples. National Poultry Improvement Plan, Conyers, GA

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, 134. doi:10.1186/1471-2105-13-134

CHAPTER 4

MAGNETIC IONIC LIQUID-BACTERIAL INTERACTIONS: CAPTURE AND RECOVERY OF *SALMONELLA* SPP. AND *ESCHERICHIA COLI* O157:H7

Stephanie A. Hice, Marcelino Varona, Jared L. Anderson, and Byron F. Brehm-Stecher

Abstract

The rapid and sensitive detection of bacterial pathogens is critical for the prevention of outbreaks associated with foods. Traditional culture-based detection is slow and provides long sample-to-answer times. Magnetic ionic liquids (MILs) have been previously demonstrated as novel extractants for *Salmonella* Typhimurium from a variety of food matrices. In this study, the extraction and recovery behavior of nine *Salmonella* serotypes (including a "deep rough" strain) and eight *E. coli* O157:H7 strains was examined for one MIL solvent. Potential cytotoxic effects of trihexyl(tetradecyl) phosphonium nickel(II) hexafluoroacetylacetonate ($[P_{66614}^+][Ni(hfacac)_3^-]$) and trihexyl(tetradecyl) phosphonium dysprosium(III) hexafluoroacetylacetonate ($[P_{66614}^+][Dy(hfacac)_4^-]$) MILs were examined by parallel plating on non-selective and selective media. Using selective media, we have determined that no detectable impacts on post-capture bacterial growth can be attributed to the ($[P_{66614}^+][Ni(hfacac)_3^-]$) MIL. Insight was gained into the previously observed antimicrobial effects of the ($[P_{66614}^+][Dy(hfacac)_4^-]$) MIL by investigating the metal and anion-complex on the growth of cells using selective media. This work suggests the broader applicability of MIL-based extractions and explores the physiological impact of the MILs on select foodborne pathogens.

4.1 Introduction

Salmonella is a ubiquitous, Gram-negative genus of bacteria that is widespread in the environment, and can be found in many different foods, food ingredients and in industrial food

processing environments (El-Gazzar & Marth, 1992). Infection typically results from ingestion of tainted food products, including consumption of contaminated poultry, eggs and dairy products (Kim & Lee, 2016). The Centers for Disease Control and Prevention (CDC) estimates that nontyphoidal *Salmonella* serotypes are annually responsible for 1.2 million cases of illness, 19,000 hospitalizations and nearly 380 deaths in the United States (Centers for Disease Control and Prevention [CDC], 2019a; Hoffman, Maculloch, & Batz, 2015). The resulting economic burden, attributed to the number of annual illnesses and their severity, is $3.4 billion USD (Hoffman *et al*., 2015). *Escherichia coli* is a Gram-negative rod-shaped bacterium that exists as part of the normal flora of the intestinal tract in humans and some animals (Meng, Feng, & Doyle, 2001). Shiga toxin-producing *E. coli* (STEC), such as *E. coli* O157:H7, are clinically significant, and result in hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and a 46.2% hospitalization rate (Hoffman *et al*., 2015; Lim, Yoon, & Hovde, 2010). HUS affects approximately 5–10% of individuals diagnosed with STEC infection (CDC, 2019b). In the United States, *E. coli* O157:H7 is responsible for 63,000 illnesses, 2,200 hospitalizations and an estimated 20 deaths, annually (Lim *et al*., 2010). The economic burden attributed to *E. coli* O157 is approximately $271 million USD per year (Hoffman *et al*., 2015). As a result, the detection of various *Salmonella* serotypes, *E. coli* O157:H7 and other foodborne pathogens is crucial for public health, while ensuring safe processing of foods. Access to rapid and accurate methods for identification of foodborne pathogens in agricultural and industrial environments will lead to improvements in outbreak traceability.

Due to low levels of bacterial contamination in foods, current detection methods employed in industrial environments rely on the use of pre-enrichment, enrichment and purification techniques prior to downstream analysis. Multi-pathogen enrichment media is well-characterized

and widely available (Garrido *et al*., 2013; Kim & Bhunia, 2008). When sampling from foods and in manufacturing environments, selective enrichment media is requisite, as it facilitates enrichment of bacterial targets while aiding in recovery of sub-lethal cellular damage (Suo & Wang, 2013; Wu, 2008). Use of enrichment media, however, adds time-to-result, consequentially delaying detection efforts. Additional approaches to purification and preconcentration of bacterial targets relies of centrifugation and filtration, which are non-selective and may lead to false-positive or false-negative results. To circumvent this, magnetic separation technologies can be applied to facilitate rapid preconcentration and isolation of bacteria. When added to a sample suspension, functionalized magnetoactive substrates rapidly preconcentrate and extract bacterial targets when an external magnetic field is applied (Clark, Purslow, Pierson, Nacham, & Anderson, 2017a; Entis *et al*., 2001; Hice, Clark, Anderson, & Brehm-Stecher, 2019). While selectivity is achieved, functionalized magnetic beads suffer from diminished extraction efficiencies due to settling and aggregation, while use of antibodies results in instability and increased cost (Clark, Varona, & Anderson, 2017b; Hice *et al.*, 2019).

Magnetic ionic liquids (MILs) are magnetoactive solvents consisting of organic/inorganic cations and anions. A paramagnetic component is integrated into either the cation or anion moiety, facilitating susceptibility to magnetic fields (Clark, Nacham, Purslow, Pierson, & Anderson, 2016; Clark *et al*., 2017a). Many classes of MILs are nonvolatile, nonflammable, and possess tunable physicochemical properties. The hydrophobic and liquid nature of MILs allows for distribution throughout a sample as liquid micro- or nanodispersions, which enables which enables capture of bacteria. Previous research suggests that MILs are a versatile and robust component of sample preparation, as they have been applied for the extraction of hormones, nucleic acids and viable bacterial cells (Clark *et al*., 2017b; Ding, Clark, Varona, Emaus, & Anderson, 2019; Merib,

Spudeit, Corazza, Carasek, & Anderson, 2018). MILs have been successfully implemented as novel extraction solvents in sample preparation for the preconcentration of viable, non-pathogenic *Escherichia coli* from fluid milk for culture- and quantitative polymerase chain reaction (qPCR)-based detection (Clark *et al*., 2017a). More recently, MILs have been combined with recombinase polymerase amplification (RPA) for the rapid preconcentration and detection of *Salmonella* Typhimurium from fluid milk, almond milk and liquid egg samples (Hice *et al*., 2019). Despite their wide use as tools for sample preparation, the antimicrobial impact MILs impart onto the bacterial cell is not well characterized.

Standard culture-based approaches are still widely adopted in manufacturing environments and are used to detect and enumerate target bacteria (Jokerst *et al*., 2012). Culture-based methods result in delayed time-to-result as they rely on pre-enrichment, enrichment and selective plating, and are therefore not considered a "rapid" approach to detection of bacterial pathogens. Recovery of viable cells is often necessary as conventional processing applications, such as heating, freezing, irradiation and high-pressure, may result in cell death, non-injury or sub-lethal injury (Wu, 2008). Recovery of stressed or injured cells is often achieved using nutrient-rich, non-selective media, as the lack of selective agents facilitates cellular resuscitation (Wu, 2008). Enrichment steps hinder processing times resulting in delayed detection timelines. The ability of the MIL to rapidly preconcentrate and extract viable bacteria from food samples provides an alternative to conventional enrichment and recovery techniques. An understanding of the physiological impact the MIL maintains on the bacterial cell is essential, and has not previously been investigated. Capture and recovery of viable bacteria using rare earth-based MILs (Dy, Gd, Nd) has not been previously reported, as decreased cell viability have been observed (Clark *et al*., 2017a). While it has been observed that some MIL structures did not impede cell growth when incubated in non-

selective Luria Bertani (LB) broth, parallel plating on non-selective and selective media as a means to screen for MIL-imparted cellular injury has not been investigated (Clark *et al*., 2017a).

By plating MIL-treated cells in parallel on non-selective and selective media, potential antimicrobial effects of the MIL can be evaluated. Cellular injury caused by exposure to deleterious chemicals or heat is often characterized by physical damage to structural or functional components of the cell, including the outer membrane (Wu, 2008). Lack of a growth differential between MIL-treated cells plated on both non-selective and selective media would suggest that exposure to the MIL does not result in cellular injury. In this study we report the evaluation of the MIL-bacterial interactions, including any potential antimicrobial effects imparted onto a panel of Gram-negative bacteria consisting of nine *Salmonella* serotypes and eight *E. coli* O157:H7 strains.

