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Bacteriophage-Based Rapid Bacteria Detection

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Bacteriophage-Based Rapid Bacteria Detection

By

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DISSERTATION

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ABSTRACT

Foodborne illness is a leading public health concern with a significant impact on food industries and society. Thus, detecting pathogens at various stages of the food supply chain is essential to reduce the risk of foodborne illness. Several new detection technologies have been developed to improve the detection of bacterial contamination in food. Nucleic acid-based and immunological-based methods are considered rapid detection methods; however, these approaches also have several limitations. These limitations include the need for expert users, the inability to distinguish the cells' viability, and the interference from food compositions and its impact on reducing the detection efficiency. Therefore, it is crucial to develop novel rapid bacteria detection methods with high sensitivity and specificity that can detect bacteria in complex food matrices with reduced resource requirements.

This research focuses on using bacteriophage as a tool for rapid and specific bacteria detection in complex food matrices to address some of the current challenges in the food industry. The research evaluated the application of engineered bacteriophage and precipitate forming enzyme substrate to enhance the sensitivity of bacterial detection. The engineered phage induces overexpression of alkaline phosphatase in infected target bacterial cells. Detection of overexpression of alkaline phosphatase activity using a precipitate forming fluorescence substrate enhanced the detection sensitivity compared to conventional soluble substrates. With fluorescence imaging and quantitative image analysis, 100 CFU/g of *Escherichia coli* could be detected within less than 6 hours. Further extending this concept, the research results demonstrated that a combination of precipitate-forming colorimetric substrate and engineered phage infection could enable visual detection of bacteria. Using this approach, the results demonstrate a simple visual

assay for detecting 10 CFU of *E. coli* in 1 ml of coconut water and 10^2 CFU of *E. coli* on 1 g of baby spinach leaves without isolation of bacteria from the food sample.

Phage-bacterial interactions are known to induce lysis of bacteria cells and thus influence bacterial cell morphology. These changes in the morphology of bacterial cells induced by specific phage-induced-lysis were detected by fluorescence imaging, and a quantitative image analysis approach was developed to indicate the presence of target bacteria in food samples. This approach detected *E. coli* at 10 CFU/ml within 8 hours in both laboratory-based cell culture medium and complex matrices. Further building on this approach, we also focused on the detection of phage amplification as an indicator of the presence of target bacteria in food systems. Phage progenies generated after phage infection and lysis bacterial cell lysis were observed by fluorescence staining, and the number of phage particles was analyzed by image analysis. This approach enables the detection of 10 CFU/ml of *E. coli* in coconut water and simulated spinach wash water within 8 hours.

Overall, this research demonstrates simple and rapid bacteriophage-based bacteria detection approaches that can be applied to detect the bacterial pathogen in complex matrices. With low-cost setup and easy-to-perform procedures, these detection methods can be beneficial in facilities with limited access to some advanced instruments. Further development of these approaches can lead to the invention of highly efficient rapid bacterial detection devices that can be applied to detect bacteria in real food samples and industrial food facilities.

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Introduction and Literature Review

1.1. Foodborne illness outbreaks

Foodborne diseases have greatly impacted public health and the economy worldwide including in the United States. Foodborne pathogens account for over 15.5 billion U.S. dollars in the economic burden on the U.S. public health each year (Hoffmann et al., 2015). In the United States, it is estimated that there are 9.4 million episodes of foodborne illness, 55,961 hospitalizations, and 1,351 deaths caused by foodborne pathogens annually (Scallan et al., 2011). The top five foodborne pathogens that contribute to the illnesses that result in hospitalization are Salmonella (35%), Norovirus (26%), Campylobacter spp. (15%), Toxoplasma gondii (8%), and STEC *E. coli* O157 (4%) (Scallan et al., 2011). This information indicates that contamination of food products with pathogenic bacteria is responsible for 54% of all foodborne illnesses and hospitalizations. Since pathogenic bacteria are considered a leading cause of foodborne illnesses, limiting the contamination of pathogenic bacteria in food/agricultural products and facilities is a vital approach to ensuring food safety.

1.1.1. Source of foodborne bacterial contamination

Foods can be considered as an ideal environment for microorganisms to grow as foods are rich in nutrients and often have optimal pH and water activity ranges that promote the growth of microorganisms (Reynolds, 1991). Consequently, once microbial contamination occurs, microbes tend to proliferate in the foods. If the microbes are pathogenic, consumption of the contaminated foods will result in foodborne diseases.

The sources of foodborne bacterial pathogen contamination are numerous depending on the type of food product. One of the most common sources of microbial contamination is animals. Foods of animal origins, like meat and dairy products, possess a high risk of contamination by microbes from animals. Farm animals such as cattle and poultries are major sources of pathogens including *Campylobacter* spp., *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes* (Heredia and García, 2018). Contamination of meat products could occur during the slaughter process when carcasses come into contact with bacterial pathogens that reside in the animal's digestive organs, feet, or hides. Poultry products are sensitive to *Salmonella* contamination which can be introduced to the food products from contaminated hands, gloves, or processing facility. Poor sanitation of the equipment in dairy production facilities can lead to contamination of dairy products by pathogenic bacteria such as *Listeria monocytogenes* (Reynolds, 1991).

Fresh produce like leafy green can be contaminated in pre- and post-harvest environments. Since fresh produce is commonly consumed raw, the contaminating microbes can persist in fresh produce. During cultivation, produce can be contaminated by contact with amended soil containing manure which is a common source of pathogens (Machado-moreira et al., 2019). The major source of pathogen contamination in fresh produce is irrigation water with poor sanitation which has been exposed to livestock or wild animals (Steele and Odumeru, 2004). For post-harvesting, the washing process can be problematic if the wash water is not properly sanitized. With contaminated wash water, the sanitation efficiency is limited leading to a high risk of cross-contamination (Murray et al., 2017).

1.1.2. Preventive policy

The rise in the cases of foodborne illness outbreaks in the United States caused significant hospitalizations, deaths, and economic losses. To alleviate it, the FDA Food Safety Modernization Act (FSMA) was implemented in 2011 with the goal to ensure the safety of the food products by focusing on preventing pathogen contaminations instead of responding to the foodborne illness incidents (FDA, 2017). To compile with FSMA, the food manufacturing sectors must implement the new regulations including monitoring and record-keeping of agricultural water treatment, soil amendments, and equipment sanitation and maintenance (FDA, 2017; Boys et al., 2015).

In 2013, FDA has released the regulation entitled 'Standards for Growing, Harvesting, Packing and Holding Produce for Human Consumption' as a science-based standard for the safety of growing, harvesting, packing, and holding produce on farms. These FSMA regulations are applied to fresh produce that is consumed raw with minimal sanitation process (FDA, 2013). The goal of these regulations is to minimize foodborne microbes that are associated with the illnesses caused by the consumption of fresh produce. Agricultural water is one of the main focuses of microbial quality control. The growers must establish a Microbial Water Quality Profile (MWQP) for agricultural water from each source based on the levels of generic *E. coli*. The rule requires no detectable generic *E. coli* in 100 mL of agricultural water used for irrigation, water applied directly to food-contact surfaces, or water used for hand-washing (Rock et al., 2019). The detection of *E. coli* in water can be conducted by using one of the methods listed by the FDA (FDA, 2014).

1.2. Bacteria detection methods

1.2.1. Culture-based methods

Culture-based methods are considered the 'Gold Standard' for bacterial detection. The detection relies on the ability of bacteria to grow on synthetic laboratory culture media. The culture-based method that is being used for estimation of the bacterial population in food samples is aerobic plate count (APC) which is also known as standard plate count (SPC). APC is performed using non-selective media such as tryptic soy agar. The result obtained from this method doesn't reflect the total number of microbes in the samples but indicates the number of microbes that grow aerobically at mesophilic temperatures (Gracias and McKillip, 2004; Mendonca et al., 2020).

In order to detect specific species of bacteria, there are three approaches of bacterial culture design that allow the isolation of specific bacterial species. The first approach is an elective culture which is when using culture media or culture conditions that promote the growth of the target bacteria without suppressing non-target bacteria. Another is to culture bacteria using selective conditions by using culturing conditions or media supplemented with reagents that inhibit the growth of non-target bacteria such as antibiotics. Selective culture may have negative effects on the growth of target bacteria which can lead to false-negative results. Lastly, the differential approach allows differentiation of colonies from different species by the integration with the compound supplemented in the media. In many cases, more than one culture approaches are adopted for the detection of specific bacteria (Betts and Blackburn, 2009). For example, MacConkey agar isolates Gram-negative lactose-fermenting bacteria by inhibiting the growth of Gram-positive using bile salts allowing the growth of only Gram-negative bacteria. For Gram-negative that ferment lactose, after fermentation of lactose, lactic acid will be generated causing a decrease in pH value and resulting in pink-red colonies (Flournoy et al., 1990).

The culture-based method for bacterial detection is relatively inexpensive, simple, and only living cells are detected. However, bacterial cells in the viable-but-not-culturable state may not be detected using this method which may cause false-negative results. Furthermore, the conventional culture-based method is time-consuming, labor-intensive, and not specific at a strain level (Benoit and Donahue, 2003; Kim and Kim, 2021).

1.2.2. Molecular-based method

Molecular-based methods are bacterial detection approaches that rely on the detection of the amplification of the specific DNA sequence of the target bacteria; therefore, these methods provide highly specific detection at the strain level. The DNA amplification is usually based on an enzymatic reaction that results in an exponential increase in the DNA copies within a few hours, which makes it a rapid detection approach (Betts and Blackburn, 2009).

1.2.2.1. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction or PCR is an enzymatic reaction that results in the multiplication of a target short DNA sequence. The reaction requires the DNA template, which is bacterial DNAs extracted from the sample, a set of oligonucleotide primers that are specific to hybridization on the DNA templates, Taq DNA polymerase, which is a temperature-resistant enzyme, and deoxynucleotide triphosphates which are the building block for DNA amplicons (Betts and Blackburn, 2009; Tamerat and Muktar, 2016). The PCR reaction consists of three different steps depending on the temperature: Denaturation, Annealing, and Elongation. Denaturation is when the double-stranded DNAs start to separate, which occurs at a high temperature of around 95°C. At the lower temperature of around 50°C, the reaction undergoes annealing, when the primers selectively hybridize to the complementary sequence on the single-

stranded DNA templates. Elongation happens when the temperature is around 72°C. In this step, with the presence of the DNA polymerase, the replication of the target DNA sequence will occur. These three steps occur repetitively. After each cycle, the target DNA doubles, and after around 30 cycles, a million copies of the target DNA fragment are obtained (Viljoen et al., 2005). For the conventional PCR method, the amplified DNA will be detected by gel electrophoresis.

Several PCR-based detection methods have been developed to improve detection sensitivity and rapidity. One of the most commonly used PCR methods in industries is Real-time PCR where, instead of detecting the amplified target DNA by gel electrophoresis, fluorescent probes such as DNA binding dyes are added to the reaction to allow real-time monitoring of the DNA products. Besides using DNA binding dyes, Taqman probe can also be utilized in real-time PCR (Valasek and Repa, 2022). The Taqman probe is an oligonucleotide sequence that is complementary to the template DNA sequence between the forward and reverse primers. The probe consists of a fluorophore reporter attached to the 5' end emitting fluorescence signal which is absorbed by the fluorophore quencher at the 3' end. Once the complementary DNA is synthesized, the Taqman probe will be displaced and cleaved by the DNA polymerase resulting in a measurable fluorescence signal from the reporter probe as the quencher is no longer in close proximity (Butler, 2012). To detect the fluorescence signal, a real-time PCR instrument that is able to provide excitation energy and detect the emission energy at desired wavelength is required (Valasek and Repa, 2022). Real-time PCR has been widely used for foodborne pathogen detection due to its specificity and rapidity over the conventional culture-based methods; however, concentration or isolation of bacteria may be required for better sensitivity (Fukushima et al., 2007; Yoon et al., 2018; Kim and Oh, 2019, 2020; Shahid et al., 2021). In case of multiple strains of pathogenic bacteria are required to be tested, multiple sets of primers complementary to different

DNA sequences from different species of bacteria can be used in the PCR reaction. This detection approach is called 'Multiplex PCR' in which multiple DNA sequences can be amplified in a single reaction; therefore, multiple strains can be detected simultaneously (Markoulatos et al., 2002). Multiplex PCR has been reported to successfully detect multiple foodborne pathogens in realistic food samples (Li and Mustapha, 2004; Li et al., 2005; Wang et al., 2007a; Fratamico and Debroy, 2010; Park et al., 2017; Wei et al., 2018). Despite the high selectivity and rapidity of the PCR-based methods, the thermocycling steps required to complete the PCR reaction may limit the usage of PCR in some remote areas with no access to the instrument.

1.2.2.2. Loop-Mediated Amplification (LAMP)

LAMP is an isothermal DNA amplification and detection technique that offers very rapid and sensitive DNA detection as an amplification of a few molecules of target DNA to 10^9-10^{10} copies can be obtained within 10-15 minutes. At the reaction temperature of 60-65 °C, this method utilizes DNA polymerase with high displacement strand activity with a set of six primers including forward inner primer, backward inner primer, forward primer, and backward primer. This method recognizes 6-8 distinct target sequences which makes it a very selective detection approach compared to the PCR method (Notomi et al., 2000; Gadkar et al., 2018). The LAMP technique has been employed to detect foodborne pathogens such as *Escherichia coli* (Wang et al., 2012; Stratakos et al., 2016; Xia et al., 2022), *Salmonella spp.* (Chen et al., 2021b; Kreitlow et al., 2021; Shang et al., 2021; Chen, 2022), and *Listeria monocytogenes* (Roumani et al., 2021; Busch et al., 2022; Lee et al., 2022; Srisawat, 2022). Despite its high rapidity and selectivity, this method suffers from the complication of primers design. In addition, multiplex detection can be challenging due to multiple sets of primers (Wong et al., 2017).

1.2.2.3. Recombinase Polymerase Amplification (RPA)

RPA is another DNA detection approach based on the amplification of target DNA. This method utilized the isothermal recombinase-driven primers targeting template material with strand-displacement DNA synthesis (Piepenburg et al., 2006; Zaghloul and El-Shahat, 2014). RPA requires only two primers for its amplification reaction; therefore, the primer design of this detection method is relatively simpler than those of the LAMP assay. RPA is also more suitable for developing multiplex detection due to fewer primers required. RPA also provides rapid detection with a turnaround time shorter than 30 minutes (Magri, 2018). RPA allows exponential DNA amplification with no need for pretreatment of the samples. Its reactions are sensitive, specific, and rapid and operate at constant low temperature. Several studies reported the detection of foodborne pathogens using RPA (Choi et al., 2016; Kim and Lee, 2017; Du and Zang, 2018; Li et al., 2019; Zhao et al., 2021, 2022). The disadvantages of RPA are that the cost of the kits can be expensive, and the modification of the reaction can be limited because the RPA kits are sold only by one company (Magri, 2018).

1.2.3. Immunological-based method

Immunological-based detection of bacteria depends on the specific affinity between antigens and antibodies, which allows the detection of very small quantities of protein, peptides, hormones, or antibodies in a fluid sample (Gan and Patel, 2016). There are numerous immunological-based methods that have been used for foodborne pathogen detection including Enzyme-linked immunosorbent assay or ELISA. This method employs enzyme-labeled antibodies (or antigens) to detect analytes such as viruses, microbial cells, or microbial toxins, by specific binding. The detection efficiency depends on the antigen-antibody complex specificity and affinity. After specific binding, the signal can be detected by enzymatic activities with substrates

which provide colorimetric or fluorescence detection (Butler, 2008; Gan and Patel, 2016). This detection approach has been developed for the detection of pathogenic bacteria such as *E. coli* (Wang et al., 2007b; Shih et al., 2015; Pang et al., 2018), *Bacillus cereus* (Zhu et al., 2016; Zeighami et al., 2020), and *Salmonella* (Di Febo et al., 2019; Gu et al., 2022).

The immunological-based methods are rapid, low-cost, and convenient compared to the culture-based and PCR-based methods. However, on a practical level, this detection approach suffers from an inability to distinguish viable from non-viable bacterial cells. Moreover, small sample volume capacity may result in low sensitivity, and cross-reactivity of polyclonal antibodies may result in reduced detection specificity (Ivnitski et al., 1999; Shen et al., 2014).

1.3. Bacteriophage for bacteria detection

Bacteriophages or phages are viruses that specifically infect viable target bacteria as hosts and can only replicate inside the living host cells. Phages can be highly specific for infecting certain strains within a single species of bacteria. Most of the all-known phages belong to the *Caudovirales* order sharing the common characteristic that their genomes are composed of double-stranded DNA stored in an icosahedral capsid. The tails and fibers proteins required for recognition of the target host are attached to the capsid (Ackermann, 2007; Paczesny et al., 2020). Phages in the *Caudovirales* order can be divided into three families depending on their tails structure. The *Siphoviridae* family, such as lambda phage, have long flexible and non-contractile tails to reach through the bacterial cell wall. Phages in the *Myoviridae* family have long and contractile tails with additional outer tail sheath such as T4 phage. *Podoviridae*, such as T7 phage, have short tails and includes enzymes that facilitate them to lyse the cell wall of bacteria. Phages that have RNA or single-stranded as their genetic material, which are less common, belong to the *Caudovirales* order (Harper et al., 2014; Richter et al., 2018).

Bacteriophages are considered a useful tool for bacteria detection due to their ability to selectively infect specific strains of bacteria. The specificity of phage infection depends on the adsorption step in which phages recognize their hosts by specific interaction between the phage tail fiber proteins and receptors on the bacterial cell surfaces such as lipopolysaccharides or proteins (Rakhuba et al., 2010; Chaturongakul and Ounjai, 2014). After phages successfully attach to the host cell, their genetic materials will be transferred through the tail tube into the bacterial cell's cytoplasm (Harada et al., 2018). Phages undergo two different life cycles: lytic and lysogenic. For the lytic life cycle, phages use bacterial cell machinery to produce hundreds of progeny phages. Phage-encoded proteins will lyse bacterial cell walls and eventually release the progeny phages to the environment (Doss et al., 2017). For the lysogenic life cycle, the phage's genetic material integrates with the host's genome and replicates as the bacterial DNA replicates. Phages in this stage are called prophages meaning that they are not virulent. Once the hosts are in stress conditions, the prophages are excised and induce bacteria to undergo the lytic cycle and produce progeny phages (Richter et al., 2018; Stone et al., 2019).

Both lytic and lysogenic phages can be utilized as a tool for bacteria detection applications. One of the criteria for phage selection is lysis time. Lytic phages lyse bacterial cells and generate progeny phages within one hour of incubation; therefore, they are not an ideal option for capturing cells for isolation purposes (Paczesny et al., 2020). On the other hand, lytic phages, in a timely manner, reproduce their progenies and lyse bacterial cells, which can be detected as signals indicating the presence of target bacteria.

Bacteriophage has been utilized for bacteria detection due to its ability to selectively infect target bacteria at the strain level. In the case of lytic phages, bacterial cells are lysed and hundreds-fold of progeny phages are produced within a short period of time, which facilitates rapid detection.