4.2 Materials and Methods

4.2.1 Reagents and Magnetic Ionic Liquids: Chemical structures of the two MIL solvents examined in this study are shown in Fig. 1. Synthesis and characterization of the MILs was performed as previously described (Pierson *et al*., 2017). MIL solvents were purified by liquid-liquid extraction with acetonitrile/hexane and dried *in vacuo*. Prior to all experiments, MILs were stored in a desiccator for at least 24 h.

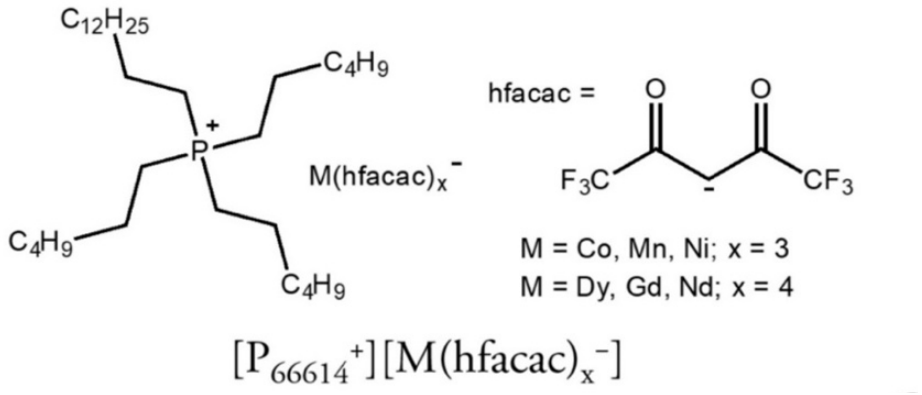

Figure 1. Chemical structures of MILs evaluated in this study.

4.2.2 Bacteria and Culture Conditions: The bacterial strains using in this study are listed in Table 1. All cultures were grown overnight in 10 mL volumes in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37 °C. Organisms were enumerated using Tryptic Soy Agar (TSA), Bismuth Sulfite Agar (BSA) or MacConkey Agar with Sorbitol (SMAC) plates (BD).

Table 1. Bacterial strains and species used in this study

Strain	Serotype	Source[a]
E. coli N886-71	O157:H7	OHA
E. coli N366-2-2	O157:H7	OHA
E. coli N549-3-1	O157:H7	OHA
E. coli N317-3-1	O157:H7	OHA
E. coli N192-5-1	O157:H7	OHA
E. coli N192-6-1	O157:H7	OHA
E. coli N336-4-1	O157:H7	OHA
E. coli N405-5-8	O157:H7	OHA
Salmonella enterica subsp. *salamae* SA 4406		SGSC
Salmonella enterica subsp. *arizonae* SA 4407		SGSC
Salmonella enterica subsp. *diarizonae* SA 4408		SGSC
Salmonella enterica subsp. *houtenae* SA 4409		SGSC
Salmonella bongori SA 4410		SGSC
Salmonella enterica subsp. *indica* SA 4411		SGSC
Salmonella Minnesota SLH 157		SLH
Salmonella Minnesota mR613		SGSC
Salmonella Typhimurium ATCC 14028		ATCC

[a] OHA, Oregon Health Authority, Public Health Division (Portland, OR, USA); SGSC, *Salmonella* Genetic Stock Centre (Calgary, Alberta, Canada); SLH, Wisconsin State Laboratory of Hygiene (Madison, WI, USA); ATCC, American Type Culture Collection (Manassas, VA, USA).

4.2.3 Instrumentation: Overnight cultures were grown in 14 mL polystyrene round-bottom tubes (Corning Inc., Corning, NY, USA) at 37 °C in a Shel Lab Shaking Incubator (Sheldon Manufacturing, Inc., Cornelius, OR, USA). Agar plates were incubated in a Lab-Line Imperial III Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C.

4.2.4 MIL-Based Whole-Cell Extraction: A universal schematic for MIL-based cell extraction protocol is depicted in Fig. 2. A 1 mL volume of diluted cell suspension was added to a 4-mL screw cap glass vial. Fifteen microliters of either the ($[P_{66614}^{+}][Dy(hfacac)_4^{-}]$) or the ($[P_{66614}^{+}][Ni(hfacac)_3^{-}]$) MIL was added and dispersed into microdroplets by vortex agitation for 30 seconds (Clark *et al*., 2017a). The aqueous phase was decanted following dispersive extraction, and the MIL was subjected to a brief wash step using 1 mL of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA) to ensure adequate removal of residual cell suspension (Clark *et al*., 2017a). Recovery of viable cells from the MIL extraction phase was carried out using a "back-extraction" step accomplished through addition of 1 mL of modified LB broth containing, 2X tryptone and 1X NaCl followed by a 120 second vortex step. After back-extraction, aliquots of the cell-enriched modified LB media were enumerated using a track dilution method on 100 x 100 x 15 mm square TSA, BSA or SMAC plates (BD).

4.2.5 Plating and Enumeration: Following back-extraction, aliquots of the cell-enriched modified LB media were serially diluted in 0.1% peptone water. A 10 µL aliquot of each dilution was applied to the appropriate lane on square TSA, BSA or SMAC plates (Siragusa, 1999). The plates were then tilted at approximately 80° for 15 min to allow the droplets to travel toward the opposite end of the plate. The plates were incubated for 24 h at 37 °C (TSA, SMAC) or for 48 h at 37 °C (BSA). Colonies were manually counted to determine the number of colony forming units (CFU) in each sample. The enrichment factor (E_F) for MIL-based extraction was calculated using Equation 1, where C_{MIL} represents the concentration of bacteria in suspension following extraction using the MIL and C_S is the initial concentration of bacteria in the sample.

$$E_F = \frac{C_{MIL}}{C_S} \quad (1)$$

4.2.6 Exposure to 1-ethyl-3-methylimidazolium thiocyanate ([$EMIM^+$][SCN^-]) **and ([P_{66614}^+][$Ni(hfacac)_3^-$]) MIL Over Time:** One milliliter of diluted *Salmonella* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. A 5% (vol/vol) or 50% (vol/vol) aqueous solution of 1-ethyl-3-methylimidazolium thiocyanate ([$EMIM^+$][SCN^-]) (IoLiTec, Tuscaloosa, AL, USA) was added and dispersed into microdroplets by vortex agitation for 30 seconds (Mester, Wagner, & Rossmanith, 2010). Aliquots of the aqueous phase were enumerated at 0, 5, 10 and 15 min using TSA and BSA. Likewise, MIL-based whole-cell extraction was performed using the ([P_{66614}^+][$Ni(hfacac)_3^-$]) MIL, and cell-enriched modified LB media were enumerated at 0, 5, 10, 15 min using TSA and BSA.

4.2.7 Comparison of Air-Displacement and Positive-Displacement Pipettes for MIL Handling: A 1 mL volume of diluted *Salmonella* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. Fifteen microliters of the ([P_{66614}^+][$Ni(hfacac)_3^-$]) MIL was added using either a Pipetman Classic P20 air-displacement pipette (Gilson, Middleton, WI, USA), or a Microman E M25E positive-displacement pipette (Gilson) and dispersed into microdroplets by vortex agitation for 30 seconds. MIL-based extraction and enumeration was carried out as previously described.

4.2.8 Exposure to ([P_{66614}^+][$Dy(hfacac)_4^-$]) MIL, $DyCl_3$ and ([NH_4^+][$Dy(hfacac)_4^-$]): One milliliter of diluted *Salmonella* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. Fifteen microliters of either the ([P_{66614}^+][$Dy(hfacac)_4^-$]) MIL or 2-10 µL of 100 mM $DyCl_3$ solution or 10 mg of the ammonium salt ([NH_4^+][$Dy(hfacac)_4^-$]) was added and dispersed by vortex agitation for 30 seconds. Aliquots of the ([P_{66614}^+][$Dy(hfacac)_4^-$]) MIL-exposed cellular suspension were enumerated using square TSA or BSA plates (BD).

4.3 Results and Discussion

4.3.1 Exposure Time-Course to the [EMIM$^+$][SCN$^-$] IL and the ([P_{66614}^+][Ni(hfacac)$_3^-$]) MIL:

To begin our investigation into the physiological characterization of the Ni(II) MIL, we selected *Salmonella* Typhimurium ATCC 14028 as a model Gram-negative bacterium of interest. Our previously reported work demonstrated successful capture and recovery of viable *Salmonella* Typhimurium cells using Ni(II) and Co(II)-based MILs. Culture-based methods depend on sample preparation steps that preserve bacterial viability; therefore, potential deleterious cytotoxic effects imparted by MIL extractants must be considered. Based on our previously reported work that demonstrated successful integration of MIL-based capture with recombinase polymerase amplification (RPA), the Ni(II) MIL was selected for further analysis. To study the effect of the Ni(II) MIL exposure on the recovery of *Salmonella* Typhimurium, a 1 mL aliquot of TSB was inoculated with 1x10^5 CFU mL^{-1} of bacteria and spiked with 15 µL of the Ni(II) MIL. A general schematic for the MIL-based extraction and recovery is shown in Fig. 2.

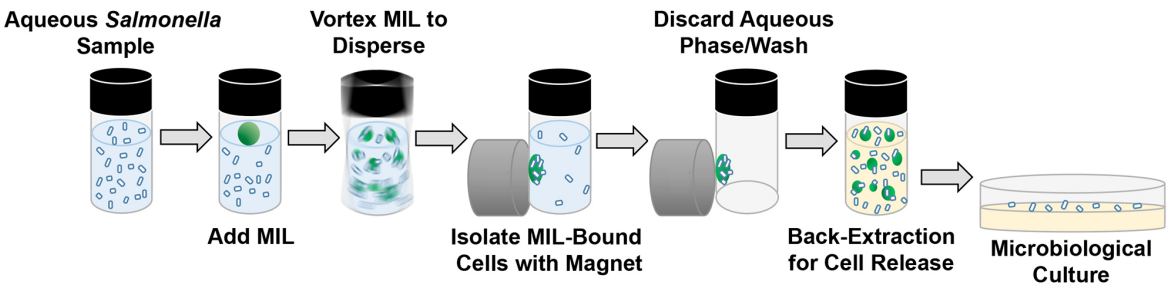

Figure 2. Schematic for the extraction, preconcentration and recovery of *Salmonella* from aqueous samples, followed by downstream analysis using microbiological culture detection methods. Panel adapted from Clark *et al*., 2017a.

Following extraction, 10 µL aliquots of the back-extraction solution were enumerated at 0, 5, 10 and 15 min using square TSA and BSA plates, as described previously. Average cell counts were compared to a standard that was not exposed to the Ni(II) MIL. As shown in Fig. 3, the Ni(II)-

based MIL had little influence on the growth of *Salmonella* Typhimurium over time when compared to a standard that had not been exposed to the Ni(II) MIL. Differences in recovered cell counts may be due to differential extraction of *Salmonella* Typhimurium by the MIL.

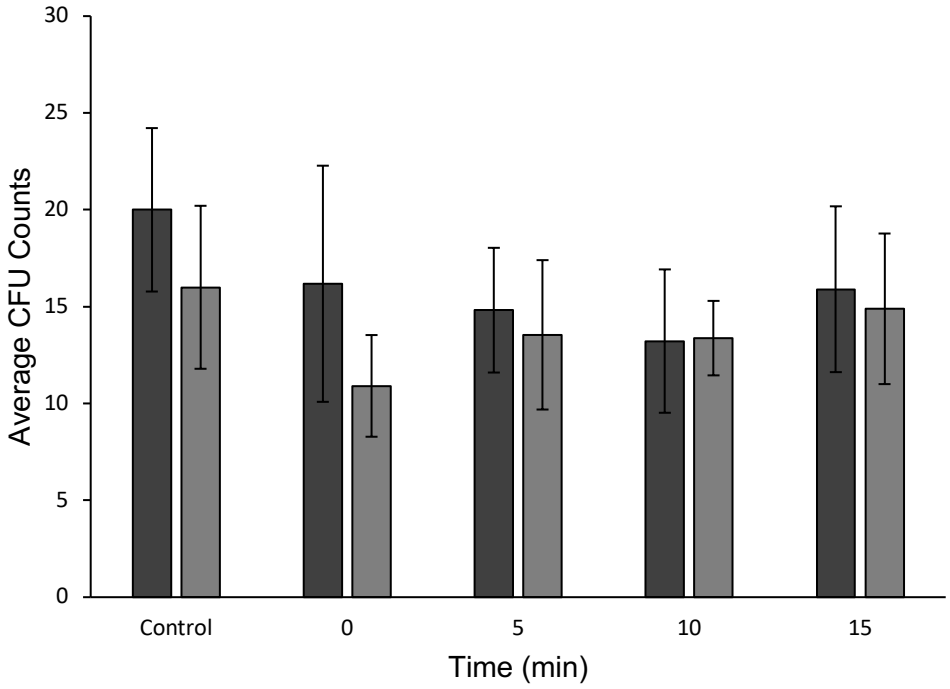

Figure 3. Recovery of *Salmonella* Typhimurium extracted with Ni(II) MIL as a function of time (min). Average colony forming unit (CFU) counts recovered from the aqueous Ni(II) MIL back-extraction phase over time (approximately 15 min). A suspension of *Salmonella* Typhimurium was prepared and captured with the Ni(II) MIL as described in the text, then back-extracted using modified LB broth containing 2X tryptone and 1X NaCl. MIL-treated cells were compared to standard that had not been exposed to the Ni(II) MIL.

Cell injury can be detected by plating MIL-treated cells in parallel on both non-selective and selective media and evaluating growth under each condition. Physiologically healthy (non-injured) Gram-negative cells are able to tolerate exposure to selective agents, such as crystal violet or brilliant green, which are inherently toxic to Gram-positive cells. Gram-negative cells possess a protective outer membrane (OM), which serves to limit the passive diffusion of molecules into the cell. Gram-positive cells do not have an OM structure. Injury to Gram-negative cells caused

by exposure to deleterious chemicals is typically characterized by damage to the OM, causing the cell to become "leaky" and allowing ingress of these selective/toxic compounds, which results in reduced growth on selective agars (Wu, 2008). Enrichment factors were calculated as a function of time and are reported in Table 2. For TSA, the resulting E_F value was 7.2±0.6 (n=4); for BSA, the E_F values was 8.2±1.0 (n=4).

Table 2. Enrichment factors for *Salmonella* Typhimurium over 15 min exposure to the Ni(II) MIL

Time (min)	Enrichment Factor (TSA)	Enrichment Factor (BSA)
0	8	7
5	7	8
10	6	8
15	8	9

The effects of 5% or 50% (vol/vol) aqueous solutions of the [$EMIM^+$][SCN^-] IL on the viability of the cells was also evaluated. Average cell counts were compared to a standard not exposed to [$EMIM^+$][SCN^-]. [$EMIM^+$][SCN^-] has been previously investigated as a sample pretreatment reagent for the solubilization of protein-rich food matrices (Mester *et al.*, 2010). The [$EMIM^+$] cation potentially acts as a detergent, while the [SCN^-] anion is chaotropic; the resulting outcome is an effective tool during matrix lysis (Mester *et al*., 2010). In our hands, when *Salmonella* Typhimurium was exposed to 50% (vol/vol) solutions of [$EMIM^+$][SCN^-], no recovery was observed on TSA or BSA after 5 min of exposure. Previously reported work demonstrates that [$EMIM^+$][SCN^-] is injurious to *Salmonella* Typhimurium during its use as an ionic liquid extractant, as observed by differential counts on non-selective and selective media (Mester *et al.*, 2010).

4.3.2 Evaluation of Capture and Recovery of Wild-Type and Mutant *Salmonella* Minnesota Strains Using the ([P_{66614}^+][$Ni(hfacac)_3^-$]) MIL: To further evaluate the importance of the OM in protecting the cell from potentially deleterious effects conferred by the Ni(II) MIL, capture and

recovery of two physiologically distinct strains of *Salmonella* Minnesota was performed. The strains compared were *Salmonella* Minnesota SLH 157 (wild-type) and *Salmonella* Minnesota mR613 (outer membrane mutant). *Salmonella* Minnesota mR613 is considered a "deep rough" mutant, possessing a truncated OM core. Compared to cells with an intact OM, cells with a truncated OM are often dramatically more susceptible to damage from antimicrobial agents or in chemically harsh environments (Nikaido, 2003). To study the effect of the Ni(II) MIL exposure on the recovery of the wild-type and mutant strains of *Salmonella* Minnesota, a 1 mL aliquot of TSB was inoculated with $1x10^5$ CFU mL^{-1} of bacteria and spiked with 15 µL of the Ni(II) MIL. MIL-based extraction was performed as previously described. Following extraction, 10 µL aliquots of the back-extraction solution were enumerated using square TSA and BSA plates. Average cell counts were compared to a standard that was not exposed to the Ni(II) MIL. Extraction and recovery of *Salmonella* Typhimurium was also assessed. Enrichment factors were calculated and are reported in Table 3.