There are several approaches developed for bacteriophage-based bacteria detections. The most conventional approaches are to use the wild-type phages as they are abundant in nature (Clokie et al., 2011). To improve the ability of bacteriophage-based detection, genetically engineered phages have been employed. According to the simplicity of bacteriophage's genetic material and the fact that its genetic material will be delivered to host bacteria followed by replication, transcription, and translation of genes using host cells' machinery, modification of bacteriophage DNA to harbor beneficial genes that generate signal inside the target bacterial cells is potentially practical.

1.3.1. Wild-type bacteriophages

1.3.1.1. Detection of phage-induced bacterial cell lysis

The most straightforward approach is to use bacteriophage as the lysing agent. After bacteriophages replicate and amplify inside the host cells, eventually, the cells will be lysed and not only progeny phages will be released but also bacterial intracellular materials. Detection of the released bacterial cell components can indicate the presence of the target bacteria. Several compounds have been proposed as indicators of cell lysis. Adenylate kinase (AK) is an essential enzyme of any cell. This enzyme catalyzes the phosphorylation of adenosine diphosphate (ADP) to Adenosine-5-triphosphate (ATP). After phage-induced cell lysis, the release of AK can be detected by adding excess ADP and detection of the catalytic product, ATP, by adding luciferase/luciferin resulting in a measurable luminescence signal (Blasco et al., 1998). ATP is another intracellular component that can be monitored directly as an indicator for bacteriophage lysis using a bioluminescent assay (Minikh et al., 2010). Chen et al., 2015 reported the detection approach involving immobilized T7 bacteriophage lysing and detection of the released intracellular β-galactosidase using a colorimetric assay (Chen et al., 2015). The release of β-galactosidase can also be detected amperometrically by using a p-aminophenyl-*β*-D-

galactopyranoside (β -PAPG) as a substrate (Neufeld et al., 2003). The release of bacterial cell components after phage-induced cell lysis also causes a change in the solution conductivity, thus conductivity variations can be measured as a signal of the presence of target bacteria (Mortari et al., 2015).

However, this detection approach may not be ideal for the detection of bacteria in complex matrices with the presence of multiple strains of non-target bacteria as the release of the endogenic enzyme might be the result of lysis of the non-target bacteria. Moreover, this approach may not be ideal for food materials where ATP from animal and plant cells may be present. These incidents can lead to false-positive results.

1.3.1.2. Detection of phage amplification

Another consequence of bacterial cell lysis due to phage infection is the release of the progeny phages which is indicative of the presence of target bacteria. After a complete lytic cycle, each phage-infected cell releases 10-100 progeny phages depending on the type of phage. The amplification of phage is relatively more rapid than the reproduction of bacteria itself since one lytic cycle of phage completes within 20-30 minutes. Therefore, in the case of the samples with a low number of bacteria, amplification of bacteriophage following infection is considered indirect enrichment which increases the detection signal within a short time allowing increased detection sensitivity. The conventional culture-based phage particle enumeration is the plaque counting assay where phages are grown on the agar media in the lawn of the target bacteria and form clear spots which can be visualized after overnight incubation. De Siqueira et al., 2003 reported the detection of phage P22 amplification as an indicator for the presence of *Salmonella* in chicken breast by plaque assay (De Siqueira et al., 2003). FASTPlaqueTB is a commercial technology by Biotec Laboratories Ltd., Ipswich, United Kingdom using phage amplification-based for *M.*

tuberculosis detection in respiratory specimens. Mycobacteriophages were used to infect *M. tuberculosis* in clinical samples and the number of phages was enumerated using plaque assay. This method allows quantitative detection of the target bacteria within a detection time of 24 – 48 hours (Albert et al., 2001). Even though the plaque counting assay yields very reliable results, overnight incubation extends the detection time. Molecular-based methods like real-time PCR have been deployed to detect DNA of amplified bacteriophage after infection and lysis of target bacteria (Tolba et al., 2008; Kutin et al., 2009; Garrido-Maestu et al., 2019; Malagon et al., 2020).

For bacteriophage amplification assay, only the progeny phages resulting from phage-infected bacteria indicate target bacteria contamination. Therefore, it is important to differentiate between parental phages and progeny phages. After the phage infection where phage DNA has been injected into the host cell, destroying free phage particles using a viricidal laser ensures that only the phages released from the infected target bacteria will be enumerated (Rodrigues et al., 2006). Another approach that can improve the detection sensitivity is to isolate the phage by specific immunomagnetic separation. This approach is reported by Martelet et al., 2015 that *E. coli* phage, T4, can be captured by superparamagnetic nanoparticles coated with anti-T4 antibodies before quantification using liquid chromatography coupled to targeted mass spectrometry (Martelet et al., 2015). The amplified phages can also be detected by lateral flow immunochromatography allowing rapid detection of *B. anthracis* within 2 hours (Cox et al., 2015). The study by Medo et al., 2018 demonstrated the detection of *E. coli* based on MS2 phage amplification using the immunological technique. The amplified phages were captured by magnetic beads coated with antibody before adding the biotinylated secondary antibody followed by streptavidin-phycoerythrin conjugate as the tracer molecule. This method was reported to be able to detect 10^2 cells/mL of *E. coli* after a 3-hour incubation (Mido et al., 2018).

Despite that detection of the phage amplification is a straightforward approach for bacteria detection, methods used for quantification of phage particles mentioned above still possess some drawbacks. The culture-based plaque counting methods are time-consuming and labor-intensive. Molecular-based methods such as PCR require expensive reagents and well-trained personnel. For immunological-based methods, the detection sensitivity may be affected by the presence of complex food samples. Therefore, methods for quantification of phage particles that are simple and inexpensive will improve the detection efficiency and can be further applied for the detection of bacteria in complex samples.

1.3.2. Genetically engineered bacteriophages

Another common approach in phage-based bacteria detection is to develop reporter phages, which are phages that have been genetically engineered to carry, in their genome, reporter genes that once expressed will yield measurable signals. One of the most commonly used reporter genes is bacterial luciferase (*lux*) responsible for protein products for bioluminescence (Loessner et al., 1997). According to the study done by Kim et al., 2014, a bacterial *luxCDABE* operon was inserted into the genome of a *Salmonella* Typhimurium phage SPC32H. Upon infection with the target bacteria, the reporter phage rapidly and sensitively emits bioluminescence allowing the detection of 20 CFU/mL of *S.* Typhimurium within 2 hours (Kim et al., 2014). The *E. coli* O157:H7-infected bacteriophage phiV10 has also been genetically modified to contain lux operon, *luxCDABE,* and used for detection of *E. coli* O157:H7 in romaine lettuce, apple juice, and ground beef by detecting bioluminescence using a luminometer (Kim et al., 2017). T7 phage was successfully genetically modified to carry *nluc* gene for expression of the new commercial nanoluciferase enzyme (NanoLuc) and *cbm* gene encoding cellulose-binding module (CBM). Upon infection with the target *E. coli* cells, NanoLuc fused with CBM were overexpressed. CBM allows immobilization

of the reporter enzyme onto the crystalline cellulosic substrate which improves the sensitivity of the bioluminescence detection (Hinkley et al., 2018a; Zurier et al., 2020). Meile et al., 2020 engineered *Listeria* phage A511 to have a gene for NanoLuc expression. This reporter phage allows the detection of 1 CFU/25g of *L. monocytogenes* in artificially contaminated milk, cold cuts, and lettuce within less than 24 h (Meile et al., 2020).

Another commonly used reporter gene is lacZ encoding β-galactosidase. *E. coli* infected with T7 bacteriophage with lacZ operon will overexpress β-galactosidase which can be detected using bioluminescent and colorimetric substrates (Burnham et al., 2014; Chen et al., 2017a, 2017b). Not only bioluminescent and colorimetric substrates were used to detect the overexpressed β-galactosidase, but also fluorescence substrates such as 4-methylumbelliferyl-β-D-galactopyranoside (Chen et al., 2021a). Instead of engineering the phage to induce host cells to overexpress β-galactosidase, phages can also be modified to display the enzyme on the capsid protein (Xu et al., 2022).

The gene that encodes alkaline phosphatase is another reporter gene that has been inserted into the T7 phage genome to induce overexpression of alkaline phosphatase in the *E. coli* cells (T7-ALP). The detection of 10^3 CFU/ml of *E. coli* was achieved within 6 hours using a chemiluminescent substrate, and 10^4 CFU/mL of *E. coli* can be detected within 7.5 hours using a colorimetric substrate (Alcaine et al., 2015). The lateral flow assays platform was applied to improve the detection sensitivity. The overexpressed ALP resulting from T7-ALP infection was captured on the test strip with anti-ALP polyclonal antibodies before detection with the 5-bromo-4-chloro-3′-indolyphosphate and nitro-blue tetrazolium (BCIP/NBT) substrate which yields dark purple precipitated product (Alcaine et al., 2016). This engineered phage for alkaline phosphatase expression was also applied to quantitatively detect *E. coli* in drinking water. By fusing the reporter

enzymes gene to gene encoding carbohydrate-binding modules specific to cellulose, the overexpress enzyme released after bacterial cells lysis will be immobilized on the cellulose filter. After adding BCIP/NBT substrate, the location of the *E. coli* cells can be visually observed and the quantification of *E. coli* can be obtained. This method was able to detect 100 CFU/mL of *E. coli* in drinking water within 10.5 hours (Hinkley et al., 2018b). Besides capturing the released reporter enzyme using filtration, the ALP with CBM fusion tag enables immobilization of the reporter to magnetic cellulose particles allowing the concentration of the reporter enzyme (Singh et al., 2019).

Even though these engineered phage approaches were reported to have high sensitivity and specificity, applying these methods to the detection of bacteria in complex matrices such as food-related samples while maintaining their simplicity and sensitivity can be challenging due to the additional steps for bacterial isolation, and the high background noise from food components. Therefore, the development of detection methods using reporter phages that can be applied to detect bacteria in food samples without complicated steps of bacteria isolation or costly devices is needed.

1.4. Overview of the dissertation study

1.4.1. Overall hypothesis of the research

The overall hypothesis of the research is the combination of phages and imaging approaches will enable rapid detection of bacteria in a complex food matrix. The unique strategies in this research will focus on applying fluorescence and colorimetric substrates with point-of-use imaging methods to enhance the detection signal of exogenously expressed enzymes induced by the infection of target bacteria by genetically modified phages. Complementary to the genetically

modified phages, the research will also develop unique imaging approaches to characterize and quantify changes in cell morphology and phage amplification number to enhance the detection of target bacteria in the presence of a food matrix. The specific objectives of the research are:

1. Enhancing the sensitivity of bacteria detection using a combination of over-expressed alkaline phosphatase induced by genetically engineered bacteriophage (T7-ALP) infection and enzymatic assay using alkaline phosphatase substrates that form precipitates fluorescence product, fluoresce imaging, and quantitative image analysis
2. Development of a colorimetric assay for a simple and rapid bacteria detection using genetically engineered bacteriophage (T7-ALP) and alkaline phosphatase substrate that yield precipitated colorimetric products
3. Development of bacteria detection methods detecting the changes in bacterial cell morphology induced by bacteriophage T7 infection and lysis using fluorescence imaging and quantitative image analysis.
4. Detection of bacteria in complex food matrices based on the amplification of phages upon infection of the target bacteria using fluorescence imaging and image analysis for quantification of phages in comparison to the conventional phage quantification method.

1.4.2. Expected impact of the research

The expected impact of this research would be to improve the sensitivity of the detection of bacteria compared to the current methods as well as to reduce the sample preparation steps and time required for bacterial detection in food systems.

1.4.3. Study outline

This dissertation consists of six chapters. The first chapter is the introduction providing background information on the importance of bacteria detection, conventional bacteria detection methods, their advantages and disadvantages, and the development of bacteriophage-based bacteria detections. Chapters 2 and 3 present the development of bacteria detection assays using engineered phage T7-ALP to induce alkaline phosphatase overexpression and detection of the enzyme by substrate yielding precipitate fluorescence (Chapter 2) and colorimetric (Chapter 3) products. In Chapter 4, bacteria detection based on cell morphology change due to phage-induced bacteria lysis using image analysis was demonstrated. Chapter 5 presents a bacteria detection assay based on the detection of bacteriophage amplification as an indicator for the presence of the target bacteria by fluorescence imaging and image analysis for bacteriophage particle enumeration. Chapter 6 provides a summary of the main outcome of this study and the potential for future research.

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CHAPTER 2:

Rapid detection of *Escherichia coli* in beverages using genetically engineered bacteriophage T7 coupled with fluorescence imaging and image analysis[1]

Abstract

Foodborne illness due to bacterial contamination is a significant issue impacting public health that demands new technology which is practical to implement by food industry. Detection of bacteria in food products and production facilities is a crucial strategy supporting food safety assessments. Bacteriophages were investigated as a tool for bacterial detection due to their ability to infect specific strain of host bacteria in order to improve sensitivity, specificity, and rapidity of bacterial detection. The results of this investigation reveal a novel method for rapid detection. The method employs a genetically engineered bacteriophage, phage T7-ALP, which expresses alkaline phosphatase. Upon infection of *Escherichia coli*, overexpression of alkaline phosphatase provides an opportunity for rapid sensitive detection of a signal indicative of bacterial presence in model beverages samples as low as 100 bacteria per gram. The method employs a fluorescent precipitated substrate, ELF-97, as a substrate for alkaline phosphatase activity coupled with fluorescence imaging and image analysis allowing single-cell imaging results in high detection sensitivity. The method is easily completed within less than 6 hours enabling it to be deployed within most large industrial food processing facilities that have routine 8-hour operational shifts.

Keyword:

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Engineered bacteriophage, *Escherichia coli* BL21, Alkaline phosphatase, ELF-97, Fluorescent microscope, Image analysis

2.1. Introduction

Over the past decades, foodborne disease outbreaks affect millions of people worldwide by causing burdens on public health and significant hindrance in socio-economic development (WHO, 2015). Each year, in the United States, approximately 9.4 million cases of foodborne illness were reported causing over $15.5 billion in economic burdens (Scallan et al., 2011; Hoffmann et al., 2015), including 64% of the foodborne-related deaths were caused by infection of bacteria (Scallan et al., 2011). Given that it is well-documented that the leading causes of foodborne illnesses are due to contamination by bacterial pathogens, detection of these microbes in food is a critical step that ensures the safety of food and beverages products before distribution to the consumers. Among all of these pathogens, *Escherichia coli* is probably one of the most prevalent pathogens during the past few years which resulted in foodborne illness outbreaks. According to the Centers for Disease Control and Prevention (CDC) surveillance of foodborne outbreaks in the U.S., several food products have been reported associated with *E. coli* contamination such as romaine lettuce, ground beef, and beverages including coconut water and apple juice (Marder et al., 2017). Therefore, the detection of *E. coli* that is applicable in a variety of food matrices is highly crucial.

Culture-based detection methods remain the "gold standard" for pathogen detection in food and animal feed because they provide highly accurate results; however, the major drawback of this method is that they require 3-5 days to complete and are relatively expensive for total material and labor costs (Koyuncu and Haggblom, 2009; Kralik and Ricchi, 2017). Economic costs of extended production storage to allow testing and loss of food quality – for short shelf-life foods – further negatively impact food manufacturers. Molecular-based methods, such as those employing polymerase chain reaction (PCR) provide alternative opportunities for rapid pathogen detection of

bacterial pathogens. Even though PCR offers the results with only a few hours of bacteria enrichment, the critical drawback of detection methods relying on PCR is that it is incapable of differentiating between viable and dead cells which leads to false-positive results (Chapela et al., 2015; Kralik and Ricchi, 2017). Economically, PCR-based methods remain accessible only to large food manufacturers that have the capacity to support the technical application of this approach and the relatively high costs. On a technical level, wide use of PCR-based methods suffers from the complexity of food matrices, many of which have been demonstrated to harbor molecules that are inhibitors of PCR, resulting in decreased sensitivity or false-negative results (Rossen et al., 1992; Schrader et al., 2012). Immunological-based methods, including variations of enzyme-linked immunosorbent assays (ELISAs), have been developed in order to provide a more rapid, economical, and simple approach compared to the culture-based and PCR-based methods; however, on a practical level, ELISAs suffer from an inability to distinguish live from dead bacteria, from low sensitivity due to small sample volume capacity, and from low specificity due to cross-reactivity of polyclonal antibodies (Ivnitski et al., 1999; Shen et al., 2014). The limitations of these foodborne pathogen detection methods provide an incentive for continued research and development into innovative novel approaches for rapid detection that have high sensitivity and specificity to detect bacterial foodborne pathogens, and can be widely adapted for use by a diversity of large and small food manufactures.

Bacteriophages or phages have drawn the attention of many researchers in the field of pathogen detection due to their high specificity to bacterial host strains which enables them to be developed as a tool for the detection of the specific bacterial pathogens. Phages are also capable of self-replication and produce progeny phages within a short period of time, this allows amplification of the signal intensity resulting in a lower limit of detection without the requirement

for any overnight enrichment (Anany et al., 2017). One significant advantage of using phages for bacterial detection is the ability to distinguish between viable and dead cells. Since phage infection and replication only occur in living bacterial cells, bacteriophage-based detection methods inherently ensure that only viable cells are targeted (Hagens and Loessner, 2007). Several strategies have been employed to develop phage-based biosensors including detection of the released cell components due to phage lysis (Griffiths, 1996; Chang et al., 2002; Chen et al., 2015), detection of labeled phages after their specific attachment to bacterial hosts (Hennes et al., 1995; Yim et al., 2009), and detection of the progeny phage amplification using nucleic acid-based detection techniques (González-Gil et al., 1998; Reiman et al., 2007; Kutin et al., 2009).

Recently, genetically engineered phages have been created in order to provide special features that allow greater possibilities in developing novel bacterial biosensors. Among the most promising approaches is 'Reporter Phage', where wild-type phages are genetically modified to harbor reporter genes that can only be activated once phage-host infection occurs. The expression of reporter genes inside host cells yields a detectable signal indicative of the presence of target bacteria (Smartt et al., 2012; Singh et al., 2013). Several gene-based reporters have been widely studied and incorporated into the phage genome, such as *lux* locus for bioluminescence (Loessner et al., 1997; Kim et al., 2017), *lacZ* encoding β-galactosidase (Goodridge and Griffiths, 2002), and *gfp* encoding a green fluorescent protein (Oda et al., 2004; Piuri et al., 2009). Alcaine et al. (2015) developed genetically engineered phage T7 (phage T7-ALP) carrying *phoA* that results in over-expression of alkaline phosphatase after infection of *E. coli* (Alcaine et al., 2015). Phage T7-ALP provides a promising opportunity but background noise could be an issue since alkaline phosphatase is a common enzyme found in a vast array of organisms including bacteria, fungi, plants, and animals (McComb, R. B., Bowers, Jr., G. N., and Posen, 1979; Alcaine et al., 2015).

Moreover, the previous research on the detection of bacteriophage-mediated alkaline phosphatase production was based on enzymatic essay using substrates that yield soluble products which distribute homogeneously in the solution resulting in a diluted signal (Alcaine et al., 2015); therefore, the signal-to-noise ratio may be reduced in the presence of food.