Table 3. Enrichment factors for *Salmonella* Typhimurium, *Salmonella* Minnesota SLH 157 (wild-type) and *Salmonella* Minnesota mR613 (mutant)

Strain Assessed	Enrichment Factor (TSA)	Enrichment Factor (BSA)
Salmonella Typhimurium	10	14
Salmonella Minnesota SLH 157	17	11
Salmonella Minnesota mR613	4	*

* For both the standard and the MIL-treated cells, no growth was observed on BSA using the *Salmonella* Minnesota mR613 "deep rough" mutant strain.

While the extraction efficiency using the Ni(II) MIL was greatly reduced for *Salmonella* Minnesota mR613, capture and recovery of viable cells was observed on TSA. Recovery of viable cells was not seen on BSA, as expected, due to the inherent susceptibility of this strain to selective agents stemming from its impaired OM barrier. Likewise, growth of the *Salmonella* Minnesota mR613 standard was observed on TSA but not on BSA.

Our ability to capture this mutant strain demonstrates two important things: (1) the capacity of the Ni(II) MIL to effectively capture and concentrate a strain of *Salmonella* Minnesota that displays a drastically different external surface and (2) the post-capture growth behavior of this physiologically sensitive strain suggests that the Ni(II) MIL capture process is not overtly antimicrobial. The lack of an observed toxic impact of the Ni(II) MIL on the "deep rough" mutant *Salmonella* Minnesota strain may result from a lack of inherent chemical toxicity of the Ni(II) MIL, from low diffusivity of the hydrophobic MIL in aqueous media—or a combination of both potential phenomena.

4.3.3 Capture and Recovery of Seven Representative DNA Subgroups of *Salmonella* and Eight Strains of *E. coli* O157:H7: Previous work has demonstrated successful capture of *E. coli* K12, *Serratia marcescens* and *Salmonella* Typhimurium using MILs (Clark *et al*., 2017a; Hice *et al*., 2019). In order to explore the versatility of MILs, evaluation of the broad applicability of MIL-based capture and recovery of a panel of viable cells is requisite. To study the effect of the Ni(II) MIL exposure on the recovery of the seven representative DNA subgroups of *Salmonella* and eight strains of *E. coli* O157:H7, a 1 mL aliquot of TSB was inoculated with $1x10^6$ CFU mL^{-1} of bacteria, and spiked with 15 µL of the Ni(II) MIL. MIL-based extraction was performed as previously described. Following extraction, 10 µL aliquots of the back-extraction solution were enumerated using square TSA and BSA plates (*Salmonella*) and TSA and SMAC plates (*E. coli* O157:H7). Average cell counts were compared to a standard that was not exposed to the Ni(II) MIL. Enrichment factors were calculated and are reported in Tables 4 and 5.

Reasons for the observed interexperimental variability in calculated enrichment factors are not clear. However, our results demonstrate capture and recovery of all seven *Salmonella* subgroups and all eight strains of *E. coli* O157:H7, with comparable recovery on non-selective and

selective media within experiments. For each strain assessed, calculated enrichment factors between media types were nearly identical (Tables 4, 5), indicating that the Ni(II) MIL did not exhibit toxicity towards the fifteen strains assessed. MIL-based extraction and recovery of *Salmonella* strains representative of the two species of *Salmonella* that comprise the genus (*S. enterica* and *S. bongori*), and of the seven DNA subgroups within the genus *Salmonella*, demonstrates the robust applicability and breadth of MIL-based capture.

Table 4. Enrichment factors for seven representative DNA subgroups of *Salmonella*

Strain Assessed	Enrichment Factor (TSA)	Enrichment Factor (BSA)
Salmonella Typhimurium	8	6
Salmonella enterica subsp. *salamae*	12	9
Salmonella enterica subsp. *arizonae*	3	2
Salmonella enterica subsp. *diarizonae*	7	11
Salmonella enterica subsp. *houtenae*	8	8
Salmonella bongori	4	4
Salmonella enterica subsp. *indica*	3	4

Table 5. Enrichment factors for eight strains of *E. coli* O157:H7

Strain Assessed	Enrichment Factor (TSA)	Enrichment Factor (SMAC)
E. coli O157:H7 N886-71	4	1
E. coli O157:H7 N366-2-2	4	1
E. coli O157:H7 N549-3-1	8	3
E. coli O157:H7 N317-3-1	4	1
E. coli O157:H7 N192-5-1	7	1
E. coli O157:H7 N192-6-1	9	4
E. coli O157:H7 N336-4-1	3	2
E. coli O157:H7 N405-5-8	2	1

E. coli, *S. marcescens* and *Salmonella* belong to a broader family of physiologically-similar Gram-negative bacteria, the Enterobacteriaceae. This family contains several other pathogenic bacteria of interest in foods, including *Cronobacter*, *Erwinia*, *Klebsiella*, *Shigella* and *Yersinia*. This study provides the foundation for further investigation into the capture and recovery of other notable

foodborne pathogens and establishes MILs as a viable platform for rapid preconcentration and extraction methodology.

4.3.4 Comparison of the Initial Wash and the Full MIL Extraction on the Recovery of *Salmonella* Typhimurium: Although all of the strains assessed were capable of being enriched by the MIL, some species were physically enriched to greater extents than others with extraction. In order to further examine the cause for this finding, the number of cells present in the wash solution was investigated. The washing step is performed after subjecting the cells to the MIL enrichment process in order to remove any loosely adsorbed bacteria prior to back-extraction. It is hypothesized that the cells with lower enrichment factors are bound with lower affinity to the MIL and, therefore, a greater number would be lost to the wash solution than those with higher enrichment factors. To test this, five different *Salmonella* strains exhibiting varying degrees of enrichment were subjected to the aforementioned analysis. Results demonstrate that the range of amount lost during the wash step for the bacteria tested ranged from 47-79%. These data indicate that the wash step is successful at removing superficially adsorbed bacteria from the MIL extraction phase. The difference in the amount lost can also be compared to the enrichment factors to aid in identifying which cells may have a higher affinity for the MIL phase (Table 6).

Table 6. Percent loss of cells in the wash solution

Strain Assessed	Percent-Loss*
Salmonella Typhimurium	53±3
Salmonella enterica subsp. *arizonae*	79±1
Salmonella enterica subsp. *diarizonae*	47±7
Salmonella Minnesota SLH 157	59±3
Salmonella Minnesota mR613	69±5

* Percent-loss was calculated by dividing the counts obtained from the wash solution by the sum of the counts of the wash and back-extraction solution, multiplied by 100.

Enrichment factors obtained for *Salmonella enterica* subsp. *arizonae* were some of the lowest (E_F=3) of the *Salmonella* strains tested. When the percent-loss is calculated for this particular strain, it is found that 79% of the bacteria present in the MIL during the extraction step are lost during the wash. This may indicate that the bacteria have a weaker affinity for the MIL compared to other strains tested. In comparison, *Salmonella* Typhimurium yielded enrichment factors twice as high as *arizonae*, and had a lower percent-loss (53%) during the wash step. This experiment provides evidence that differences in E_F values for the various bacteria tested could be due to differential affinity for the MIL extraction phase. If this is true, bacteria with lower affinities for the MIL may be weakly bound, and are therefore easily removed by the washing step compared to the strains with higher observed enrichment factors.

4.3.5 Investigation of the [P_{66614}^+][Dy(hfacac)$_4^-$] MIL: Incorporation of a rare-earth metal into the MIL structure is of significant interest as these metals possess greater magnetic moments. In principle, this allows for easier manipulation with an external magnet compared to transition metal-based MILs. However, when the [P_{66614}^+][Dy(hfacac)$_4^-$] MIL was previously explored for the capture of bacteria, recovery of viable cells was not observed (Clark *et al*., 2017a). In order to understand these results and to determine if they might be related to antimicrobial activity exerted by this MIL, bacterial suspensions of *Salmonella* Typhimurium were exposed to various structural components of the MIL.