In order to overcome this obstacle, instead of colorimetric and chemiluminescent soluble substrates, our research focused on heterogeneous detection using an alkaline phosphatase substrate 2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone (ELF-97) that, when hydrolyzed, gives a precipitated fluorescent product that localized at the site of reaction. Deposition of the precipitated ELF-97 product allows concentration of the signal which results in a higher signal-to-noise ratio and increased detection sensitivity. ELF-97 is a non-fluorescent water-soluble substrate that once cleaved with alkaline phosphatase will yield the product, ELF-97 alcohol, that precipitates at the site of the reaction (Huang et al., 1993; Telford et al., 1999; Duhamel et al., 2009). This outstanding feature of ELF-97 results in signal deposition allowing detection of enzyme activity at the single-cell level within a few minutes after the reaction (González-Gil et al., 1998). ELF-97 has been applied for detecting mRNA *in situ* hybridization (Paragas et al., 1997), cytological labeling and imaging of mammalian cells (Paragas et al., 2002), and detection of alkaline phosphatase activity in marine plankton at the single-cell level (González-Gil et al., 1998; Nedoma et al., 2003; Peacock and Kudela, 2012). To the best of our knowledge, none of the previous studies have investigated the application of ELF-97 for microbial detection approaches in food systems.

With the benefit of phage T7-ALP to induce alkaline phosphatase over-expression in *E. coli* and the potential of ELF-97 to endogenously detect a small amount of alkaline phosphatase enzyme, this research aims to develop a novel detection method for *E. coli* in authentic beverages

samples. With fluorescence imaging and image analysis, we hypothesized that this approach will provide a method able to detect low concentrations of *E. coli* in a variety of different beverage matrices within less than six hours.

2.2. Materials and Methods

2.2.1. Bacteriophage and bacterial strains

Engineered bacteriophage T7-ALP was kindly provided by Dr. Sam Nugen (Cornell University). Bacteriophage T7-ALP has been amplified by inoculation of phage 10^5 PFU/ml into 10^8 CFU/ml of log-phase *E. coli* BL21 followed by 15 minutes of incubation at 37°C for initial infection and 10 minutes of centrifugation at 16,100 × *g* to harvest infected bacteria. The bacteria pellet was resuspended in 15 ml of sterile tryptic soy broth (TSB, Sigma-Aldridge, St. Louis, MO, USA) before incubation at 37°C with 200 rpm constant shaking for further infection. After there was no visible turbidity, 3 ml of chloroform was added and the mixture was kept at 4°C overnight to lyse intact cells. To separate cell debris, the mixture was centrifuged at 16,100 × *g* for 10 minutes. The upper liquid phase which contains free phage was collected and centrifuged at 16,100 × *g* for 10 minutes. The supernatant was discarded and the phage pellet was resuspended in sterile phosphate buffer saline (Fisher Scientific, Pittsburg, CA, USA) and stored at 4°C. The titer of the phage stock was enumerated by a standard plaque counting assay.

The model bacterial strain for this research was *E. coli* BL21 (ATCC BAA-1025) obtained from American type culture collection. Two strains of bacteria were used as controls: *Listeria innocua* (ATCC 33090) kindly provided by Dr. Linda Harris (University of California, Davis), and *Pseudomonas fluorescens* (ATCC 13525) from American type culture collection. All bacterial strains were stored in TSB containing 15% (vol/vol) glycerol at -80°C. For short-term storage, the

glycerol stock of bacteria was streaked onto tryptic soy agar (Sigma-Aldridge, St. Louis, MO, USA) plates then incubated overnight at 37°C for *E. coli* and at 30°C for *Listeria innocua* and *Pseudomonas fluorescens*. The agar plates were then stored at 4°C for further experiment.

2.2.2. Beverage sample preparation

Coconut water (Vita coco, 100% coconut water) and apple juice (Signature select, 100% apple juice) were purchased from the local grocery store. Prior to the experiment, coconut water was filtered through a 0.22-micron syringe filter. To support the growth of bacteria and phage infection, TSB with double concentration was added to coconut water and apple juice at a ratio of 1:1. The pH of the apple juice-TSB mixture was adjusted to 7 using 1M tris before filtering through a 0.22-micron syringe filter.

2.2.3. Bacterial inoculation, enrichment, and phage infection

For overnight culture preparation, a single colony of bacteria from the agar plate was transferred to 10 ml of TSB and incubated at 37 °C with constant shaking at 200 rpm for 16 hours. For inoculum preparation, 1 ml of the overnight culture (10^9 CFU/ml) was centrifuged at 16,100 × g for 1 minute then the cell pellet was washed twice by resuspending in 1 ml of sterile phosphate buffer saline. The cell suspension was serial diluted and inoculated in 10 ml of TSB and beverages-TSB mixture; coconut water and apple juice, to the final concentration of 10^2 and 10^3 CFU/ml and incubated at 37 °C with constant shaking (200 rpm) for 4 hours. Bacteriophage T7-ALP was added to the mixture to the final concentration of 106 PFU/ml and incubated in the same condition for 15 and 30 minutes.

2.2.4. Filtration and optical detection of alkaline phosphatase activity

To capture the infected bacterial cells, all 10 ml of the mixtures were filtered through 0.22-micron white polycarbonate membrane discs with a 19 mm diameter (Nucleopore Polycarbonate Whatman). After filtration, the filters were stained with ELF-97 alkaline phosphatase substrate using ELF™ 97 Endogenous Phosphatase Detection Kit (Molecular Probes, Eugene, OR, USA). The reaction mixture was prepared by diluting the ELF-97 phosphatase substrate 1:20 in ELF-97 developing buffer (provided in the kit) and then spotted 20 µl of the reaction mixture on a microscopic slide. The filters were removed from a vacuum filtration system and put into the reaction mixture on the slides then another 20 µl of the reaction mixture was added onto the top of the filter and spread evenly on the filter surface using sterile pipette tips. The slides with filters were incubated at room temperature in the dark for 30 minutes. To stop the enzymatic reaction, the filters were removed from the slides and put on the cellulose filter paper saturated with 1% formaldehyde for 5 min. All filters were counterstained with red fluorescent nucleic acid stain SYTO 60 (Molecular Probes, Eugene, OR) to stain all bacterial cells present on the filter. The ELF-97-stained filters were placed on the microscopic slides pre-spotted with 10 µl of 20 µM SYTO 60. Another 10 µl SYTO 60 was dropped on top of the filters and covered with cover glasses then observed under the Leica TCS SP8 STED 3X confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a white light laser for excitation. ELF-97 was excited by 405 nm STED laser and the fluorescent emission was collected at 452 - 560 nm. SYTO 60 was excited at 647 nm white light laser and the fluorescent emission was collected at 654 - 752 nm. All images were taken with a 100 × oil-immersion objective (NA 1.4) with a laser power of 5%. For negative control, the experiment was performed as described earlier with *E. coli* BL21 without adding phage and *L. innocua* and *P. fluorescens*. Experiments for all conditions were performed in triplicates.

2.2.5. Image analysis for mean fluorescence intensity

Image analysis was performed using ImageJ (available for download at https://imagej.nih.gov/ij/ download.html). All images were binarized to have images of cells in black and the background in white. The intensity threshold for each image was set at 70 when the maximum intensity was 225. The mean density of each cell particle was obtained using ImageJ's particle analysis package. To eliminate the noise background signal, only particles with a size larger than 0.5 pixels were analyzed. The analysis of multiple images was repeated for each condition (N=15).

2.2.6. Alkaline phosphatase assay using FDP soluble fluorescent substrate

Fluorimetric detection of alkaline phosphatase was performed using Amplite™ Fluorimetric Alkaline Phosphatase Assay Kit (Green Fluorescence) (AAT Bioquest, Biomol, Hamburg, Germany). *E. coli* BL21 with concentrations of 10^2, 10^3, and 10^4 CFU/ml were inoculated in 1 ml of beverage samples-TSB mixture before incubation at 37°C with constant shaking (200 rpm) for 4 hours. For phage infection, 10^6 PFU/ml of T7-ALP phage was inoculated and then incubated under the same condition for 30 minutes. The mixtures were centrifuged (16,100 × *g*) for 10 minutes to separate the cell debris. Fifty microliters of the samples were mixed with the FDP substrate provided from the kit in a black flat-bottom 96-well plate. The fluorescence was measured using a microplate reader TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria) with excitation at 488 nm and emission at 520 nm. The experiments were performed in parallel with the blank which is 1 ml of beverage sample without *E. coli* and phage inoculation and negative controls of *E. coli* without phage infection.

2.2.7. Statistical analysis

All experiment was done in triplicate independently for each condition. For image analysis, the total number of 15 images were analyzed (N=15). The mean and standard deviation values were calculated within the samples in all cases. The Tukey's HSD test was performed on the mean intensity of the fluorescent images and the signal/noise ratio of the fluorometric assay of alkaline phosphatase using FDP substrate in order to define the limit of detection. All statistical analysis was done using the R software.

2.3. Results

2.3.1. Schematic diagram of bacterial detection methods based on T7-ALP phage infection

The protocols of *E. coli* detection based on the assay of phage-induced alkaline phosphatase activity using soluble and precipitated fluorescent substrates are shown in Figure 2.1 Both methods started with 4 hours of enrichment of *E. coli*. The initial concentrations of *E. coli* in both beverage samples were 10^2 CFU/ml and 10^3 CFU/ml. After 4 hours of enrichment, engineered phage T7-ALP was added to the sample allowing phage infection and expression of alkaline phosphatase. Both methods focus on the detection of phage-induced alkaline phosphatase activity. The first method involves filtration to capture bacterial cells on the filter and detection of phage-induced alkaline phosphatase using ELF-97 substrate which is cell-permeable and yields a bright precipitated fluorescent product that can be visualized under the fluorescent microscope. Another approach is based on using FDP alkaline phosphatase substrate to detect alkaline phosphatase activity. The soluble fluorescent product of FDP was detected by measuring the fluorescent signal using a microplate reader.

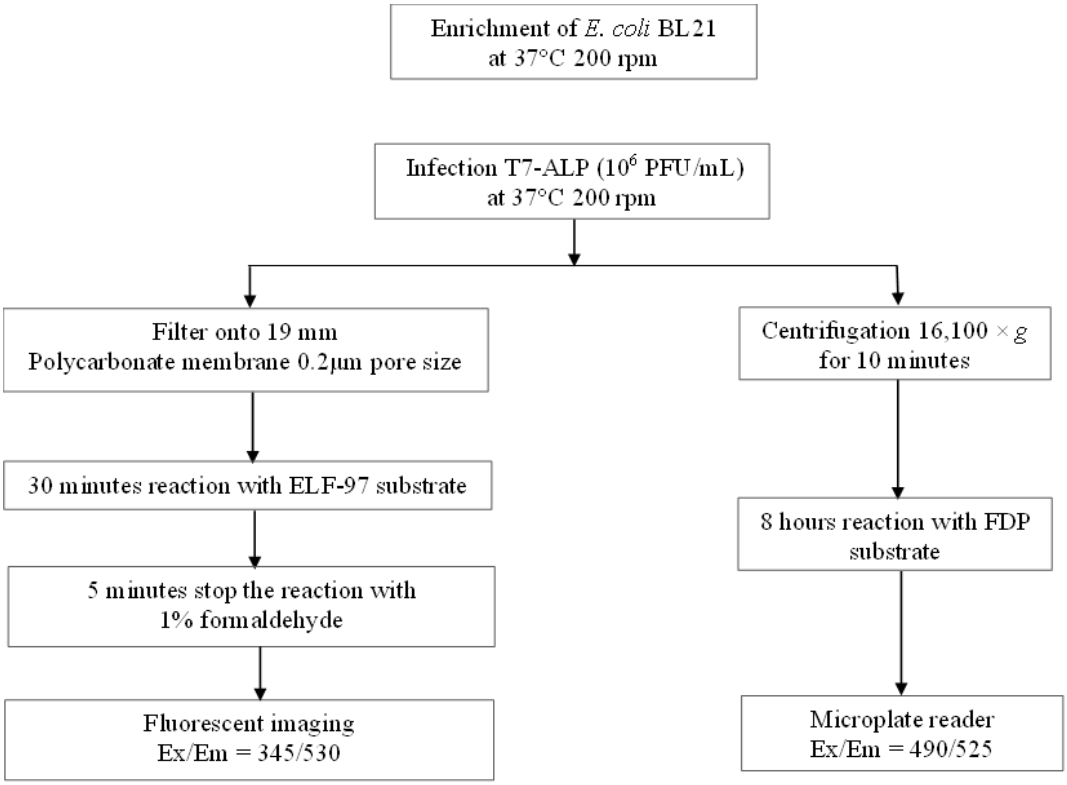

Figure 2.1. Schematic diagram of detection protocol based on alkaline phosphatase activity assay using soluble alkaline phosphatase substrate (FDP) and insoluble alkaline phosphatase substrate (ELF-97)

2.3.2. Optical detection of E. coli using ELF-97 fluorescent substrate in TSB

Before investigating the detection method in authentic beverage samples, detection of *E. coli* in TSB was first performed. In 10 mL of sterile TSB, 10^2 CFU/mL and 10^3 CFU/mL were inoculated and incubated at 37°C for 4 hours with constant shaking at 200 rpm. T7-ALP phage with the final concentration of 10^6 PFU/mL was added then the mixture was incubated under the same condition for 15 and 30 minutes. Ten milliliters of the mixture were filtrated onto a 0.2 µm white polycarbonate filter. *E. coli* BL21 in the same concentrations but without phage infection served as negative controls. Detection of alkaline phosphatase activity was performed by adding ELF-97 substrate directly onto bacterial cells captured on the filter. After counterstaining with SYTO™ 60, the filters were observed under a confocal fluorescent microscope.

Representative fluorescent images are illustrated in Figure 2.2 The total bacterial cells present on the filter appeared in red due to SYTO-60 red-fluorescent nucleic acid stain. Without phage infection, Figures 2.2a and d reveal that *E. coli* has no alkaline phosphatase activity after 4 hours of enrichment without phage infection at a concentration of 10^2 CFU/mL and 10^3 CFU/mL, respectively. Figures 2.2b and c represent the fluorescent images of *E. coli* cells after 15 and 30 minutes of infection by phage T7-ALP when the initial bacterial concentration was 10^2 CFU/ml. At both time points, at least one *E. coli* cell cluster per image was observed to have a fluorescent signal localized inside the cell showing that the cell was infected and alkaline phosphatase was produced but the cell was not yet lysed by the phage. Figures 2.2e and f represent images of *E. coli* cells after 15 and 30 minutes of infection by phage T7-ALP when the initial bacterial concentration was 10^3 CFU/ml. After 15 minutes of phage infection, few cells exhibit green fluorescence as shown in Figure 2.2e but after the infection continues to 30 minutes, a representative image shows an increase in the number of cells that exhibit green fluorescence as shown in Figure 2.2f.

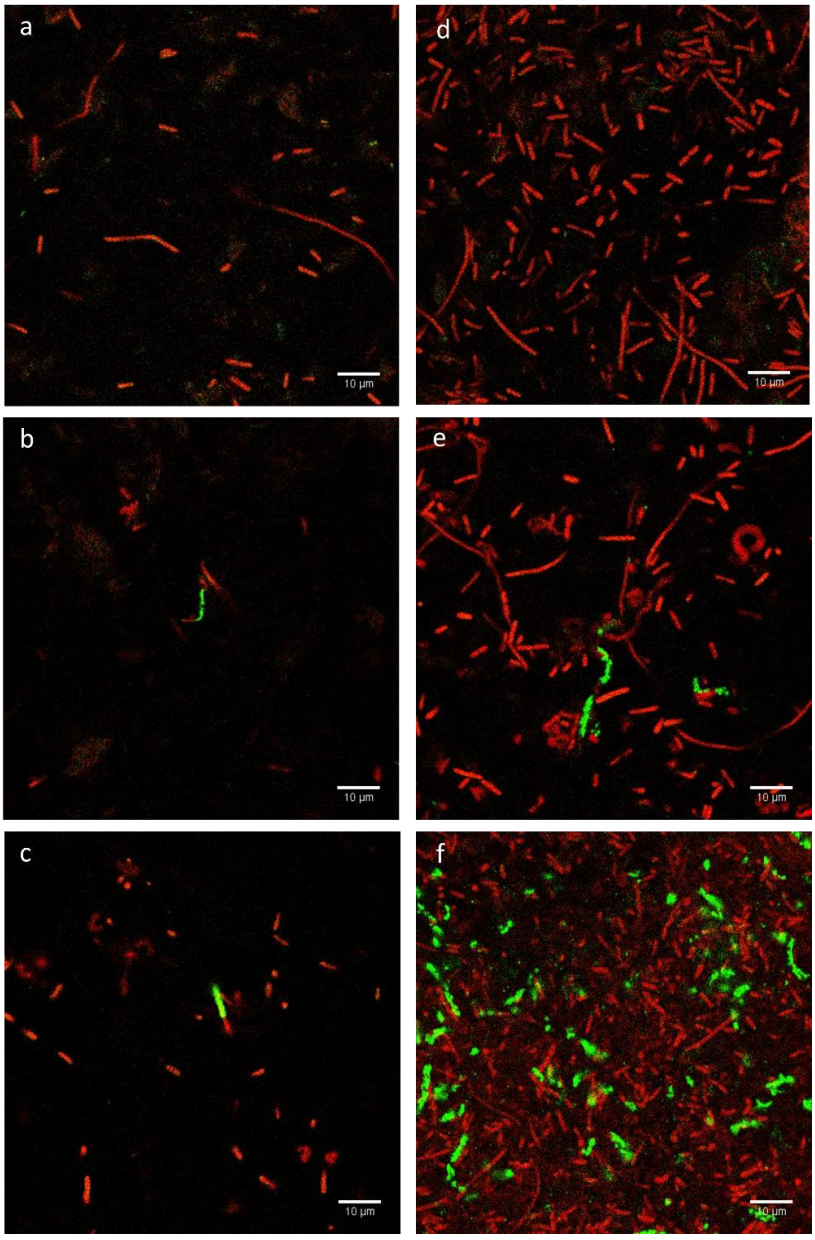

Figure 2.2. Representative fluorescent images of *E. coli* BL21 10^2 CFU/mL after 4 hours enrichment in TSB: (a) no phage infection, (b) after 15 minutes of T7-ALP infection, (c) after 30 minutes of T7-ALP infection. Fluorescent images of *E. coli* BL21 10^3 CFU/mL after 4 hours enrichment in TSB: (d) no phage infection, (e) after 15 minutes of T7-ALP infection, (f) after 30 minutes of T7-ALP infection

2.3.3. Evaluation of issue of potential false-positive identification of E. coli

Phage T7 infection is specific to *E. coli*; therefore, phage T7 will not be able to infect other bacterial strains or species thereby eliminating false positive results if deployed as a bacterial detection method. However, to more rigorously investigate this assumption, the specificity of this phage approach to *E. coli* detection was challenged using two food-related bacteria: *L. innocua* and *P. fluorescens*. Both strains with an initial concentration of 10^3 CFU/mL were enriched in TSB for 4 hours before infection with 10^6 PFU/mL of phage T7-ALP for 30 minutes. After filtration and reaction with ELF-97, there was no significant green fluorescent signal was detected (Figure 2.3).