Initially, the addition of 15 µL of [P_{66614}^+][Dy(hfacac)$_4^-$] MIL to a cell suspension was evaluated. However, after 30 sec of vortex, visual examination of the suspension solution revealed significant flocculation. Upon plating on selective and non-selective media, no observable growth was observed on either type of media. Due to the visual appearance of the suspension after MIL exposure and lack of growth on plates, it is possible that the MIL is capable of lysing the bacteria.

To gain further insight into this phenomenon, the effect of dysprosium was evaluated by subjecting the cells to 0.1 mM and 1.0 mM solutions of $DyCl_3$. The cells were exposed to the metal solutions for 30 seconds and subsequently plated on selective and non-selective media. Results shown in Fig. 4 demonstrate that growth can be observed on both types of plates. However, significantly lower counts are observed on BSA than TSA, particularly at the higher $DyCl_3$ concentration. This suggests that the coordinated metal may be partially responsible for the deleterious effects of the MIL.

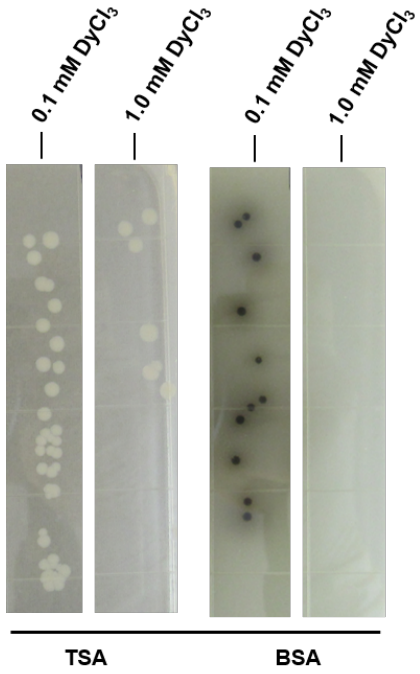

Figure 4. Recovery of *Salmonella* Typhimurium following 30 sec exposure to 0.1 mM and 1.0 mM $DyCl_3$. Viable cell counts recovered on non-selective TSA (left) and selective BSA (right) from the $DyCl_3$-exposed cells following initial 30 sec exposure.

Although the metal showed some cytotoxicity, it is not completely responsible for the MIL's effect on the cells, as growth was still observed. Since both the Ni(II) and the Dy(III) MILs contain identical cations ($[P_{6,6,6,14}^+]$), the effect of the anion structure was evaluated. The anion of the Dy(III) MIL contains one additional hexafluoroacetylacetonate ligand than the Ni(II) MIL,

making the coordination geometry of the two complexes different. To test the effects of the anion structure, cells were subjected to 10 mg of the ammonium salt ([NH_4^+][Dyhfacac$^-$]). After 30 sec vortex, similar flocculation was observed as when the cells were exposed to the Dy(III) MIL. After plating and 24 hr incubation, no growth was seen on either BSA or TSA. These results provide strong evidence that the anion structure is largely responsible for the antimicrobial effects of the MIL. Ongoing work is focused on the design and synthesis of a non-toxic Dy(III)-based MIL whose strong paramagnetism can be exploited.

4.3.6 Assessment of Positive and Air Displacement Pipettes: The Ni(II) MIL used in this study possesses one of the lowest viscosities (927.9 centipoise (cp)) of MILs previously reported. While it is less viscous than many previously synthesized MILs, its viscosity is only slightly lower than glycerol (950 cp) at room temperature. This makes pipetting with traditional air displacement (AD) pipettes a challenge, as the MIL must be drawn up very slowly in order to prevent any aspiration of air. Furthermore, the high viscosity prevents all of the MIL from being completely dispensed as some remains adhered to the walls of the pipette tip. These drawbacks prevent the MILs from being utilized by end-users with minimal training and can lead to significant amounts of unused MIL from the adherence to the pipette tip.

In order to circumvent these challenges associated with the manipulation of the MIL, the use of a positive displacement pipette for dispensing the MIL was explored. Positive displacement (PD) pipettes use piston-driven displacement as the mechanism for fluid delivery. This type of pipette is popular for applications with highly viscous liquids, as the mechanism allows for faster draw up, as well as ensuring virtually no liquid is left on the inside of the tip after dispensing. To compare the performance of both pipettes, in terms of quantity and reproducibility of MIL delivery, 15 μL of MIL originating from each pipette was weighed (n=3). From the results shown

in Fig. 5, it is obvious that the PD pipette is able to deliver a significantly greater mass of MIL (17.1±0.6 mg) compared to the AD pipette (11.3±0.4 mg) with similar reproducibility. This difference is most likely attributed to the MIL remaining on the walls of the AD tip as no MIL was visually observed to remain within the PD tip following pipetting.

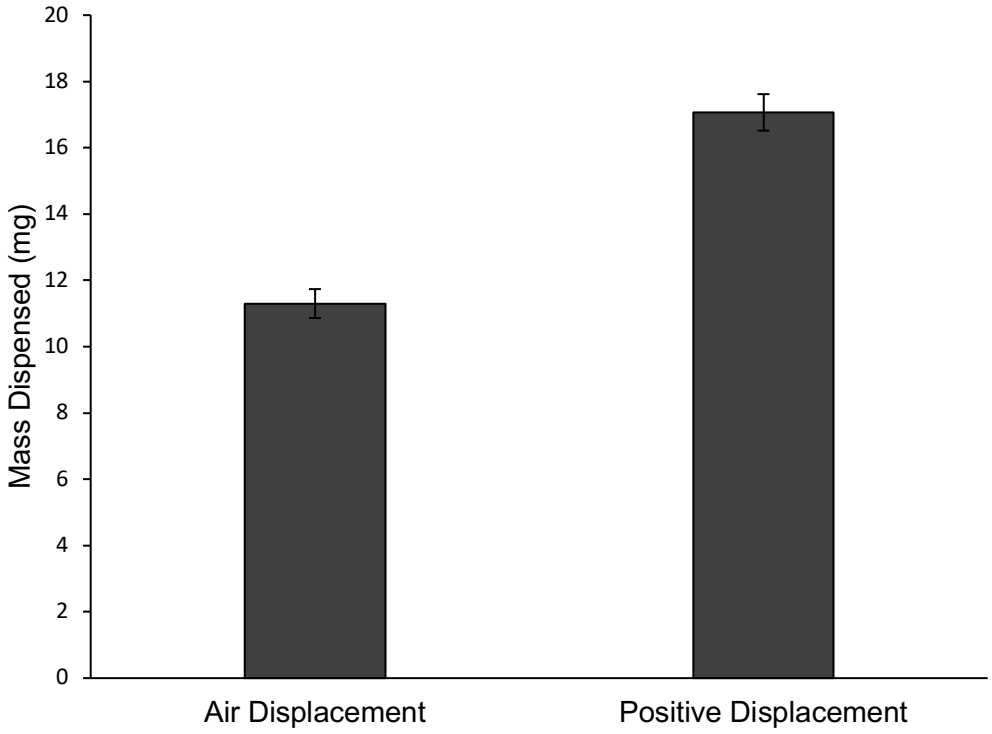

Figure 5. Evaluation of Ni(II) MIL delivery using air displacement (AD) and positive displacement (PD) pipettes. Average mass in mg of Ni(II) MIL dispensed using the AD and PD pipettes. The PD pipette is able to deliver a significantly greater mass of Ni(II) MIL (17.1±0.6 mg) compared to the AD pipette (11.3±0.4 mg).

To test whether or not this difference resulted in a noticeable increase in extraction performance, extractions were performed as previously described using each pipette to dispense 15 μL of MIL. The results demonstrate no discernable difference in the enrichment factors obtained from using either pipette. While no difference was observed, the PD offers the following advantages over AD: (1) dispensing the MIL is much faster and easier with PD; using the AD requires slower draw-up and dispensing as it relies on air to displace a viscous liquid, (2) although

the extraction performance did not appear to differ between the two pipettes, there is a significant difference in the amount of MIL dispensed. Using a PD can be economically advantageous as smaller volumes can be used to dispense similar quantities as AD without any loss of MIL to the walls of the tip.