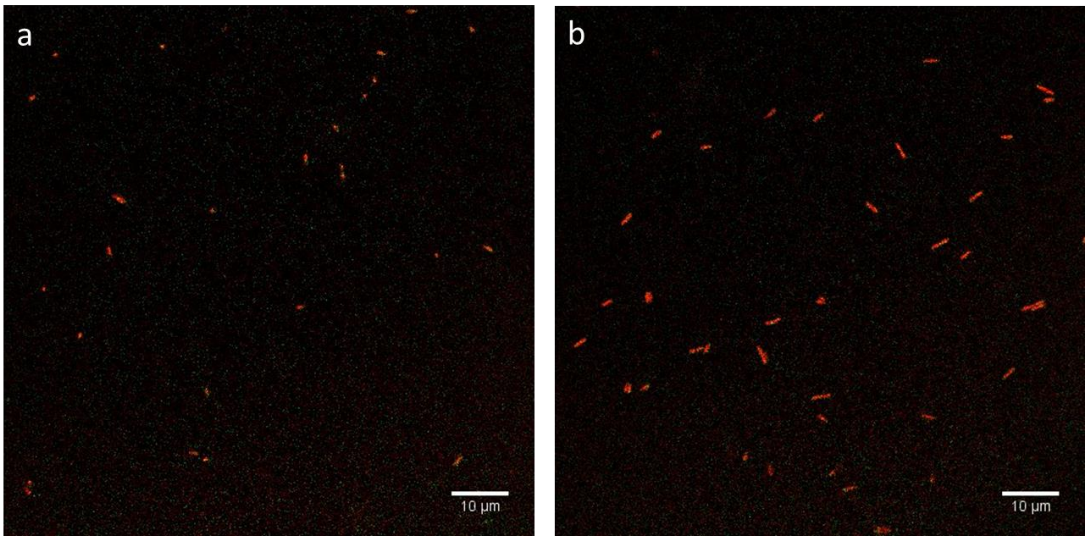

Figure 2.3. *L. innocua* (a) *P. fluorescens* (b) 4 hours enrichment in TSB and coconut water after 15- and 30-minutes infection with bacteriophage T7-ALP

2.3.4. Optical detection of E. coli in realistic challenging beverage matrices

This detection method was also studied for the detection of *E. coli* in an authentic complex beverage matrix. First, coconut water was examined, which predominantly contains sugar and minerals (Prades et al., 2012). The coconut water was filtered through a 0.22 µm syringe filter to ensure no contamination of other bacteria then mixed with double-concentrated TSB to support the bacterial growth of inoculated bacteria. *E. coli* with the concentration of 10^2 CFU/ml and 10^3 CFU/ml was inoculated into the coconut water-TSB mixture and incubated at 37°C with 200 rpm constant shaking for 4 hours for enrichment. For phage infection, 10^6 PFU/ml was added and co-incubated at the same condition for 15 and 30 minutes. The mixtures were filtered through a 0.2 µm white polycarbonate membrane before adding ELF-97 substrate and looked under the microscope. Representative fluorescent images were shown in Figures 2.4. a and d show the fluorescent images of 10^2 CFU/ml and 10^3 CFU/ml of *E. coli*, respectively. After 4 hours of enrichment with no phage infection, the images show total cells with red fluorescence without visible green fluorescence of cell or background noise indicating that coconut water-TSB does not induce alkaline phosphatase production by *E. coli*.

Figure 2.4b illustrates a fluorescent image of bacteria 10^2 CFU/mL of *E. coli* after 4 hours of enrichment and 15 minutes of phage T7-ALP infection. There was at least one cluster of cells per image that has green fluorescence of ELF-97 product. Even though the infection time was extended to 30 minutes, the number of cells with alkaline phosphatase activity did not increase. This result is similar to the result observed for phage T7-ALP infection of *E. coli* grown in TSB (Figure 2.2).

In the case of *E. coli* 10^3 CFU/ml, phage T7-ALP infection occurred a significant green fluorescent signal was detected. Interestingly, the signal occurred as multiple small particles

scattered outside the cells as can be seen in Figure 2.4e After the infection continued to 30 minutes, there was an increase in the number of small punctate green fluorescence particles (Figure 2.4f).

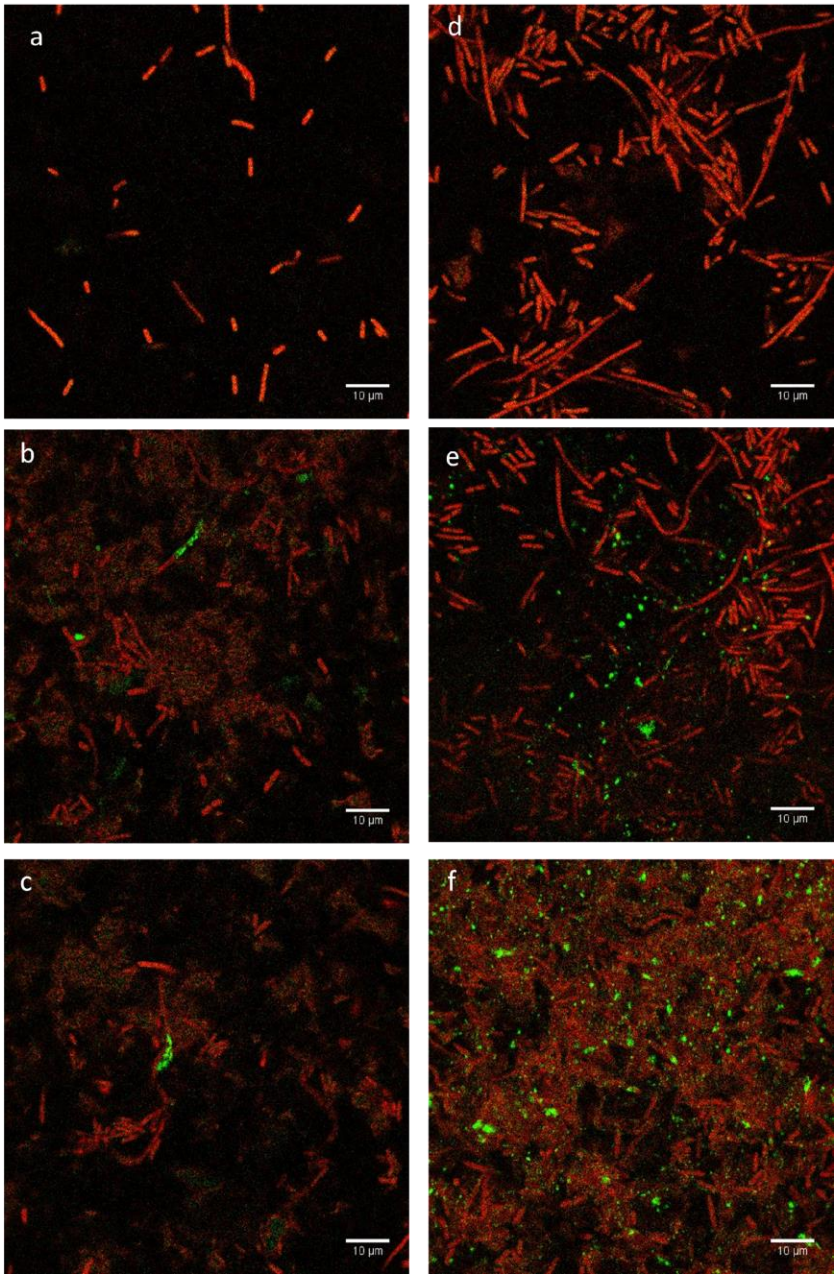

Figure 2.4. Representative fluorescent images of *E. coli* BL21 10^2 CFU/mL after 4 hours enrichment in coconut water-TSB mixture: (a) no phage infection, (b) after 15 minutes of T7-ALP infection, (c) after 30 minutes of T7-ALP infection. Fluorescent image of *E. coli* BL21 10^3 CFU/mL after 4 hours enrichment in TSB: (d) no phage infection, (e) after 15 minutes of T7-ALP infection, (f) after 30 minutes of T7-ALP infection.

As an alternative beverage sample, apple juice was tested for *E. coli* using this methodology. Apple juice predominantly has sugars and minerals but has an acidic pH lower than 6. *E. coli* contaminated apple juice was used to set up an enrichment. An apple juice-TSB mixture was adjusted to pH 7 before filtration through a 0.22-micron syringe filter and bacterial inoculation. The detection method using engineered phage T7-ALP and alkaline phosphatase substrate ELF-97 was performed as described earlier. Representative fluorescent images of *E. coli* in apple juice-TSB after reaction with ELF 97 are shown in Figure 2.5a and d show the results of *E. coli* after 4 hours of enrichment without phage infection with an initial concentration of *E. coli* of 10^2 CFU/mL and 10^3 CFU/mL, respectively. After 4 hours of enrichment, the bacteria appeared to have filamentous morphology; however, there was no cell with the visible green fluorescent signal from the precipitated ELF-97 fluorescent product.

Following 15 minutes of phage T7-ALP infection, images of bacteria originating from a concentration of 10^2 and 10^3 CFU/mL were observed as shown in Figure 2.5b and e, respectively. This revealed a green fluorescent signal marking the location of significant alkaline phosphatase activity and the signals were very bright and easy to distinguish from the background. When the infection was extended to 30 minutes prior to imaging, there was more than one cell with green fluorescence and the signal became more intense and appeared as a long filamentous arrangement, Figure 2.5c, and f. For Apple juice, a signal was always confined to cells, with no apparent small green fluorescent particle, suggesting that in this experiment situation there was no loss of bacterial cell integrity.

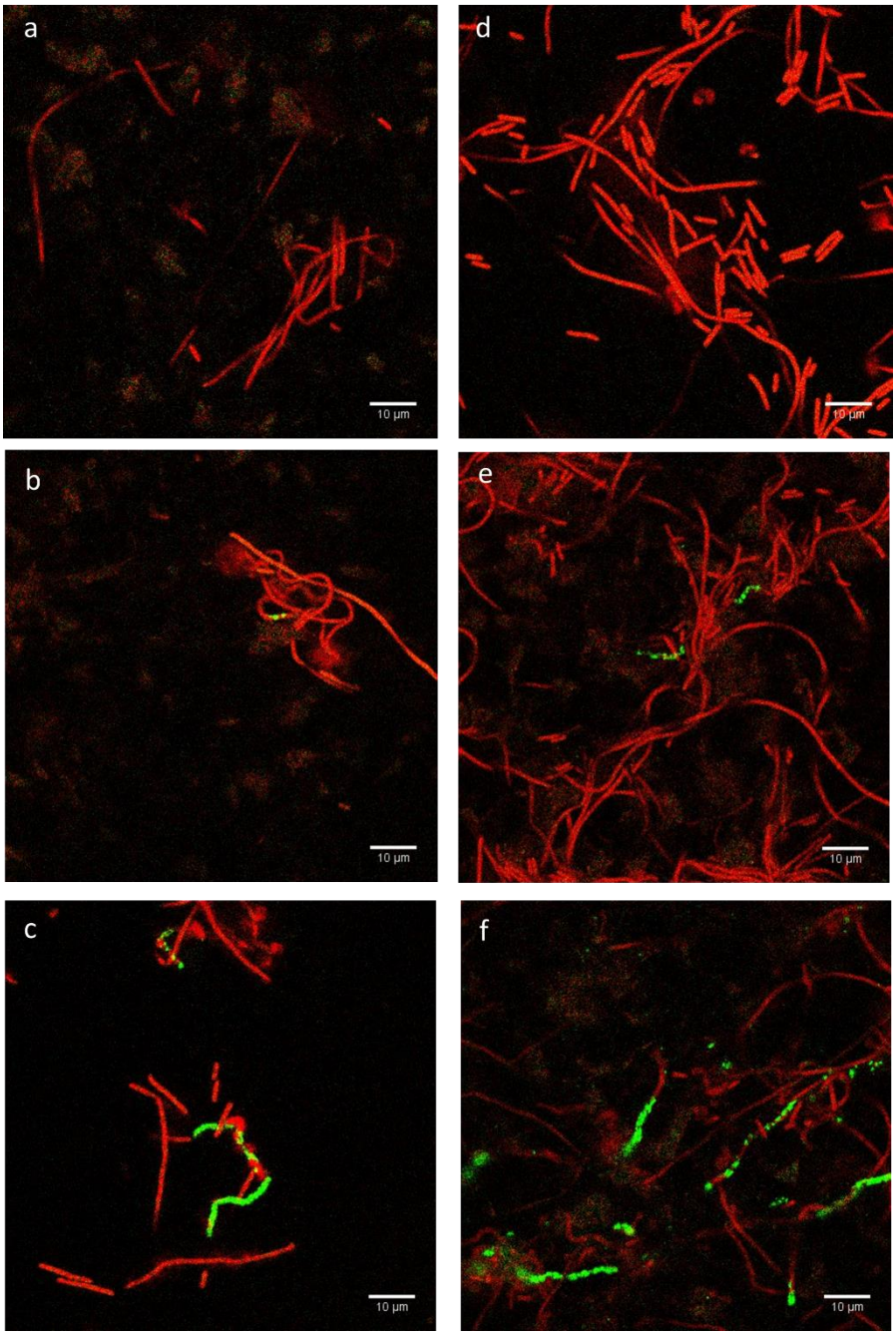

Figure 2.5. Representative fluorescent images of *E. coli* BL21 10^2 CFU/mL after 4 hours enrichment in apple juice-TSB mixture: (a) no phage infection, (b) after 15 minutes of T7-ALP infection, (c) after 30 minutes of T7-ALP infection. Fluorescent images of *E. coli* BL21 10^3 CFU/mL after 4 hours enrichment in TSB: (d) no phage infection, (e) after 15 minutes of T7-ALP infection, (f) after 30 minutes of T7-ALP infection.

2.3.5. Image analysis of T7-ALP infected E. coli fluorescent images

Figures 2.6a, b, and c show the level of mean intensity of the fluorescent signal of ELF-97 precipitated fluorescent product in TSB, coconut water-TSB mixture, and apple juice-TSB mixture, respectively. In all media, initial bacteria concentrations of 10^2 CFU/ml and 10^3 CFU/ml gave a significantly higher mean intensity compared to negative control which was bacteria with no phage infection. However, there is no significant difference ($P < 0.05$) between the mean intensity of 10^2 CFU/ml and 10^3 CFU/ml. However, after 30 minutes of infection in coconut water, the result indicates a decrease in the mean fluorescence intensity. This is probably due to the fact that, in coconut water, after 30 minutes of infection most bacterial cells were lysed causing the release of alkaline phosphatase thus the signal was no longer localized in bacterial cells. Therefore, it is critical that the bacteria-phage mixture was filtered before the lysis of bacterial cells in order to maintain the high intensity of the fluorescent signal.

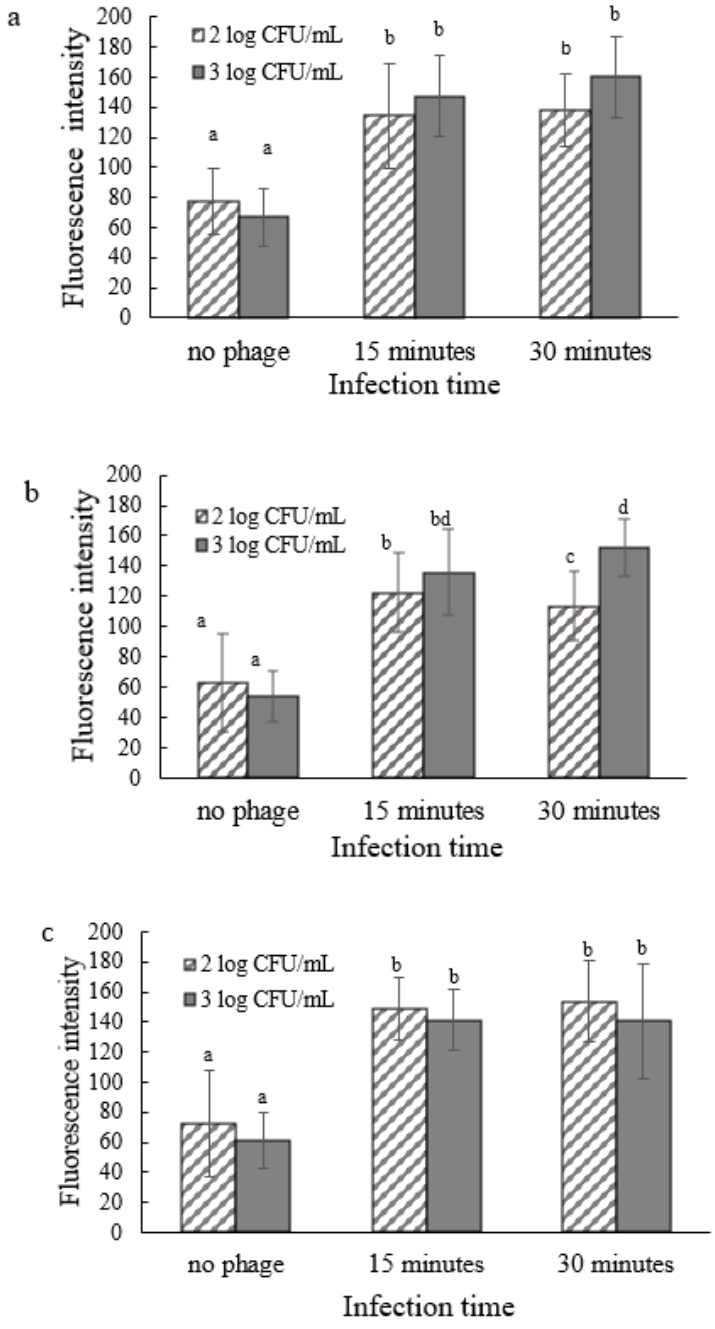

Figure 2.6. Mean gray value of fluorescent images of *E. coli* BL21 10^2 CFU/mL and 10^3 CFU/mL 4 hours enrichment in TSB (a), coconut water (b), and apple juice (c) after 15- and 30-minutes infection with bacteriophage T7-ALP. Treatments with different letters are significantly different ($P < 0.05$). Error bars indicate ± standard deviation of means.

2.3.6. Detection of E. coli based on alkaline phosphatase activity using a soluble FDP fluorescent substrate

The signal-to-noise ratio of the fluorescent signal when the infection took place in TSB, coconut water-TSB mixture and apple juice-TSB mixture is shown in Figure 2.7a, b, and c, respectively. In TSB, the detection limit of *E. coli* was 10^4 CFU/ml after 4 hours of reaction with FDP substrate. For detection in beverage sample, initial cell concentration of 10^4 CFU/ml in coconut water-TSB mixture can be detected after 8 hours of reaction, and after 12 hours in apple juice-TSB mixture while there was no significant difference between negative control of no bacteria and *E. coli* 10^3 and 10^2 CFU/ml. To detect 10^4 CFU/ml, it requires a reaction time of 4 hours, 8 hours, and 12 hours in TSB, coconut water, and apple juice, respectively.