3.4 Conclusions

In summary, the extraction performance and physiological effects of the $[P_{66614}^+][Ni(hfacac)_3^-]$ MIL on nine *Salmonella* serotypes and eight *E. coli* O157:H7 strains was explored. Potential damage to the cells was assessed by comparing cell-counts on non-selective (TSA) and selective (BSA, SMAC) media, as damaged cells are expected to show impaired growth on selective media. Virtually no difference was observed in the counts between media types for all *Salmonella* and *E. coli* O157:H7 strains tested, indicating no detectable antimicrobial effects were imparted by the Ni(II) MIL. Furthermore, physical enrichment was achieved for all of the strains tested (E_F from 1-12), demonstrating the broader applicability of MIL-based capture of bacterial pathogens.

We determine experimentally a possible reason for the difference in the enrichment factors for the various cell strains by analyzing the back-extraction solution. The strains that resulted in lower enrichment factors were found to lose a higher number of cells during the washing process, indicating a weaker interaction between the cells and the MIL. The cytotoxic effects of the $[P_{66614}^+][Dy(hfacac)_4^-]$ MIL were also investigated. It was found that the anion played a large role in the cytotoxic behavior of the MIL. Ongoing work is focused on further understanding the role of the Dy(III) anion in order to better design a Dy(III)-based MIL with reduced cytotoxic effects.

Author Contributions

Stephanie A. Hice and Marcelino Varona contributed equally to this work.

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References

Centers for Disease Control and Prevention (CDC). (2019a). *Salmonella*. Retrieved March 30, 2019, from http://www.cdc.gov/salmonella/index.html

Centers for Disease Control and Prevention (CDC). (2019b). *E. coli*. Retrieved March 30, 2019, from https://www.cdc.gov/ecoli/index.html

Clark, K. D., Nacham, O., Purslow, J. A., Pierson, S. A., & Anderson, J. L. (2016). Magnetic ionic liquids in analytical chemistry: A review. *Analytica Chimica Acta, 934*, 9-21. doi:10.1016/j.aca.2016.06.011

Clark, K. D., Purslow, J. A., Pierson, S. A., Nacham, O., & Anderson, J. L. (2017a). Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR amplification and culture-based diagnostics. *Analytical and Bioanalytical Chemistry, 409*(21), 4983-4991. doi:10.1007/s00216-017-0439-y

Clark, K. D., Varona, M., & Anderson, J. L. (2017b). Ion-tagged oligonucleotides coupled with a magnetic liquid support for the sequence-specific capture of DNA. *Angewandte Chemie - International Edition, 56*(26), 7630-7633. doi:10.1002/anie.201703299

Ding, X., Clark, K. D., Varona, M., Emaus, M. N., & Anderson, J. L. (2019). Magnetic ionic liquid-enhanced isothermal nucleic acid amplification and its application to rapid visual DNA analysis. *Analytica Chimica Acta, 1045*, 132-140. doi:10.1016/j.aca.2018.09.014

El-Gazzar, F. E., & Marth, E. H. (1992). Salmonellae, salmonellosis, and dairy foods: A review. *Journal of Dairy Science, 75*(9), 2327-2343. doi:10.3168/jds.S0022-0302(92)77993-4

Entis, P., Fung, D. Y. C., Griffiths, M., McIntyre, L., Russell, S., Sharpe, A. N., & Tortorello, M. L. (2001). Rapid methods for detection, identification, and enumeration. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 89–125). Washington, DC: American Public Health Association.

Garrido, A., Chapela, M.-J., Román, B., Fajardo, P., Vieites, J. M., & Cabado, A. G. (2013). In-house validation of a multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and *Listeria monocytogenes*. *International Journal of Food Microbiology, 164*(1), 92-98. doi:10.1016/j.ijfoodmicro.2013.03.024

Hice, S. A., Clark, K. D., Anderson, J. L., & Brehm-Stecher, B. F. (2019). Capture, concentration, and detection of *Salmonella* in foods using magnetic ionic liquids and recombinase polymerase amplification. *Analytical Chemistry, 91*(1), 1113-1120. doi:10.1021/acs.analchem.8b04751

Hoffman, S., Maculloch, B., & Batz, M. (2015). Economic burden of major foodborne illnesses acquired in the United States. *Current politics and economics of the United States, Canada and Mexico, 17*(4).

Jokerst, J. C., Adkins, J. A., Bisha, B., Mentele, M. M., Goodridge, L. D., & Henry, C. S. (2012). Development of a paper-based analytical device for colorimetric detection of select foodborne pathogens. *Analytical Chemistry, 84*(6), 2900-2907. doi:10.1021/ac203466y

Kim, H., & Bhunia, A. K. (2008). SEL, a selective enrichment broth for simultaneous growth of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. *Applied and Environmental Microbiology, 74*(15), 4853-4866. doi:10.1128/AEM.02756-07

Kim, J. Y., & Lee, J.-L. (2016). Rapid detection of *Salmonella enterica* serovar Enteritidis from eggs and chicken meat by real-time recombinase polymerase amplification in comparison with the two-step real-time PCR. *Journal of Food Safety, 36*(3), 402-411. doi:10.1111/jfs.12261

Lim, J. Y., Yoon, J., & Hovde, C. J. (2010). A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *Journal of Microbiology and Biotechnology, 20*(1), 5-14.

Meng, J., Feng, P., & Doyle, M. P. (2001). Pathogenic *Escherichia coli*. In F. P. Downes & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 331–341). Washington, DC: American Public Health Association.

Merib, J., Spudeit, D. A., Corazza, G., Carasek, E., & Anderson, J. L. (2018). Magnetic ionic liquids as versatile extraction phases for the rapid determination of estrogens in human urine by dispersive liquid-liquid microextraction coupled with high-performance liquid chromatography-diode array detection. *Analytical and Bioanalytical Chemistry, 410*(19), 4689-4699. doi:10.1007/s00216-017-0823-7

Mester, P., Wagner, M., & Rossmanith, P. (2010). Use of ionic liquid-based extraction for recovery of *Salmonella* Typhimurium and *Listeria monocytogenes* from food matrices. *Journal of Food Protection, 73*(4), 680-687.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews, 67*(4), 593-656. doi:10.1128/MMBR.67.4.593-656.2003

Pierson, S. A., Nacham, O., Clark, K. D., Nan, H., Mudryk, Y., & Anderson, J. L. (2017). Synthesis and characterization of low viscosity hexafluoroacetylacetonate-based hydrophobic magnetic ionic liquids. *New Journal of Chemistry, 41*(13), 5498-5505. doi:10.1039/C7NJ00206H

Siragusa, G. R. (1999). Statistical validation of the track-dilution plating method from ground beef and carcass surface samples. *Journal of Rapid Methods and Automation in Microbiology, 7*(3), 155-161. doi:10.1111/j.1745-4581.1999.tb00385.x

Suo, B., & Wang, Y. (2013). Evaluation of a multiplex selective enrichment broth SEL for simultaneous detection of injured *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Brazilian Journal of Microbiology, 44*(3), 737-742.

Wu, V. C. (2008). A review of microbial injury and recovery methods in food. *Food Microbiology, 25*(6), 735-744. doi:10.1016/j.fm.2008.04.011

CHAPTER FIVE

GENERAL CONCLUSIONS

The second chapter of this dissertation describes an approach to paper-based enzymatic colorimetric assays for the identification of metabolic state in *Salmonella* Typhimurium and *E. coli* from environmental samples. Non-specific bacterial enzymes, including oxidoreductases and alkaline phosphatases, are present at different levels depending on metabolic state of the cell. Respectively, these enzymes act by reducing tetrazolium salts to purple formazans, or cleaving para-nitrophenyl-phosphate salts to yellow para-nitrophenol. The developed INT-PMS (metabolically active) and INT-PNPP (metabolically inactive) assays resulted in visible color change on paper within 60 min. Capture using *Salmonella* or *E. coli*-specific bacteriophage (P22 and T4) allows for specific detection. Our approach to detection of bacterial pathogens using non-specific bacterial enzymes is inexpensive, portable and rapid (30 min), allowing a semi-quantitative determination of the concentration of bacteria at each metabolic state. The assays facilitate the rapid determination of the metabolic state of bacteria, which is important in preventing deleterious environmental and industrial effects induced by rapid bacterial growth.