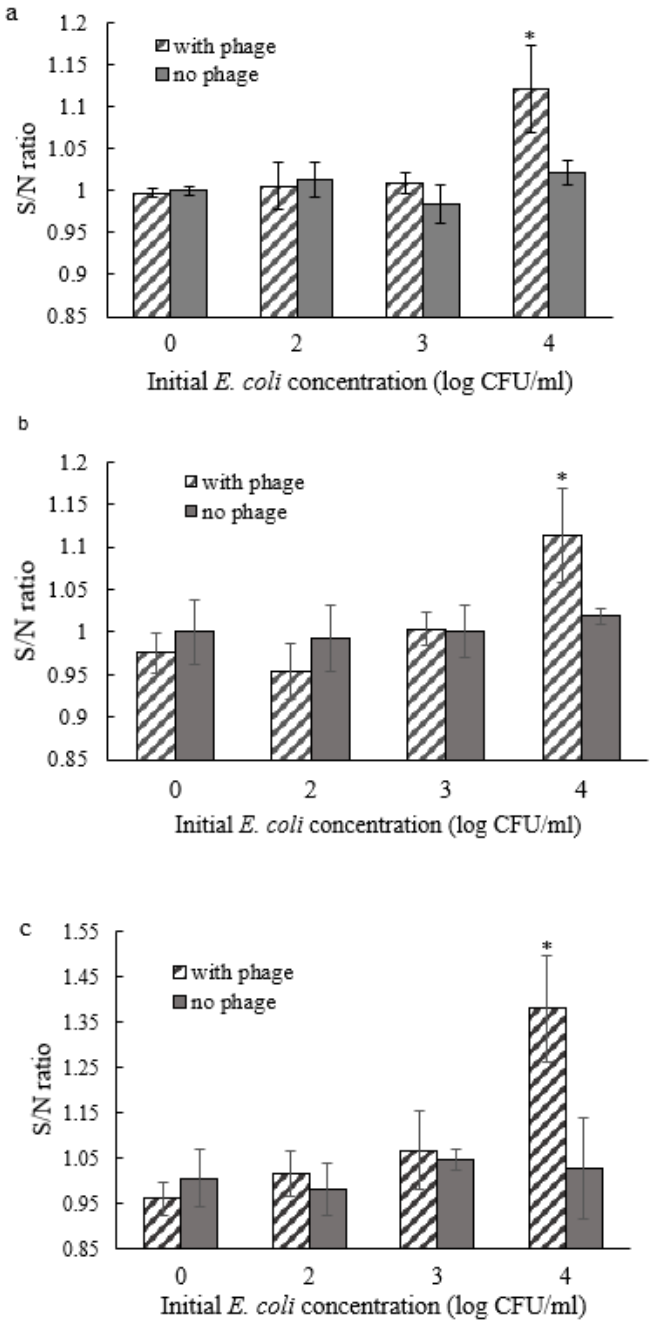

Figure 2.7. Signal to noise ratio of Alkaline phosphatase over-expressed by *E. coli* BL21 after 4 hours of enrichment in TSB (a), coconut water (b), and apple juice (c) after infection with phage T7-ALP for 30 minutes. '*' indicates a significant difference ($P < 0.05$) from the negative control. Error bars indicate ± standard deviation of means.

2.4. Discussion

Bacteriophage has been continuously employed as the valuable tools for bacteria detection due to its specificity and rapidity of the infection cycle, robustness, and low-cost preparation (Richter et al., 2018). Bacteriophage T7 and its host, *E. coli*, is one of the most widely studied models for bacteria detection. The simplicity of the bacteriophage T7 genome has enabled genetically engineering for the insertion of the reporter gene. Several bacteriophage-based bacteria detection approaches have been recently implemented in order to improve bacteria detection sensitivity (Table 2.1). These previous studies developed the detection method based on colorimetric, bioluminescent, and electrochemical detection of enzymatic activity using substrates that yield soluble products; therefore, their detection sensitivity relies on the cell concentration and/or immobilization of the reporter enzyme on biomaterial substrates.

Table 2.1. Short summary of bacteriophage-based rapid bacteria detection studies using genetically engineered bacteriophage T7

Method description	Detection limit	Assay time	Matrices	Potential limitations	References
Detection of T7-induced alkaline phosphatase activity using chemilumiscent methods	10^3 CFU/ mL	6 h	Luria Broth	Detection of enzymatic activity can be affected by background noise	(Alcaine et al., 2015)
Detection of phage T7 amplification using lateral flow assays with phage-based enzymatic reporter	10^2 CFU/100 mL	9 h	river water	Reduced detection efficacy in complex matrices may occur due to non-specific binding to antibody	(Alcaine et al., 2016)
Detection of T7-induced β-galactosidase using colorimetric substrate	10^2 CFU/mL	7 h	drinking water, skim milk, orange juice	Additional cost and complexity due to lyophilization	(Chen et al., 2017)
Detection of T7-induced β-	10^2 CFU/mL	7 h	drinking water,	Low sample volume (1 ml)	(Wang et al., 2017)

galactosidase using electrochemical method			skim milk, orange juice		
Detection of T7-induced luciferase and alkaline phosphatase by filter-based colorimetric and bioluminescence method	1 CFU/100 mL	10 h	drinking water	Reduced detection efficacy in complex matrices may occur due to background color and large food particles	(Hinkley et al., 2018b)
Detection of T7-induced luciferase immobilized on microcrystalline cellulose	<10 CFU/100 mL	3 h	drinking water	Reduced detection efficacy in complex matrices may occur due to non-specific binding to cellulose	(Hinkley et al. 2018)

This study demonstrates the novel approach of enhancing the detection sensitivity by detection of reporter enzyme using precipitated substrate followed by fluorescent imaging and image analysis. From the results, we established that infection of engineered bacteriophage T7-ALP coupled with the use of ELF-97 as alkaline phosphatase substrate was a viable option that could be developed into a food safety application. Localization of the fluorescent precipitated signal inside the cell resulting in increased signal intensity allowing visualization of a single bacterial cell. Further, they are consistent with other studies. ELF-97 was used for detection of alkaline phosphatase activity as a phosphate stress marker in marine phytoplankton with fluorescent imaging and flow cytometry (González-Gil et al., 1998; Dyhrman, 1999; Nedoma et al., 2003; Van Wambeke et al., 2008). ELF-97 yielded a highly sensitive detection of a single cell of phosphate-stressed marine bacteria (Duhamel et al., 2009). Huang et al., (1998) successfully used ELF-97 to detect alkaline phosphatase expression of bacteria colonies and biofilm in phosphate starvation condition (Huang et al., 1998).

Developing a robust pathogen detection system demands that the method is ultimately specific without unexpected false positive results. As bacteriophage is highly specific to bacterial host strain, other bacterial strains will not be infected by bacteriophage T7-ALP and will not overexpress alkaline phosphatase thus no fluorescent signal is detected. Two strains of foodborne bacteria, *L. innocua*, and *P. fluorescens*, were tested to evaluate the detection specificity. *L. innocua* is a nonpathogenic surrogate of *Listeria monocytogenes* which is a Gram-positive foodborne pathogenic bacterium commonly found in food and agricultural product (Friedly et al., 2008). While *P. fluorescens* is representing Gram-negative bacteria with rod shape that are commonly found in food system to cause problems with spoilage (Rajmohan et al., 2002). The results indicated no significant green fluorescent signal was detected which reinforced the potential

to distinguish target bacteria from other foodborne bacterial strain. Although it might not be strictly defined as a false positive, it remains possible that the resulting alkaline phosphatase activity observed by *E. coli* following phage T7-ALP infection is due to a stress condition resulting in induction of the endogenous *E. coli phoA*. To test this possibility 10^3 CFU/mL of *E. coli* was infected by wildtype T7 phage. This alternative possibility of *phoA* expression was not supported by the experimental results, which showed no significant green fluorescent signal.

Detection of *E. coli* in the complex beverage matrices is the essential step to evaluate the effect of food component on bacteriophage infection and expression of *phoA* gene. When the 30-minutes infection took place in coconut water (Figure 2.4f), green fluorescence particles were not in the cell shape yet appeared in small punctate particles. We interpret that these results show that some of the integrity of some infected cells was compromised which caused the release of alkaline phosphatase. Assuming a loss of cell integrity is due to phage-mediated cell lysis following replication, then indicating that phage T7-ALP conditions provided by cultivation in coconut water-TSB may shorten the phage replicative cycle.

E. coli cells, after 4 hours of enrichment in apple juice, displayed filamentous morphology (Figure 2.5) which may be due to the SOS response noted previously by other investigators (Justice et al., 2006). Apple juice contains high content of phenolic compounds which show antibacterial activity against *E. coli* (Kahle et al., 2005; Alberto et al., 2006), which may account for the induction of the filamentous cell structure. However, there was no cell with the visible green fluorescent signal from precipitated ELF-97 fluorescent product showing that these apparently stressed conditions have an impact on the expression of endogenous *E. coli phoA* and, therefore, no significant green fluorescent signal was detected. After bacteriophage T7-ALP infection (Figure 2.5b, c, e, and f), the signal was confined to distinctive segments of the filamentous cellular

arrangement, revealing that phage T7-ALP replicated at this timepoint within a subset of *E. coli* cells forming the filament.

Image analysis results indicated that green fluorescence can represent the activity of phage-induced alkaline phosphatase since the signal intensity of phage-infected bacteria is significantly different from the auto-fluorescent of uninfected bacteria. Infection time has no influence on alkaline phosphatase activity when the media were TSB and apple juice as there are no changes in the mean fluorescence intensity. However, there was a decrease in fluorescence intensity after 30 minutes of infection in coconut water which may be due to the release of alkaline phosphatase after cell lysis. This finding indicated that the natural properties and compositions of food matrices may influence the infection rate of bacteriophages which results in different optimal infection times for different food samples.

For detection of alkaline phosphatase activity using a soluble substrate, FDA, the length of reaction time depends on the concentration of enzyme in the solution. Since the infection time is 30 minutes, the cells were not completely lysed; therefore, phage-mediated alkaline phosphatase was not released into the solution. Unlike the signal of the precipitated fluorescent substrate, the signal from a soluble fluorescent substrate is dissolved in the solution resulting in lower detection sensitivity. The results also indicate the difference in reaction time required to detect 10^4 CFU/ml in different infection media. More complexion of media resulted in more time required for *E. coli* detection using this approach as the composition of coconut water and apple juice may interfere with the reaction of alkaline phosphatase.

Overall, the results of this study indicated that the detection of alkaline phosphatase activity induced by engineered bacteriophage infection using a substrate that yields precipitated fluorescence product coupled with fluorescence imaging and quantitative image analysis is a

promising approach for rapid and highly specific bacterial detection. This proof-of-concept system can be applied in the real complex beverage matrices.

2.5. Conclusion

The detection of alkaline phosphatase overexpression induced by the infection of engineered T7-ALP phage by using a substrate that yields precipitated fluorescence product can be performed by fluorescence imaging and quantitative image analysis. This detection approach enables the single cell-level detection of 100 CFU/ml of *E. coli* in model beverages, coconut water, and apple juice within a total of 8 hours of operation time.

2.6. Potential future work

2.6.1. Broaden the range of the target bacteria by using a phage combination

T7 phages have been used for specific detection of *E. coli* BL21 as they will not infect other bacterial strains or species thereby eliminating the false-positive results (Wisuthiphaet et al., 2019). Using a phage cocktail composed of multiple phages with different host strains could be a potential approach to broaden the target bacterial strains (Ramirez et al., 2018; Korf et al., 2020). To broaden the range of target *E. coli* strains, phages that can infect multiple hosts can be employed instead of using a single phage. The infectivity of other *E. coli* phages such as T4, T6, and MS2 phages can be tested against multiple strains of *E. coli*. In addition, phages infecting other pathogenic bacterial species can also be tested. For example, *Listeria* phage A511 is a wild-type host-specific phage with rapid amplification, which has been utilized for *Listeria monocytogenes* detection (Stambach et al., 2015), and phage 12600 which is specific to infecting *Staphylococcus aureus* (Byeon et al., 2015). Combining several phages may need further optimization of the

infection time as different phage-bacteria systems may have the different times required for induction of the alkaline phosphatase expression.

2.6.2. Detection of E. coli infected with T-ALP using ELF-97 fluorescent substrate by flow cytometry

In principle, the imaging approach shall be able to detect the expression of alkaline phosphatase in individual bacteria using the ELF-97 probe. It is envisioned that bioanalytical tools such as benchtop flow cytometry and other single-molecule detection approaches can further enhance the sensitivity.

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CHAPTER 3:

Colorimetric rapid detection of *Escherichia coli* in Food Matrices using engineered bacteriophage T7[2]

Abstract

Detection of pathogens in a food matrix is challenging due to various constraints including the complexity and cost of sample preparation for microbial analysis from food samples, the time period for the detection of pathogens, and the high cost and specialized resources required for advanced molecular assays. To address some of these key challenges, this study illustrates a simple and rapid colorimetric detection of target bacteria in distinct food matrices, including fresh produce, without prior isolation of bacteria from a food matrix. This approach combines bacteriophage-induced expression of an exogenous enzyme, alkaline phosphatase, a specific colorimetric substrate that generates insoluble color products, and a simple filtration method to localize the generation of the colored signals. Using this approach, this study demonstrates the specific detection of inoculated *E. coli* in coconut water and baby spinach leaves. Without isolation of bacteria from the selected food matrices and using a food sample size that is representative of industrial samples, the inoculated samples were added to the enrichment broth for a short period (5 hours) and incubated with an engineered bacteriophage T7 with a *phoA* gene. The incubation period with the engineered bacteriophage was 30 minutes for liquid samples and 2 hours for fresh

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produce samples. The samples were then filtered through a 0.2-micron polycarbonate membrane and incubated with a colorimetric substrate, i.e., nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). This substrate forms a dark purple precipitate upon interactions with the released enzymes on a filter membrane. This approach successfully detected 10 CFU/ml of *E. coli* in coconut water and 10^2 CFU/g of *E. coli* on baby spinach leaves with 5 hours of enrichment. The success of this approach illustrates the potential for detecting target bacteria in food systems using a simple visual assay and/or quantitative colorimetric measurements.

Keywords: Engineered bacteriophage, *Escherichia coli*, Colorimetric detection, Alkaline phosphatase, 5-bromo-4- chloro-3-indolyl phosphate

3.1. Introduction

Worldwide, human illness due to foodborne pathogens is a major cause of morbidity and mortality. Even within the United States, which has advanced sanitation systems, an estimated 48 million people experience foodborne illness each year (Centers for Disease Control and Prevention, 2018). The leading cause of foodborne illnesses has been the contamination of food such as fresh produce, dairy, and meat products with microbial pathogens, for example, among those pathogens of most concern are Shiga toxin-producing *Escherichia coli* (STEC) which are reported as a cause for an estimated 265,000 illnesses and 3,600 hospitalizations annually in the United States (Scallan et al., 2011). Therefore, rapid and efficient detection of microbial contaminants plays an important role in mitigating the risk of foodborne outbreaks and improving sanitary controls in the food supply chain. Several approaches for pathogen detection have been deployed in the industry. However, rapid and sensitive detection of bacteria, especially in complex food matrices, continues to be a challenge.

In the food industry, multiple detection methods are used for the food safety assessment process. The conventional culture-dependent detection method is considered the gold standard for bacterial detection; however, it is both time-consuming and labor-intensive. These limitations can be acute for food industries such as the fresh produce industry as products have limited shelf life (Cho and Ku, 2017). Therefore, more advanced rapid detection methods, including nucleic acid-based and immunological-based methods, are currently being employed in the food industry. Polymerase chain reaction (PCR) is a nucleic acid-based technique offering rapid and specific pathogen detection. This approach has high sensitivity enabling simultaneous amplification and quantification of specific nucleic acid sequences (Kralik and Ricchi, 2017). The most commonly

used immunological-based method is the Enzyme-Linked Immunosorbent Assay (ELISA) which can be automated to enable rapid detection of pathogens with reduced labor.

However, these advanced methods also have some drawbacks. PCR has suffered from complicated sample preparation, costly reagents, and technical support requirements. Using nucleic acid-based methods to detect pathogens in food samples can be challenging since foods are highly complex biomolecular matrices with diverse arrays of biomolecules such as proteins, carbohydrates, fats, oil, polyphenolics, and other small molecules. These compounds may act as inhibitors of the enzymatic reactions in PCR resulting in false-negative results or limiting the sensitivity of the assay (Jaykus, 2003). The use of PCR in food is also limited by high sample volumes (≥25 ml or g) compared to small amplification volumes (10–50 µl) used for the PCR detection (Stevens and Jaykus, 2014). In the case of ELISA, reduced specificity due to cross-reactivity of polyclonal antibodies with closely related antigens can be a limitation (Hornbeck, 2015). Therefore, constraints for the detection of foodborne pathogens by ELISA include a large number of background microflora present in food samples. Even though these microbes may not cause any health problems, they can interfere with the selective identification and isolation of target pathogenic bacteria which are usually found in relatively low numbers. In order to improve the sensitivity of these detection methods, at least 4-5 hours of enrichment is still required for both PCR and ELISA assays which prolongs the overall turnaround time. Despite these enrichment steps, the detection sensitivity of typical RT-PCR assays using isolation and detection of bacteria from food matrices ranges from 10^2-10^4 CFU/ml and for ELISA assays ranges from 10^3-10^5 CFU/ml (Sharma and Mutharasan, 2013). In addition, the standard PCR and ELISA methods provide limited specificity for differentiating live vs dead microbes (De Boer and Beumer, 1999; Liu et al., 2017).

Considering the above challenges, there is still a significant unmet need for rapid, cost-effective, and easy-to-perform bacterial detection methods that can be applied to detect specific bacteria in the presence of complex food samples. Bacteriophages have become a valuable tool for developing bacterial detection assays due to their ability to recognize and infect specific strains of bacteria with the time of analysis ranging from 15 minutes to 8 hours (Paczesny et al., 2020). For the detection of bacteria in complex food matrices, the common approach is to genetically engineer bacteriophages to express reporter genes upon infecting the target host bacteria. One of the most commonly used reporter genes is bacterial luciferase (*lux*) which generates a bioluminescence signal (Loessner et al., 1997; Kim et al., 2014), for example, the *E. coli* O157:H7-infecting bacteriophage phiV10 was genetically modified to express the *lux* operon and used for the detection of *E. coli* O157:H7. This approach was reported to detect 10 CFU/cm^2, 13 CFU/ml, and 17 CFU/g of *E. coli* O157:H7 in romaine lettuce, apple juice, and ground beef, respectively, by detecting bioluminescence using a luminometer with 5-hour pre-enrichment (Kim et al., 2017). Another commonly used reporter gene is *lacZ* encoding β-galactosidase. Using this approach, *E. coli* infected with T7 bacteriophage with the *lacZ* operon overexpresses β-galactosidase and allowed the detection of 10 CFU/mL of *E. coli* using a colorimetric substrate within 7 hours and enables the detection of 10^2 CFU/mL of *E. coli* in food samples (Chen et al., 2017a, 2017b). The gene that encodes alkaline phosphatase (*phoA*) is another reporter gene that has been engineered into the bacteriophage T7 genome to induce overexpression of alkaline phosphatase. Overexpression of alkaline phosphatase gene has been detected using both soluble colorimetric substrate, p-nitrophenyl phosphate (pNPP), which allows for the detection of 10^4 CFU/ mL within 7.5 hours and 10^3 CFU per mL of *E. coli* in 6 hours using a chemiluminescent substrate (Alcaine et al., 2015). Enzyme-Labeled Fluorescence-97 (ELF-97), an alkaline phosphatase substrate that

gives insoluble fluorescent precipitated products, has been used to detect the engineered bacteriophage-induced alkaline phosphatase activity at the single-cell level using fluorescent imaging and image analysis. This approach has been tested in beverage samples and was able to detect 10^2 CFU/ml of target bacteria within 6 hours (Wisuthiphaet et al., 2019). Even though these methods have been proven to detect a low number of the specific bacteria in complex matrices, maintaining both rapidity and simplicity of the procedure can be challenging due to a range of issues related to sample preparation including bacteria isolation steps, instruments required for signal analysis, and distinguishing signal from high background noise in food samples.

The goal of this study was to develop a rapid bacteriophage-based colorimetric bacterial detection that is low-cost, easy to perform, and can be applied to detect bacteria in complex food samples without isolation of bacteria from food matrices. Isolation of bacteria from food samples is a time, equipment, and labor-intensive process (Stevens and Jaykus, 2014). The approach evaluated in this study does not use any isolation of bacteria from food samples and thus reduces the complexity of the assay. Furthermore, the colorimetric detection approach was selected as it provides a simple read-out for a visual analysis as well as the quantitative measurement using a camera. For colorimetric detection, we evaluated both an insoluble substrate, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and commonly used soluble substrate, pNPP to detect the enzymatic activity. NBT/BCIP yields insoluble dark purple precipitate that is localized at the site of the reaction and can be visualized by the naked eye. After applying this substrate to the bacterial cells with overexpressed alkaline phosphatase, the precipitated product was accumulated inside the cells. After the separation of bacterial cells from the combined culture medium and the food samples by filtration, the signal was concentrated and enabled the detection of visible color change. The efficiency of bacterial detection using NBT/BCIP and engineered

bacteriophage T7 was evaluated in coconut water and baby spinach leaves and compared with those of the commonly used soluble substrate, pNPP. Overall, the results of this study demonstrate the potential of visual detection of bacterial contaminants in food samples using engineered bacteriophages without extensive sample preparation steps.

3.2. Materials and Methods

3.2.1. Bacteriophage and bacterial strain

The engineered bacteriophage used in this study, designated bacteriophage T7-ALP, was bacteriophage T7 that has been genetically modified to carry the gene for alkaline phosphatase production, *phoA*. The bacteriophage T7-ALP strain was kindly provided by Dr. Sam Nugen (Alcaine et al., 2015). *Escherichia coli* BL21 (ATCC BAA-1025) obtained from the American type culture collection was used as a host for the bacteriophage T7-ALP. Bacteria were stored in tryptic soy broth (TSB) (Sigma-Aldridge, St. Louis, MO, USA) containing 15% (vol/vol) glycerol at -80°C. For short-term storage, the glycerol stock was streaked onto tryptic soy agar (Sigma-Aldridge, St. Louis, MO, USA) plates. After incubation at 37°C for 24 hours, the culture plates were stored at 4°C. Overnight culture of *E. coli* BL21 was prepared by inoculation a loop-full of culture on an agar plate in TSB, after 16 hours of aerobic incubation at 37°C, bacteria with the concentration of 10^9 CFU/ml were obtained.

3.2.2. Sample preparation and bacterial inoculation

The overnight culture of *E. coli* BL 21 was centrifuged at 16100 × *g* for 1 minute. The cell pellet was washed twice and resuspended in sterile phosphate buffer saline (PBS) (Fair Lawn, NJ, USA) at the population of 10^9 CFU/ml. The serial dilutions were performed using sterile PBS to obtain bacterial concentrations of 10^3 and 10^2 CFU/ml.

Sterile TSB was portioned to 10 ml in a 50 ml sterile centrifugal tube. Pasteurized coconut water (Vita coco 100% coconut water) purchased from a local grocery store 5 ml was mixed with 5 ml of double concentrated TSB in a 50 ml sterile centrifugal tube. *E. coli* BL21 with the final concentration of 10 CFU/ml was inoculated in 10 ml of the liquid media. The fresh produce sample was represented by baby spinach leaves. Store-bought baby spinach leaves were weighted 25 g in a sterile sampling bag before inoculation with *E. coli* BL21 to achieve the final concentration of 10^2 CFU/g. The bags were tightly sealed and kept at 4°C for 24 hours. Then 225ml of TSB was added to 25g inoculated spinach in the sampling bags.

3.2.3. Enrichment and bacteriophage infection

The enrichment of all samples was carried out at 37°C with constant shaking at 200 rpm for 5 hours. For spinach leaf samples, 10 ml of the TSB was collected in a 50 ml centrifugal tube. Genetically engineered bacteriophage T7-ALP was added to 10 ml of the enriched samples with a concentration of 10^6 PFU/ml. For liquid samples, the infection time was fixed at 30 minutes at 37°C with constant shaking at 200 rpm. For 25-gram spinach leaf samples, the infection time was fixed at 2 hours at 37°C with constant shaking at 200 RPM. Negative controls of each experiment were *E. coli* inoculated samples without bacteriophage infection and samples without bacteria inoculation with bacteriophage added.

3.2.4. Colorimetric assay of alkaline phosphatase activity using NBT/BCIP

After phage infection, 10 ml of TSB and coconut water samples, and 2 ml of spinach leaf samples were filtered through a 0.22-micron white polycarbonate membrane discs with a 19-mm diameter (Nucleopore Polycarbonate, Whatman) using a vacuum filtering system in order to capture infected bacterial cells harboring bacteriophage-induced alkaline phosphatase. After the

filters were completely dry, 20 µl of 1-Step™ NBT/BCIP substrate solution (Thermo Scientific, Rockford, IL, USA) was spotted on a petri dish. The filter was then transferred directly onto the NBT/BCIP drop with topside down. The visible color change due to the formation of black-purple precipitated product was observed and compared with those of negative controls and tryptic soy broth without bacteria and bacteriophage. The Hunter's color values (L*, a*, b*) of the filters were measured at 5 locations of each filter using the ColorFlex EZ Spectrophotometer (Hunter Lab, Reston, VA, USA). The delta E (dE) value was calculated using equation 1.

$$Equation\ 1: \qquad \Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

3.2.5. Colorimetric assay of alkaline phosphatase activity using pNPP

After bacteriophage infection, 10 ml of all samples were centrifuged at 4025 × g for 10 minutes at room temperature. The liquid media were discarded and the precipitated cells were resuspended with 50 µl of sterile PBS. Amplite™ Colorimetric Alkaline Phosphatase Assay Kit (AAT Bioquest, Sunnyvale, CA) was used to perform the assay. After adding the substrate, the absorbance at 400 nm was measured every 10 minutes for 4 hours using a SpectraMax 340 spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA, USA). The experiments were performed in parallel with the blank which was TSB without *E. coli* and bacteriophage inoculation and negative control of *E. coli* without bacteriophage inoculation.

3.2.6. Statistical analysis

All experiments were repeated three times. Color value was measured at 5 random positions on the filter and the mean and standard deviation values were calculated within the samples. The Tukey's HSD test was used to determine significant differences ($\alpha = 0.05$) between mean values. All experimental data were analyzed using the R software.

3.3. Results

3.3.1. Colorimetric detection of E. coli using engineered bacteriophage T7-ALP and alkaline phosphatase assay

The schematic diagram (Figure 3.1) depicts the detection procedure developed in this study. One of the most challenging aspects of the detection of bacteria from a complex food system is the presence of food components and non-pathogenic microbes associated with the food. These elements can influence both the sensitivity and accuracy of the detection method. Developing a general protocol to separate and purify target cells from different food matrices is difficult as food matrices vary significantly in composition and structure (Frederick et al., 2013). In order to overcome these constraints, the bacteriophage-based detection method developed in this study requires no complicated sample preparation and bacterial separation or concentration steps. The food samples, coconut water, and baby spinach leaves were simply mixed with the TSB before enrichment and used for detection of the bacteria using a simple colorimetric approach. The steps for sample preparation are illustrated in Figure 3.1 The steps include enrichment of bacteria (step 1), incubation with T7-ALP (step 2), filtration (step 3), and incubation with ALP substrate to form colorimetric precipitate (step 4). The details of these steps are described in the material and methods section. Among these steps, the incubation and infection time was a critical factor in this assay, as our goal was to overexpress and entrap the expressed enzyme inside the bacterial cells, thus the infection time should be long enough for the expression of the enzyme but not too long to have complete lysis of the bacterial cells and release of the enzyme. This approach helps localize the colorimetric signal on a filter and increases the sensitivity of the detection. According to a previous study, after 30 minutes of co-incubation with bacteriophage T7-ALP, *E. coli* BL21 cells were infected and alkaline phosphatase was produced while the majority of the cells were still

intact (Wisuthiphaet et al., 2019). By filtration (Figure 3.1 step3), bacterial cells with alkaline phosphatase were captured on the filter and the colorimetric signal was concentrated on the filter membrane for visual detection.

Alkaline phosphatase activity was analyzed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) which forms a dark purple precipitated product. The color development was enhanced by nitro blue tetrazolium (NBT) yielding an insoluble black-purple precipitate as shown in the following reaction equation 2 (Jékely and Arendt, 2007; Kundu, 2014) (Figure 3.1 step 4).

Equation 2:

This enzymatic reaction took place on the filter. Therefore, the change in filter color to dark purple indicates the presence of the target bacteria. The color formation was observed visually as well as using a colorimeter (Figure 3.1 step5). The detection method using a precipitated colorimetric substrate was compared to the soluble colorimetric alkaline phosphatase substrate, pNPP. In this method, infected cells with alkaline phosphatase were harvested by centrifugation before resuspension in sterile PBS prior to the enzymatic reaction. Colorless pNPP was hydrolyzed to yellow p-nitro-phenol (pNP) in the presence of alkaline phosphatase and was quantified at 400 nm (Jackson et al., 2016).

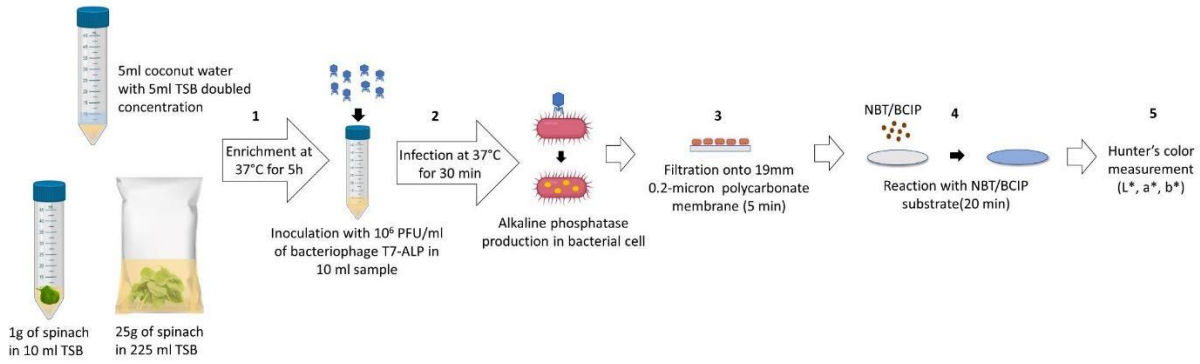
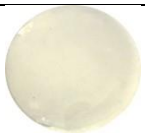


Figure 3.1. Schematic diagram of the detection protocol based on alkaline phosphatase activity assay using colorimetric alkaline phosphatase substrates: NBT/BCIP. 1) Enrichment of bacteria in spinach leaves without separation step (5 hours), 2) Adding bacteriophage T7-ALP 10^6 PFU/ml for infection of *E. coli* and alkaline phosphatase expression (30 minutes – 2 hours), 3) Filtration to harvest the infected *E. coli* cells (5 minutes). 4) Adding NBT/BCIP substrate for enzymatic reaction and forming of precipitated products (5-30 minutes), 5) Color measurement using a colorimeter.

3.3.2. Detection of bacteria using bacteriophage T7-ALP and alkaline phosphatase substrate NBT/BCIP

For the proof-of-concept demonstration, the detection of bacteria was first investigated in TSB which is the media for supporting bacterial growth and bacteriophage infection. Table 3.1 shows the filters after adding NBT/BCIP substrate and incubating the filter at room temperature for 0, 5, 10, 20, and 30 minutes. For the samples with bacteriophage infection, after 10 minutes of incubation with the substrate, a visible dark purple color was observed and the color intensity increased with an increase in incubation time. In contrast, the negative control sample, i.e. filtered bacteria without bacteriophage infection showed no color change after 30 minutes of incubation with the substrate. The negative control of TSB with bacteriophage but without bacteria also showed no visible color change. In order to quantify the color change, the filters were also characterized using Hunter's color scale measurements using a colorimeter. In this measurement, the L*, a*, and b* color values were measured at random 5 locations of the filter and the dE values were calculated using equation 1. The dE value of the samples with T7-ALP infected bacteria and the negative control of bacteria without bacteriophage infection are shown in Figure 3.2 After 5 minutes with the substrate, the dE value of the sample with T7-ALP infected bacteria is significantly higher than those of the controls and the dE value increases dramatically with an extended reaction time. On the other hand, the negative controls showed no significant change in the dE value after 20 minutes of incubation with the substrate.

Table 3.1. Filters with 5-hours enriched 10 CFU/mL *E. coli* BL21 and 30 minutes with and without infection with bacteriophage T7-ALP in TSB and bacteriophage T7-ALP in TSB after enzymatic reaction with NBT/BCIP for 5, 10, 20, and 30 minutes.

Reaction condition		Reaction time with substrate (minutes)				
E. coli inoculation	T7 infection	0	5	10	20	30
+	+					
+	-					
-	+					

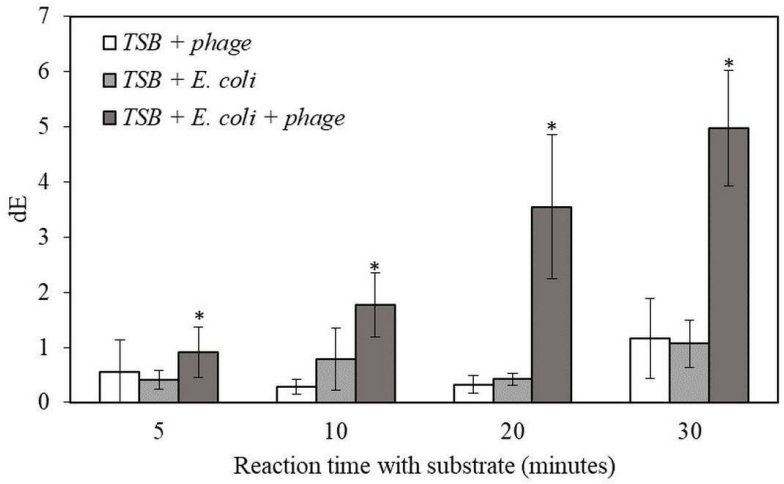


Figure 3.2. The dE value of the filter with bacteriophage T7-ALP in TSB, 5-hours enriched 10 CFU/mL *E. coli* BL21 and 30 minutes with and without infection with bacteriophage T7-ALP in TSB after enzymatic reaction with NBT/BCIP for 5, 10, 20, and 30 minutes. Treatments with '*' are significantly different ($p < 0.05$). Error bars indicate ±standard deviation of means.

The results indicate that, after 30 minutes of infection, the *E. coli* cells were still intact and the alkaline phosphatase induced by T7-ALP infection was produced inside the cells which were captured on the filter after filtration. The results of the negative control sample, i.e., bacterial cells without T7-ALP infection, demonstrate a lack of significant endogenous alkaline phosphatase activity in the bacteria since this enzyme is usually produced by the bacteria only during phosphate starvation (Rani et al., 2012). When the bacteria are enriched in TSB, a nutrient-rich environment for bacterial growth, endogenous bacterial alkaline phosphatase enzyme is not highly expressed in bacterial cells. Overall, these results demonstrate that using a combination of engineered bacteriophage T7-ALP and colorimetric substrate NBT/BCIP, *E. coli* cells can be specifically detected at the initial inoculation levels of 10 CFU/ml within 6 hours using a simple visual analysis or quantitative color imaging.

In order to validate this method using complex food materials, the detection of target bacteria in coconut water and baby spinach leaves was evaluated. Coconut water was selected as it represents a beverage product with sugars, fatty acids, and amino acids (Prades et al., 2012). Before bacterial inoculation, coconut water was mixed with a double concentrated TSB in order to provide nutrients to support bacterial growth. The samples were then inoculated with *E. coli* BL21 10 CFU/ml and incubated at 37°C for enrichment for 5 hours followed by infecting the enriched sample with the T7-ALP bacteriophage for 30 min and detection of the alkaline phosphatase activity after filtration of the coconut water sample. As shown in Table 3.2, after adding NBT/BCIP substrate for 10 minutes, the color change to dark purple color was observed and the color intensity increased with extended reaction time while the negative controls showed no color change after 30 minutes with the substrate.

Table 3.2. Filters with 5-hours enriched 10 CFU/mL *E. coli* BL21 and 30 minutes with and without infection with bacteriophage T7-ALP in TSB-coconut water mixture and bacteriophage T7-ALP in TSB-coconut water mixture after enzymatic reaction with NBT/BCIP for 5, 10, 20, and 30 minutes.

Reaction condition		Reaction time with substrate (minutes)				
E. coli inoculation	T7 infection	0	5	10	20	30
+	+					
+	-					
-	+					

The result indicates that this detection method can be applied to detect 10 CFU/ml of the target bacteria in coconut water samples within 6 hours. The samples with infected *E. coli* gave significantly higher dE values compared to negative controls. The dE value increased with a longer reaction time while there was no increase in dE value for the negative controls (Figure 3.3). The results for the detection of bacteria inoculated in coconut water were similar to those of the detection of bacteria inoculated in pure TSB (Figure 3.2). However, the dE values of the bacteria inoculated in coconut water samples were lower than those of bacteria inoculated in TSB, indicating reduced alkaline phosphatase expression or activity in coconut water. Therefore, the composition of coconut water may have an influence on the signal from alkaline phosphatase enzymatic reaction and the formation of the precipitated colorimetric product.

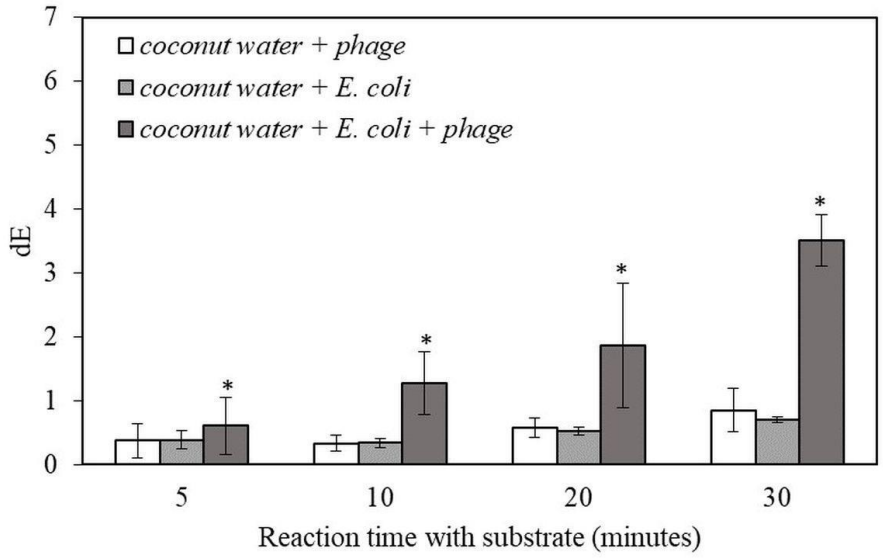


Figure 3.3. The dE value of the filter with bacteriophage T7-ALP in TSB-coconut water mixture, 5-hours enriched 10 CFU/mL *E. coli* BL21 and 30 minutes with and without infection with bacteriophage T7-ALP in TSB after enzymatic reaction with NBT/BCIP for 5, 10, 20, and 30 minutes. Treatments with '*' are significantly different ($p < 0.05$). Error bars indicate ±standard deviation of means.