The third chapter of this dissertation describes the combined use of magnetic ionic liquids (MILs) and recombinase polymerase amplification (RPA) for the detection of *Salmonella* Typhimurium in aqueous food samples. Ni(II) and Co(II)-based MILs were investigated for the extraction and recovery of viable *Salmonella*, which had not been previously reported. Use of the Ni(II) and Co(II)-MILs resulted in rapid (5 min) preconcentration of cells approximately 12 times the initial concentration. Following extraction, a 20 minute isothermal RPA assay was employed, and results were visualized using gel electrophoresis or nucleic acid lateral flow immunoassay

(NALFIA). Sodium acetate heat packs were used as a chemical heat source during amplification. Our combined MIL-RPA-NALFIA method demonstrates a low-cost, portable and rapid (30 min) approach to bacterial pathogen detection. The simple, streamlined approach to single-tube detection facilitates ease of operation and interpretation for end-users. Presently, this work is intended to benefit food processors, enabling rapid, in-plant testing of foods and environments.

The fourth chapter of this dissertation describes the investigation into the physical properties of the Ni(II) and Dy(III)-based MILs, including an evaluation of potential antimicrobial effects. A multi-strain panel of *Salmonella* and *E. coli* O157:H7 were surveyed, including nine serotypes of *Salmonella* and eight strains of *E. coli* O157:H7. Non-selective (TSA) and selective (BSA, SMAC) media was employed to identify any potential deleterious effects of the MILs, including cell damage, over time (15 min). By plating MIL-treated cells on non-selective and selective media in parallel, evaluation of the potentially antimicrobial effects of the MIL is achieved. Cellular injury is frequently characterized by damage to the outer membrane. MIL-based exposure was compared to 1-ethyl-3-methylimidazolium thiocyanate ($[EMIM^+]SCN^-$), which has been reported to induce cellular injury. While the Ni(II) MIL did not impart any observable antimicrobial effects onto the cell, exposure to the Dy(III) MIL did not result in recovery of viable cells on both non-selective and selective media. Investigation into the individual components of the Dy(III) MIL suggested that the ammonium salt ($[NH_4^+][Dyhfacac^-]$) was responsible for the cytotoxic effects observed.

Throughout this dissertation the described approaches to bacterial pathogen detection encompassed four key advantages, including: **1)** time-to-result, **2)** low-cost, **3)** portability and **4)** ease of operation/interpretation. Our described approaches to rapid detection may enable simple and low-cost sampling of bacterial pathogens, which can be used for agricultural testing efforts

and to improve industrial efficiency in food processing environments. MILs provide a unique approach to the purification and preconcentration of bacterial pathogens, and are a versatile, robust sample preparation technique with a variety of applications, including a particle-free approach to capture of sequence-specific DNA. Future work will emphasize extending MIL-based capture and recovery of notable foodborne pathogens, including members of the Enterobacteriaceae (*Cronobacter*, *Erwinia*, *Klebsiella*, *Shigella*, *Yersinia*) and other important foodborne pathogens, such as *L. monocytogenes*. While our research suggests that weak electrostatic interactions drives MIL-based capture, use of fluorescently-labeled cells may facilitate observation of the interaction that exists between the MIL and the cells. Labeling with Syto-9, followed by washing and dispersal in a model medium, may allow observation of MIL-bound bacteria under a fluorescent microscope, with the option for magnetic focusing of the cell-charged MIL to improve visualization. A deeper understanding of the MIL capture process will ultimately provide insights into design of new MIL structures for improving the purification and enrichment of pathogenic bacteria.

APPENDIX A

SUPPORTING INFORMATION ACCOMPANYING CHAPTER 2

Table *S*1.- Chemical composition of INT/PMS/L-malic Assay Solutions. Different combination of INT (0 to 2.5 mM), PMS (0 to 1 mM) and L-malic (0 to 10 mM) were dissolved in PBS and tested with 10^4 CFU of *E. coli* LLV per 38.5 mm^2. Color intensity is reported in the green channel as an average of 5 samples.

INT (mM)	PMS (mM)	L-malic (mM)	color intensity	two standard errors
0.5	0.00	0.0	0.4922	0.0129
0.5	0.00	2.5	0.4723	0.0105
0.5	0.00	5.0	0.5311	0.0247
0.5	0.00	10.0	0.5152	0.0181
0.5	0.25	0.0	0.6807	0.0169
0.5	0.25	2.5	0.5924	0.0688
0.5	0.25	5.0	0.5499	0.0382
0.5	0.25	10.0	0.5560	0.0139
0.5	0.50	0.0	0.5609	0.0131
0.5	0.50	2.5	0.5774	0.0231
0.5	0.50	5.0	0.5621	0.0172
0.5	0.50	10.0	0.5521	0.0171
0.5	0.75	0.0	0.4893	0.0050
0.5	0.75	2.5	0.4929	0.0136
0.5	0.75	5.0	0.4649	0.0047
0.5	0.75	10.0	0.4831	0.0085
0.5	1.00	0.0	0.5401	0.0301
0.5	1.00	2.5	0.5883	0.0113
0.5	1.00	5.0	0.5174	0.0185
0.5	1.00	10.0	0.5568	0.0190
1.0	0.00	0.0	0.4875	0.0176
1.0	0.00	2.5	0.5069	0.0419
1.0	0.00	5.0	0.5427	0.0314
1.0	0.00	10.0	0.5761	0.0202
1.0	0.25	0.0	0.5286	0.0156
1.0	0.25	2.5	0.5400	0.0141
1.0	0.25	5.0	0.5481	0.0224
1.0	0.25	10.0	0.5729	0.0161
1.0	0.50	0.0	0.5127	0.0200
1.0	0.50	2.5	0.5796	0.0316
1.0	0.50	5.0	0.5469	0.0157
1.0	0.50	10.0	0.5329	0.0246

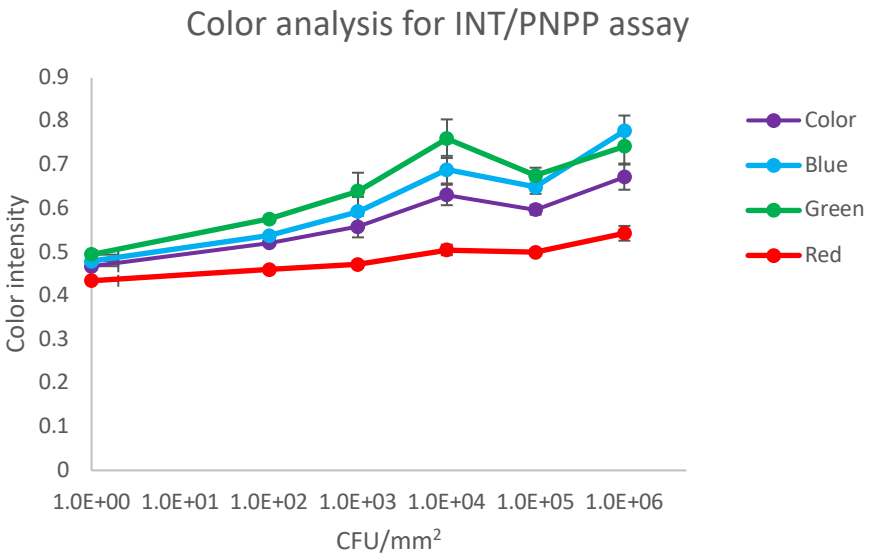
INT (mM)	PMS (mM)	L-malic (mM)	color intensity	two standard errors
1.0	0.75	0.0	0.5544	0.0345
1.0	0.75	2.5	0.5515	0.0158
1.0	0.75	5.0	0.4917	0.0192
1.0	0.75	10.0	0.5301	0.0217
1.0	1.00	0.0	0.5488	0.0168
1.0	1.00	2.5	0.5122	0.0088
1.0	1.00	5.0	0.5154	0.0263
1.0	1.00	10.0	0.5403	0.0294
1.5	0.00	0.0	0.5830	0.0291
1.5	0.00	2.5	0.5366	0.0077
1.5	0.00	5.0	0.5616	0.0088
1.5	0.00	10.0	0.5446	0.0129
1.5	0.25	0.0	0.5171	0.0134
1.5	0.25	2.5	0.5300	0.0163
1.5	0.25	5.0	0.5387	0.0111
1.5	0.25	10.0	0.5507	0.0165
1.5	0.50	0.0	0.6186	0.0506
1.5	0.50	2.5	0.5506	0.0207
1.5	0.50	5.0	0.5349	0.0267
1.5	0.50	10.0	0.4865	0.0200
1.5	0.75	0.0	0.5207	0.0168
1.5	0.75	2.5	0.5327	0.0288
1.5	0.75	5.0	0.5076	0.0164
1.5	0.75	10.0	0.5041	0.0125
1.5	1.00	0.0	0.5120	0.0191
1.5	1.00	2.5	0.5075	0.0270
1.5	1.00	5.0	0.5196	0.0157
1.5	1.00	10.0	0.5198	0.0145
2.0	0.00	0.0	0.5454	0.0268
2.0	0.00	2.5	0.5363	0.0196
2.0	0.00	5.0	0.5639	0.0145
2.0	0.00	10.0	0.5152	0.0129
2.0	0.25	0.0	0.5361	0.0366
2.0	0.25	2.5	0.5425	0.0115
2.0	0.25	5.0	0.5610	0.0404
2.0	0.25	10.0	0.5294	0.0232
2.0	0.50	0.0	0.6002	0.0128
2.0	0.50	2.5	0.5673	0.0042
2.0	0.50	5.0	0.5701	0.0185