Fresh produce is one of the food samples that is considered challenging for bacterial detection since the plant samples contain solid particles, pigments, and diverse microflora. In order to validate this detection method, baby spinach was selected to represent leafy greens. The sample size of baby spinach leaves was 25 g which represents a more realistic sample size used in the industry. After inoculation with *E. coli* BL21, the sampling bags containing inoculated leaves were sealed and stored at 4°C for 24 hours to simulate a scenario in the industry where the harvested spinach leaves are stored at refrigerated temperature prior to washing and packaging. After 5 hours of enrichment, spinach samples were then incubated with the bacteriophage T7-ALP for 2 hours. The extended infection time was selected to reduce the influence of non-target microbes and the plant exudate from the spinach leaves. The release of plant exudate from the wound and cut of spinach leaves results in a green color that may interfere with the color change measurement induced by overexpression of alkaline phosphatase and its colorimetric substrate. Moreover, an increase in the level of non-target bacteria may slow the growth of *E. coli* during enrichment and may physically obstruct the binding of bacteriophage and the target bacteria. An increase in infection time to 2 hours allows additional growth of *E. coli* during incubation with bacteriophages and increases the infection efficiency. Detection of *E. coli* on a small scale of 1-gram spinach leaves was also conducted. With less interference from the plant exudate, the optimal coincubation time was 30 minutes.

Table 3.3. Filters with 5-hours enriched 10 CFU/mL *E. coli* BL21 and 30 minutes with and without infection with bacteriophage T7-ALP in TSB-spinach and bacteriophage T7-ALP in TSB-spinach after enzymatic reaction with NBT/BCIP for 0 and 30 minutes.

Reaction condition		Reaction time with substrate (minutes)	
E. coli inoculation	T7 infection	0	30
+	+		
+	-		
-	+		

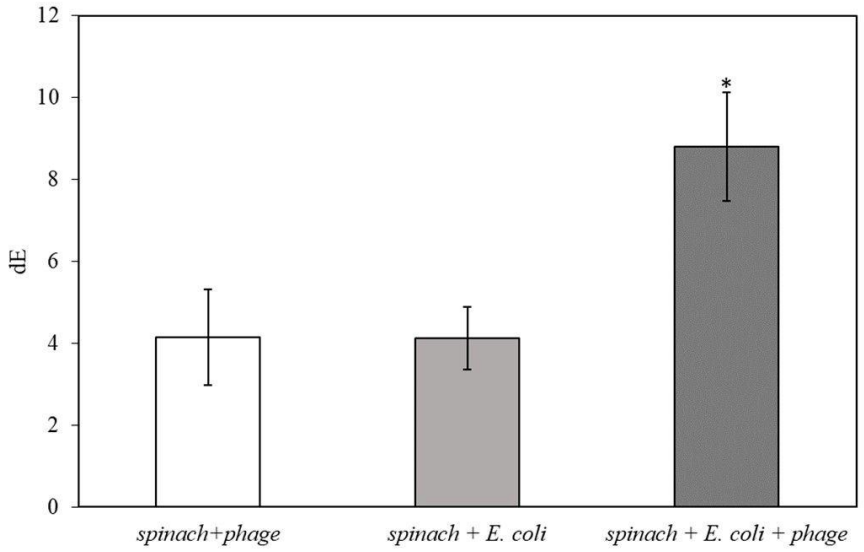


Figure 3.4. The dE of the filter with 5 hours-enriched 10 CFU/mL *E. coli* BL21 and 2 hours infection with bacteriophage T7-ALP in 225ml TSB with 25g spinach leaves after enzymatic reaction with NBT/BCIP for 30 minutes. Treatments with '*' are significantly different ($p < 0.05$). Error bars indicate ±standard deviation of means.

After 2 hours of infection with bacteriophage, 2 ml of enriched TSB was filtered through a 0.2-micron polycarbonate filter. Since the spinach samples contain solid plant particles and also native microflora from plant tissue that would clog the pores of the filter, the volume of the sample subjected to filtration was limited to 2ml. As shown in Table 3.3, the filters after filtration appeared slightly green due to the presence of released plant pigments. After 30 minutes of enzymatic reaction with the NBT/BCIP substrate, the filter appeared darker and the dE value of inoculated spinach samples with bacteriophage infection on a filter was significantly higher than the negative controls (Figure 3.4). The results demonstrate that this detection method can be applied to detect bacteria using a realistic spinach sample size without isolation of bacteria from the inoculated plant samples.

3.3.3. Detection of bacteria using bacteriophage T7-ALP and alkaline phosphatase substrate pNPP

To detect the expression of alkaline phosphatase using colorimetric assays, often substrates such as pNPP that generate a soluble colorimetric signal are used. The advantage of the homogeneous assay is the ease of detection using a simple UV-Vis measurement. In this study, one of our objectives was to compare the sensitivity of colorimetric detection of target bacteria using both insoluble and soluble enzymatic substrates. For this comparison, the experimental conditions for the enrichment and infection steps were maintained the same as in the case when using the substrate that yields precipitated products, NBT/BCIP. The only difference was that after initial infection, the cells were harvested using a centrifuge, and then the isolated cell pellet was resuspended in PBS and incubated with the pNPP substrate as described in the materials and methods section. The absorbance of the resulting solution was measured at 400 nm using a UV-Vis spectrophotometer.

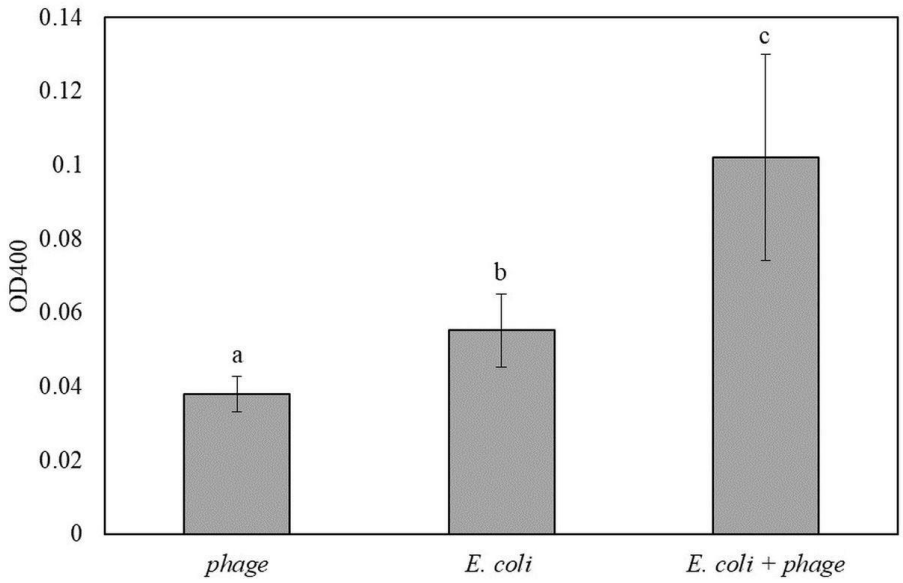


Figure 3.5. The OD400 of 5 hours-enriched 10 CFU/mL *E. coli* BL21 and 30 minutes infection with bacteriophage T7-ALP in TSB after enzymatic reaction with *p*NPP for 2 hours. Treatments with different letters are significantly different ($p < 0.05$) error bars indicate ± standard deviation of means.

Figure 3.5 shows the results for the detection of *E. coli* in TSB that was enriched for 5 hours and infected with bacteriophage T7-ALP for 30 minutes. After 2 hours of incubation with pNPP substrate, *E. coli* infected with bacteriophage had significantly higher absorbance compared to the negative controls. For the soluble substrate assay, the bacteria are incubated in PBS for 2 hours and this incubation of bacteria in a low nutrient environment can increase the endogenous expression of alkaline phosphatase compared to bacteria incubated with TSB. An increase in endogenous expression of alkaline phosphatase can reduce the sensitivity of the detection using the soluble substrate.

Similar to the results in Figure 3.5, coconut water was used as a model liquid food system for the detection of *E. coli*. The mixture of 1:1 coconut water and double-concentrated TSB inoculated with 10 CFU/ml of *E. coli* was enriched for 5 h followed by 30 minutes of infection with bacteriophage T7-ALP. Then the bacterial cells were incubated with the soluble substrate for 2 hours. The inoculated samples with bacteriophage infection had the highest OD400 followed by coconut water with *E. coli* without bacteriophage infection and coconut water and bacteriophage without *E. coli*, respectively (Figure 3.6). The trend was similar to those of bacteria in TSB alone.

This method using soluble substrate was not applicable for the detection of *E. coli* in 25g of spinach samples and a larger volume of media since enrichment steps results in the growth of other local microbes and centrifugation also harvested plant particles along with microbial cells resulting in high turbidity after resuspension which interfered with the absorbance measurement.

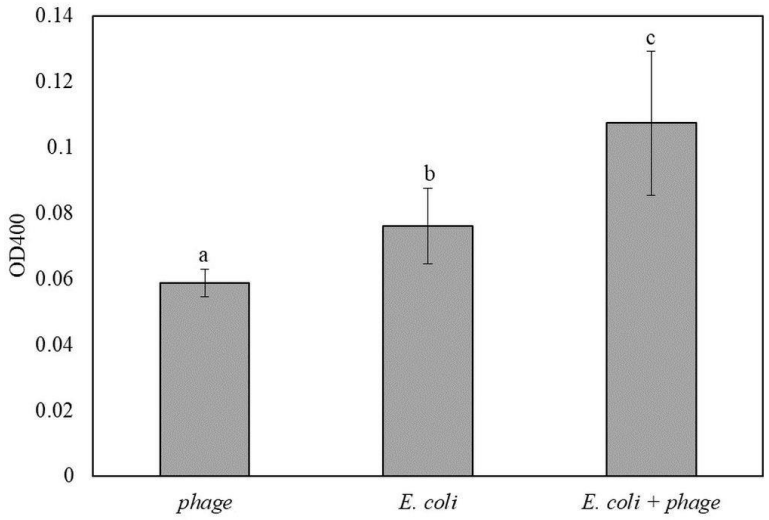


Figure 3.6. The OD400 of 5 hours-enriched 10 CFU/mL *E. coli* BL21 and 30 minutes infection with bacteriophage T7-ALP in coconut water-TSB after enzymatic reaction with *p*NPP for 2 hours. Treatments with different letters are significantly different ($p < 0.05$) error bars indicate ±standard deviation of means.

3.4. Discussion

Detection of specific bacteria in food matrices is a challenging task. This challenge results because of low counts of target bacteria in food systems and also the interference due to the food matrix and other microbes. To address these challenges various processes have been designed to isolate, concentrate, and separate target bacteria from the food matrix using both physical and biochemical methods. Physical methods such as filtration and centrifugation can improve the detection sensitivity but their drawback is that the solid food debris will also be concentrated along with bacteria (Benoit and Donahue, 2003) and can further limit detection sensitivity. Immunomagnetic separation has been used coupled with several bacterial detection methods in order to specifically separate target bacteria from food debris and other endogenous microbes. The significant challenge results due to the binding affinity and specificity of antibodies that can be reduced by food components due to the diversity of mechanisms (Van Aken and Lin, 2011). Moreover, the attachment of bacteria to food surfaces can significantly limit the overall capture efficiency using both physical and biochemical methods. In addition to these constraints, sample preparation for the detection of bacteria in food materials is often one of the key labor and resource-intensive steps with a series of manual handling steps to prepare samples for enrichment and detection. These handling practices can lead to contamination of the samples especially when multiple samples are being processed simultaneously (Benoit and Donahue, 2003). To overcome these limitations, the detection methods developed in this study require no sample preparation steps to isolate bacteria from diverse food matrices. Coconut water and baby spinach leaf samples, representing a liquid and a fresh produce model system inoculated with bacteria were added directly to the bacterial enrichment media that can enrich a diversity of bacteria, thus providing a general framework for the detection of bacteria. Furthermore, the time required for bacterial

enrichment step was comparable to or less than the average enrichment time used prior to other detection methods such as RT-PCR and other nucleic acid or ELISA based methods (De Boer and Beumer, 1999) and was achieved in less than a single work shift in the industry. In addition, unlike PCR or other nucleic acid-based methods, the detection approach developed in this study does not require isolation and purification of nucleic acids. Thus, overall, the simple workflow of the method described in the study significantly reduces the labor and resources typically required for the sample preparation and detection of bacteria. These simplifications combined with visual detection of bacteria using colorimetric analysis provides a simple yet effective approach to detect bacteria in food systems.

Colorimetric detection provides simple and low-cost operations since it doesn't require any advanced instruments, which allows portable and easy-to-use diagnostic. Prior studies have developed colorimetric assays for *E. coli* detection tested in culture media, drinking water, and liquid food matrices as summarized in Table 3.4.

Table 3.4 Summary of the previous studies on calorimetric bacteriophage-based detection of *E. coli*

Sample Matrices	Colorimetric detection approach	Detection limit	References
Drinking water	Chromogenic soluble substrate detection of β-galactosidase released after phage-induced lysis of *E. coli*	10 CFU/mL with 6 h of pre-enrichment	(Chen *et al.*, 2015)
Culture medium	Detection of alkaline phosphatase overexpression induced by engineered bacteriophage T7 using pNPP	10^4 CFU/mL within 7.5 hours	(Alcaine *et al.*, 2015)
Culture medium	Detection of β-galactosidase induced by engineered T7 phage infection on *E. coli*	10 CFU/mL within 7 hours	(Chen *et al.*, 2017a)
Drinking water, skim milk, orange juice	Detection of β-galactosidase induced by freeze-dried engineered T7 phage infection on *E. coli*	10^2 CFU/mL within 7 hours	(Chen *et al.*, 2017b)

Culture medium 100 mL	Detection of alkaline phosphatase overexpression induced by engineered bacteriophage T7 infection on *E. coli*	< 10 CFU/mL within 8 h	(Singh *et al.*, 2019)

To the best of our knowledge, most of these studies have focused on using soluble substrates whose chromatic products will be dissolved in the bulk solution. This limits the detection sensitivity compares to using substrates that yield localized enzymatic products inside the infected cells. Moreover, using soluble substrates requires the release of enzyme from cells to the aqueous phase, which may not be efficient as alkaline phosphatase have high molecular weight of 94 kDa. In our prior research, we have observed significant retention of cellular content including DNA and cell membrane after bacteriophage lysis of cells (Yang et al., 2020). Thus, extended time of incubation may result in more efficient release of enzymes from residual cellular content after lysis as well as multiple repeat cycles of phage infection, and expression of exogenous enzyme can increase the effective enzyme concentration.

In order to increase the sensitivity and simplicity of the detection, the substrate that forms a colorimetric precipitated product after the enzymatic reaction, NBT/BCIP, was investigated in this study. This substrate has been used as the substrate for alkaline phosphatase in the in-situ hybridization for gene expression study (Jékely and Arendt, 2007; Trinh et al., 2007). The dark purple insoluble precipitated product is the result of the enzymatic reaction and it can be visualized by the naked eye. The use of this substrate to detect bacterial alkaline phosphatase was supported by the study of Hinkley et al. (2018) which reported that BCIP substrate can be used to visualize bacterial colonies infected by engineered bacteriophage with alkaline phosphatase. This method allows quantitative analysis of bacteria within 10 hours of operation (Hinkley et al., 2018). For the detection method developed in this study, the samples after bacteriophage infection were filtered to capture bacterial cells with alkaline phosphatase. Since the product of NBT/BCIP is water-insoluble and will localize inside bacterial cells, the release of the enzyme was not required. The enzymatic product can be concentrated simultaneously with the bacterial cells by filtration

which can improve the detection sensitivity. The substrate can be applied directly to the filter and the color formation can be visualized within 10 minutes without any instruments or imaging system. As bacteriophage T7 is highly specific to *E. coli* infection; therefore, other bacteria present in the sample will not be infected thereby there is no overexpression of alkaline phosphatase which eliminates the cause of the false-positive result. Prior to this study, the T7-ALP has been tested against food and agriculture-related bacterial strains including *P. fluorescens* and *L. innocua* and the results indicated no significant increase of alkaline phosphatase after bacteriophage T7-ALP infection (Wisuthiphaet et al., 2019).

Compared to the previous calorimetric bacteriophage-based detections, the detection approach developed in this study offers a rapid detection method that can be applied to detect target bacteria in beverage and fresh produce samples with a more realistic sample size. The results indicated the successful detection of bacteria 10 CFU/ml and 10^2 CFU/g in coconut water and spinach leaves with 5-hour enrichment. However, for the food samples containing solid particles and pigments like spinach, the filtration step could limit the detection efficiency. Due to the solid plant particles, the sample volume for filtration is limited which affects the detection sensitivity. Moreover, the leaf pigments can interfere with the color readout. The higher sample volume results in more food debris and local microbes which leads to reduced filtration volume and detection sensitivity. Therefore, the reaction time was extended to 2 hours to increase the alkaline phosphatase level to achieve the detection limit of 10^2 CFU/g of bacteria. The detection using pNPP substrate is not applicable since the centrifugation step did sediment all the debris and local microbes that might outgrow the target bacteria causing high turbidity of the samples and preventing an accurate absorbance measurement.

Further refinement of the detection procedure is still needed in order to develop more efficient detection methods that can be applied to detect bacteria in complex food samples by focusing on eliminating the background noise from food particles and non-target microbes. Multistep filtration can be introduced to remove coarse suspended solids in the samples before harvesting the bacteriophage-infected bacteria. Selective media can be applied during the enrichment step to support the growth of the target bacteria and reduce the proportion of other non-target microbes. In addition, quantitative analysis of bacteria using this detection method can be further investigated as well as combining this detection approach with digital imaging and image processing also improve the detection specificity and sensitivity.

3.5. Conclusion

This study demonstrates an isolation-free rapid bacterial detection strategy using engineered bacteriophage to induce alkaline phosphatase expression. The NBT/BCIP substrate of alkaline phosphatase allows the rapid colorimetric bacterial detection. The proposed method was validated to detect *E. coli* in complex food samples, coconut water and baby spinach. The detection limit of 10 CFU/ml of bacteria in coconut water was achieved within 6 hours of operation. For baby spinach leaves, 10^2 CFU/g of bacteria can be detected with an extended detection time of 8 hours. Further study may be conducted in order to address some of the drawbacks including limitations of filtering large volume samples with food particulate matter and analyzing large number of samples.

3.6. Potential future works

3.6.1. Improving the detection sensitivity in complex matrices by using cerium oxide nanoparticles

One of the limitations of the colorimetric detection methods is that the color of the food samples may interfere with the color generated by the enzymatic reaction of alkaline phosphatase and the substrates which results in reduced detection sensitivity. Enhancing the intensity of the color formation is an approach to improve the detection of alkaline phosphatase. Besides the colorimetric substrates used in this chapter, alkaline phosphatase can be detected based on the surface reactivity and optical properties of redox-active nanoparticles of cerium oxide, or nanoceria (Hayat et al., 2014). Substrates of alkaline phosphatase are phosphate compounds whose phosphate ester functional group can be hydrolyzed by the enzymatic reaction yielding respective alcohol products (Preechaworapun et al., 2008). The alcoholic products are then oxidized by nanoceria and form a brownish complex (Hayat et al., 2014).