INT (mM)	PMS (mM)	L-malic (mM)	color intensity	two standard errors
2.0	0.50	10.0	0.5526	0.0221
2.0	0.75	0.0	0.5167	0.0265
2.0	0.75	2.5	0.4975	0.0233
2.0	0.75	5.0	0.5351	0.0153
2.0	0.75	10.0	0.5100	0.0260
2.0	1.00	0.0	0.6007	0.0076
2.0	1.00	2.5	0.4953	0.0186
2.0	1.00	5.0	0.5042	0.0114
2.0	1.00	10.0	0.4884	0.0216
2.5	0.00	0.0	0.4870	0.0153
2.5	0.00	2.5	0.4826	0.0330
2.5	0.00	5.0	0.5488	0.0118
2.5	0.00	10.0	0.4695	0.0115
2.5	0.25	0.0	0.5121	0.0146
2.5	0.25	2.5	0.4977	0.0064
2.5	0.25	5.0	0.4910	0.0185
2.5	0.25	10.0	0.4540	0.0045
2.5	0.50	0.0	0.5263	0.0190
2.5	0.50	2.5	0.5508	0.0171
2.5	0.50	5.0	0.4678	0.0198
2.5	0.50	10.0	0.5196	0.0107
2.5	0.75	0.0	0.5218	0.0250
2.5	0.75	2.5	0.5168	0.0185
2.5	0.75	5.0	0.5020	0.0116
2.5	0.75	10.0	0.5217	0.0100
2.5	1.00	0.0	0.7746	0.0447
2.5	1.00	2.5	0.5918	0.1119

Table S2 Chemical composition of INT/PNPP Assay Solutions. Different combination of INT (0 to 3 mM) and PNPP (1 to 45 mM) were dissolved in TRIS 0.5 M and tested with 10^4 CFU of *E. coli* per 38.5 mm^2. Color intensity was measured in the blue channel. The reported values are an average of 5 samples.

INT (mM)	PNPP (mM)	color intensity	two standard errors
0.0	1	0.4427	0.0033
0.0	5	0.4424	0.0051
0.0	10	0.4372	0.0040
0.0	15	0.4311	0.0025
0.0	20	0.4594	0.0044
0.0	30	0.4594	0.0044
0.0	45	0.4617	0.0068
0.5	1	0.4838	0.0089
0.5	5	0.4838	0.0067
0.5	10	0.4765	0.0245
0.5	15	0.4571	0.0049
0.5	20	0.4661	0.0070
0.5	30	0.4661	0.0070
0.5	45	0.4722	0.0083
1.5	1	0.4633	0.0043
1.5	5	0.4712	0.0073
1.5	10	0.4787	0.0075
1.5	15	0.4659	0.0024
1.5	20	0.4849	0.0078
1.5	30	0.4849	0.0078
1.5	45	0.4687	0.0039
3.0	1	0.4613	0.0037
3.0	5	0.5097	0.0156
3.0	10	0.4982	0.0070
3.0	15	0.4799	0.0066
3.0	20	0.5052	0.0131
3.0	30	0.5052	0.0131
3.0	45	0.5109	0.0094

A)



B)

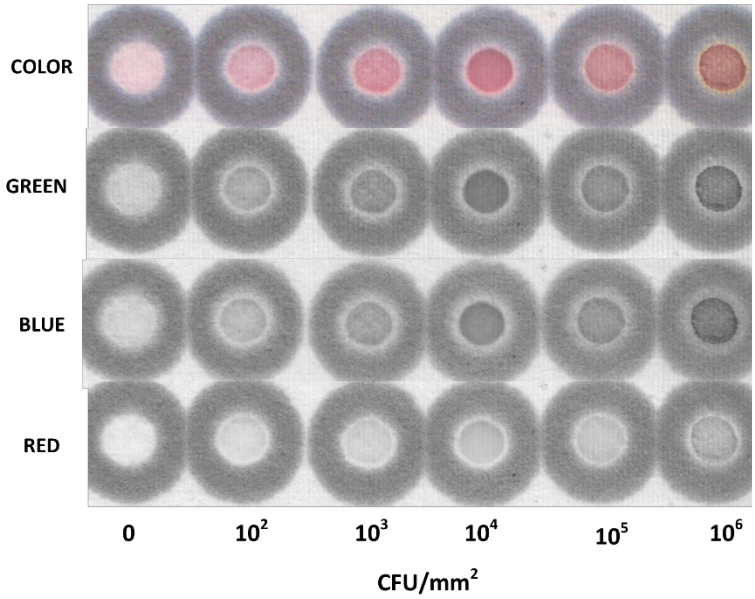


Figure *S*1.- Color analysis performed in samples with dormant *E. coli* and INT/PNPP assay. The color image was analyzed by ImageJ™ as well as each color channel from the same image. (**Panel a**) Blue and green channel demonstrated the highest color intensity values however blue channel had lowest background on samples without bacteria. Blue channel was chosen for analyses since it captured both enzymatic activities (oxidoreductases and alkaline phosphatases) from the same sample. (**Panel b**) Sample of visual images utilized for analysis.

APPENDIX B

SUPPORTING INFORMATION ACCOMPANYING CHAPTER 3

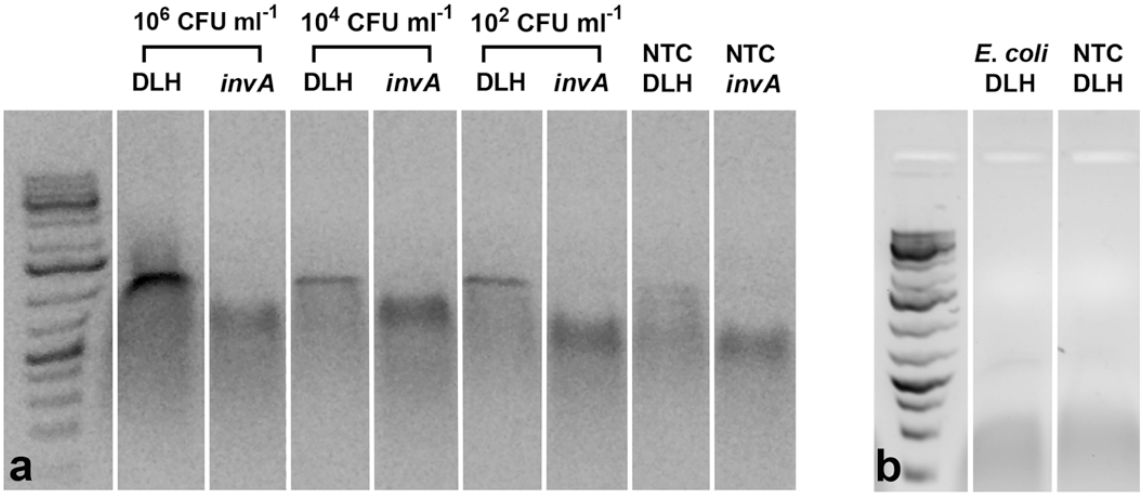


Figure S1. Comparison of DLH and invA primers for detection of MIL-extracted *Salmonella* Typhimurium using gel electrophoresis. Suspensions of *S.* Typhimurium in aqueous media at three different levels (10^6, 10^4 and 10^2 CFU mL^{-1}) were extracted using the Ni(II) MIL, subjected to a 20 min RPA using the DLH and *invA* primers and examined on an agarose gel stained with SYBR Safe DNA Gel Stain. (**Panel a**) Bands for DLH primers consistently showed higher fluorescence (greater product yield) across all cell concentrations used. (**Panel b**) Evaluation of DLH RPA with MIL-extracted *E. coli* supports *in silico* results demonstrating the specificity of the DLH primers for *Salmonella* spp.