This approach can be applied for the detection of alkaline phosphatase induced by T7-ALP phage infection. A substrate of alkaline phosphatase such as ascorbic 2-phosphate substrate can be added to the samples after phage infection. After the enzymatic reaction, the product which is ascorbic acid interacts with nanoceria and generates a brown complex that can be visualized by the naked eye. The sensitivity of this detection approach can be evaluated in comparison with the results reported in this chapter. The improvement in the detection sensitivity will provide the potential for *E. coli* detection in more complex food products with an intense background color.

3.6.2. Machine learning for color classification

The complexity of the food samples and their natural color may create background noise that results in reduced detection sensitivity. One approach that could improve the detection sensitivity is to applied machine learning algorithms for color classification. The images of filters after adding colorimetric substrate will be acquired using a smartphone. The RGB value of the color images can be extracted using MATLAB and used as a data set to train machine learning classification models. There are numerous classification models that can be applied in this approach. For example, a support vector machine (SVM), a supervised classification model with a nonparametric statistical learning technique used to distinguish data from different classes by constructing a hyperplane (Mutlu et al., 2017; Castro et al., 2019). Multi-layer neural network (NN) is another classifier that has been used for paper chromogenic array (PCA) color classification for the detection of bacteria in food (Yang et al., 2021).

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CHAPTER 4:

Rapid detection of bacteria based on bacterial cell morphology change induced by bacteriophage infection[3]

Abstract

Rapid detection of bacterial pathogens is a critical unmet need for both food and environmental samples such as irrigation water. As a part of the Food safety Modernization Act (FSMA), The Produce Safety rule has established several requirements for testing for the presence of generic *Escherichia coli* in water, but the current method available for testing (EPA M1603) demands specified multiple colony verification and highly trained personnel to perform these tests. The purpose of the study was to assess phage-induced bacterial lysis using quantitative image analysis to achieve rapid detection of *E. coli* at low concentrations within 8 hours. This study aimed to develop a simple yet highly sensitive and specific approach to detecting target bacteria in complex matrices. In the study, *E. coli* cells were first enriched in tryptic soy broth (TSB), followed by T7 phage-induced lysis, concentration, staining, and fluorescent imaging. Image analysis was conducted including image pre-processing, image segmentation, and quantitative analysis of cellular morphological features (area, eccentricity, and full width at half maximum). Challenge experiments using realistic matrices, including simulated fresh produce wash water, coconut water, and spinach wash water, demonstrated the method can be applied for use in situations that occur

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in food processing facilities. The results indicated *E. coli* cells that are lysed by T7 phages demonstrated significantly ($P < 0.05$) higher extracellular DNA release, altered cellular shape (from rod to circular), and diffused fluorescent signal intensity. Using this biosensing strategy, a sensitivity to detect *E. coli* at 10 CFU/ml within 8 hours was achieved, both in laboratory medium and in complex matrices. The proposed phage-based biosensing strategy enables rapid detection of bacteria and is applicable to the analysis of food systems. Furthermore, the steps involved in this assay can be automated to enable the detection of target bacteria in food facilities without extensive resources.

Keywords: Fluorescence imaging, Escherichia coli, Bacterial detection, Bacteriophage, Image analysis

4.1. Introduction

As part of the Food Safety Modernization Act (FSMA), The Produce Safety rule has established several standards for testing for the presence of generic *Escherichia coli* in water. For instance, no *E. coli* shall be detected in water that is directly used to contact any fresh produce after harvest or food-contact surfaces. In addition, agricultural water that is applied for irrigation produces crops should contain equal to or less than 126 colony-forming unit (CFU) per 100 ml of tested water (FDA, 2015). To comply with The Produce Safety rule, sensitive, cost-effective and rapid methods for *E. coli* detection are desired. Ideally, a method for *E. coli* detection should be completed within 8-hours to match the typical work-shift schedule of most fresh produce packaging operations with a product that has a relatively short shelf-life (NRDC, 2017). The current method for generic *E. coli* detection in agricultural water is based on the U.S. Environmental Protection Agency Method 1603 (EPA M1603). This method requires specified multiple colony verification and highly trained personnel to perform a test. Furthermore, there is significant subjectivity in evaluating the false-positive results (EPA/US EPA (US Environmental Protection Agency), 2007).

Complementary to conventional detection approaches, lytic bacteriophages (phages) have also been evaluated as a bio-sensing element for the detection of bacteria. The extraordinary host specificity of phages for their host provides a naturally occurring event that can be co-opted into a method for bacterial pathogen detection (Bergh et al., 1989). In addition, the rapidity of phage multiplication provides a "built-in" amplification step that can be detected within a time frame of hours (Brovko et al., 2012). Based on these advantages, a variety of phage-based biosensing technologies have been developed. Phage typing is a classical phage-based culture method for detecting specific bacterial pathogens. The formation of a visually observed clear plaque indicates

the presence of a bacterial host specific to the phages supplemented (Baggesen et al., 2010). The optical approach to detection involves monitoring the decrease of turbidity by using a spectrophotometer (Anany et al., 2018). However, both visual and optical methods need a relatively high number of host cells, which could be time-consuming. Detection of phage-bacteria complex formation is another phage-based biosensing strategy. The phage-bacteria complex forms upon infection with high specificity and stability. One approach to detect the complex is to fluorescently label phages, followed by absorption of phages onto bacteria. Then, by using flow cytometry or fluorescent microscopy, the phage-bacteria complex can be detected (Hermes and Suttle, 1995; Parish and Goodrich, 2005; Lee et al., 2006). The assay is simple and straightforward, but the fluorescent signal from only phages has a low signal-to-noise ratio (Edgar et al., 2006). Another phage-based biosensing technique is through the utilization of reporter phages, which carry genetically modified reporter genes to manipulate the host bacteria's metabolic process (Brovko et al., 2012). Several studies have exploited this concept to develop genetically modified reporter phages to overexpress β-galactosidase or alkaline phosphatase (Jackson et al., 2016; Chen et al., 2017; Wisuthiphaet et al., 2019). However, the current approaches based on reporter phages may add more complexity and capital cost to the detection process.

As described above, a variety of phage-based biosensing approaches have been developed, but each method has some limitations. The current study was aimed at developing a novel, rapid and sensitive detection for *E. coli* through microscopy and image analysis. Imaging using fluorescence microscopy combined with automated image analysis is a promising alternative approach that allows improved sensitivity and reduced complexity of bacteria detection method protocols.

Microscopic imaging is able to focus on the fluorescence signal from an individual bacterial cell; therefore, it provides a single-cell level of detection sensitivity (Cortesi et al., 2017). Implementing imaging and image analysis for bacteria detection has the potential to develop detection methods that require simple instrumental setups such as fluorescence microscopes or even the miniaturized versions of microscopes for field detection. The imaging procedure and image analysis can be automated which allows a simple and user-friendly detection protocol with no specialized trained personnel required, and the simplified protocol results in more time-effective detection.

Moreover, imaging and image analysis processes can be performed in the field since the aseptic technique environment is not required, unlike nucleic acid-based detection approaches which need to be conducted in a molecular laboratory. In the food industry, imaging and image analysis may not be commonly employed in food safety and quality control assay, but this approach has been widely used for medical diagnostic purposes. Currently, imaging procedures have been applied to detect the malaria parasite, *Plasmodium*, infection of red blood cells, based on cellular morphology using simple cellphone-based microscopy which is applicable to low-cost in-the-field optical diagnostics of malaria (Agbana et al., 2018).

The biosensing strategy in this study focuses on the detection of *E. coli* through phage-induced lysis in authentic food samples and artificial wash water supplemented with organic chemicals simulating a chemical oxygen demand (COD) of wash water. Phages are well characterized for producing endolysin which could induce explosive host cell lysis (Loessner, 2005). As shown in Figure 4.1, the life cycle of lytic T7 phages starts when the specialized adsorption structures, called T7 tail fibers, bind to receptor molecules on *E. coli* BL21 (Step 1). Injection of phage DNA occurs when the phage tail tube punctures the membrane layers of *E. coli*,

which is followed by injection of the viral genome into the cytoplasm of the host cell (step 2) (Kostyuchenko et al., 2003, 2005). Immediately after injection of the T7 phage genome, replication of the phage genome occurs; a process that involved co-opting of host cellular machinery. During this stage, numerous copies of the phage genomic DNA are synthesized (step 3). Subsequently, protein subunits of the phage particle are synthesized and assembled into procapsids. During the final step, a copy of the phage genomic DNA is packaged procapsids (step 4). Mature phages are then liberated by host cell lysis with the aid of two specific enzymes, holin and lysin. Bacterial host cells that undergo phage-induced lysis in Figure 4.1 illustrate a significant change in their cellular morphology, proposed as a "rod-to-round" transition (Turnbull et al., 2016), followed by intracellular to extracellular DNA release, defined as environmental DNA (eDNA). Cellular morphology changes and eDNA release from host bacteria can be visualized under a microscope, coupled with simple DNA stains and further fluorescence image analysis using BacFormatics v0.7 developed in MATLAB (Turnbull et al., 2016). The key advantage of fluorescence image analysis is the higher signal-to-noise ratio and increased detection sensitivity, which hypothetically allows low detection limit at low concentrations of *E. coli* in a variety of different food matrices.

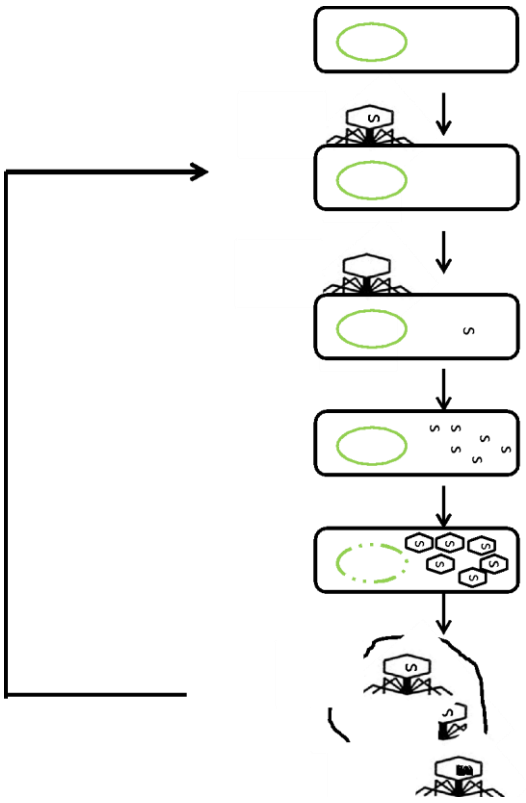

Figure 4.1. Life cycle of lytic T7 phages. Step 1, attachment of T7 phage on the host; step 2, phage DNA injection into the bacterial host; step 3, phage hijack bacteria metabolism and multiplication of phage DNA; step 4, production of new capsids and assembly of phages; step 5, host cell lysis and eDNA release.

4.2. Materials and Methods

4.2.1. Chemical reagents

SYBR Green I nucleic acid stain, 10,000× concentrate was purchased from Invitrogen, USA, and a working solution of 10× SYBR Green I was prepared in Milli-Q water. p-Phenylenediamine and chloroform were obtained from Acros Organic (Fair Lawn, NJ, USA) and a 10% (wt/vol) stock solution of p-phenylenediamine was prepared in Milli-Q water. Whatman® Anodisc inorganic filter membrane (13 mm, 0.02 µm pore size) was obtained from GE Healthcare (Buckinghamshire, UK). Microscopic slides were obtained from VWR international (Radnor, PA, USA). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The filtration system, cover glass, and Luria Bertani (LB) broth were obtained from Fisher Scientific (Pittsburgh, PA, USA). Phosphate buffered saline (PBS) was purchased from Fisher Bioreagents (Fair Lawn, NJ, USA). Polycarbonate filters (20 um pore size, 47 mm diameter) were purchased from Maine Manufacturing (ME, USA). Milli-Q water was produced by QPAK® 2 purification system (EMD Millipore, Billerica, MA, USA).

4.2.2. Bacterial cultures and phage preparation

Both *E. coli* BL21 (ATCC BAA-1025) and bacteriophage T7 (ATCC BAA-1025-B2) were obtained from American Type Culture Collection. *E. coli* BL21 was cultured in TSB broth at 37 °C for 16 hours before use.

Bacteriophages were propagated as the following procedure. Bacteriophages were first inoculated into log-phase *E. coli* BL21 culture at the ratio of 1:100 (phage:bacteria). The mixture was then incubated at 37 ºC for 15 min for initial infection and then centrifuged at 16100 × *g* for 10 min. The supernatant was discarded and the same volume of TSB was added to

resuspend the pellet, followed by incubating at 37 ºC with 200 rpm shaking until no visible turbidity was observed. Chloroform was then added to the final concentration at 20% (vol/vol) and incubated at 4 ºC overnight. Then, the chloroform added mixture was centrifuged at 5,000 × *g* for 10 min and the water phase was collected. The water phase which contained free phages, TSB medium, and bacterial host lysis debris was then centrifuged again at 16100 × *g* for 10 min. Pellet was washed for one time and resuspended in PBS at the population of 10^9 PFU/ml phage concentration.

4.2.3. T7 phage-induced E. coli cell lysis.

T7 phage-induced *E. coli* cell lysis was conducted in two modes: low titer co-incubation lysis (LTCL) and high titer two-step lysis (HTTL). LTCL refers to co-incubation of low initial phage concentration (10^2 PFU/ml) with *E. coli* in TSB. LTCL allows for the initial growth of *E. coli* with delayed lysis. HTTL refers to enriching *E. coli* first in TSB, followed by inoculation of high titer phage concentration (10^7 PFU/ml) to induce lysis for 20 min.

4.2.4. E. coli cellular morphological change and eDNA release during LTCL.

The dynamic morphological change of *E. coli* and release of eDNA were analyzed through LTCL during extended lysis period as reported earlier (Anany et al., 2018; Tilton et al., 2019). Briefly, overnight *E. coli* BL21 cultures were precipitated, washed, and resuspended in PBS. A 50-ml falcon tube containing 10 ml TSB pre-warmed at 37 °C was prepared and inoculated with 10^3 CFU/ml *E. coli* BL21 and 10^2 PFU/ml T7 phage. The inoculated tube was then incubated at 37 °C in a shaking incubator for 2, 3, and 4 hours and 2 ml of the aliquot was taken at each time point, respectively. Then, aliquots from each time point were filtered through Anodiscs and subsequently stained with SYBR Green I. The filtration and staining process was conducted

according to a published protocol (Patel et al., 2007). Briefly, the filtration system was made up of a filtering flask, a fritted glass base, and a polycarbonate filter. The Anodisc filter was directly located on top of the polycarbonate filter, with the glass base and filtering flask attached underneath. An aliquot from each tube was gently pipetted onto their respective Anodisc for filtration. After filtration, the Anodisc was carefully removed from the polycarbonate filter onto a microscopic slide pre-spotted with 20 µl of SYBR Green I stock solution. The Anodisc was then transferred directly onto the SYBR Green I drop with the backside down since the stain can easily pass through the Anodisc to stain the microorganisms on the topside. The Anodisc was later stained in a dark laboratory bench drawer for 20 min before the dye was removed by gently rubbing the backside Anodisc filter against a Kimwipe. In the meanwhile, 1% of *p*-phenylenediamine was prepared from the 10% stock solution as an anti-fading reagent. After staining and removal of excess dyes, the Anodisc was transferred directly onto a new microscopic slide with a 20 µl anti-fading reagent drop. The Anodisc was eventually covered with a cover glass and observed under an Olympus IX-71 inverted research fluorescence microscope with a ×100 (1.25 NA) objective lens, a CCD (charge-coupled device) camera (Model C4742-80-12AG, Hamamatsu, Tokyo, Japan) and Metamorph imaging software (version 7.7.2.0, Universal Imaging Corporation). An average of 10-12 images were taken for each Anodisc sample. The fluorescence excitation/emission wavelength of SYBR Green I stain was 480 ± 30 and 535 ± 40 nm, respectively. Negative controls were performed at the same condition except for adding phages. All conditions including negative controls were performed in triplicates.

4.2.5. Characterization of E. coli morphological change and eDNA release through LTCL.

A similar experimental procedure was followed as described in the previous section. Three 50 ml falcon tubes (A, B, and C) containing 10 ml TSB were prepared and inoculated with 10^2

PFU/ml T7 phages in each tube. Tube A received *E. coli* inoculation at 10^2 CFU/ml, tube B received *E. coli* inoculation at 10^3 CFU/ml while tube C received *E. coli* inoculation at 10^4 CFU/ml. All tubes were then incubated at 37 °C with 200 rpm shaking for 2, 3, and 4 hours, respectively. After incubation, contents from each tube were filtered onto their respective Anodisc, and subsequently stained and observed under a fluorescence microscope as described earlier. Negative controls were conducted at the same condition without phage inoculation. Both phage-supplemented groups and negative controls were performed in triplicates.

4.2.6. Characterization of E. coli morphological change and eDNA release through HTTL.

To start with, three 50 ml falcon tubes (A, B, and C) each containing 5 ml of TSB were prepared and pre-warmed in a 37 °C water bath before inoculation. Overnight *E. coli* BL21 cultures were precipitated, washed, and resuspended in PBS, followed by inoculation in tubes A, B, and C at 10^1, 10^2, and 10^3 CFU/ml, respectively. All tubes were then incubated at 37 °C with 200 rpm shaking for 5, 4, and 2 hours, respectively. After incubation, T7 phages were inoculated in all tubes at 10^7 PFU/ml, followed by lysis under the same condition for 20 min. Then, aliquots from each tube were filtered through Anodiscs, and were subsequently stained with SYBR Green and observed under the microscope as described earlier. Negative controls were performed at the same condition without phage addition. All conditions including negative controls were performed in triplicates.

4.2.7. Validation of the biosensing approach in realistic food items.

HTTL was chosen to be a more robust, consistent, and sensitive approach for analyzing morphological change and eDNA release of *E. coli* in realistic food matrices, including artificial wash water, coconut water, and spinach wash water. Coconut water was selected as a challenging

matrix as it contains sugars, amino acids, vitamins, and phytohormones (Yong et al., 2009). Artificial wash water was created by adding LB broth to sterile water to achieve a final COD at 1,000 ppm, which is comparable to fresh-cut produce wash water in the industry (Elizaquível et al., 2012). Both coconut water and artificial wash water were inoculated with *E. coli* at 10 CFU/ml, followed by mixing with double strength TSB at 1:1 (vol/vol) ratio for pre-enrichment. The mixture was then aerobically incubated at 37 °C for 5 hours and subsequently lysed with 10^7 PFU/ml T7 phages for 20 min. Content after lysis was filtered onto Anodisc, stained, and visualized under a microscope. Negative control without phage lysis was also conducted and both conditions were performed in triplicates. For spinach wash water, detailed sample preparation is described in the supporting information.

4.2.8. Image analysis - preprocessing.

All images were subjected to analysis by Matlab™ 2017a software (Mathworks, Natick, Mass., USA) to generate binary images, followed by quantification of morphological features. Before binary images conversion, a series of image pre-processing functions were applied to enhance image visual experience, remove uneven background illumination and adjust for fluctuation in data acquisition. Specifically, to best represent the visual appearance of images, *strel* function was applied to create a disk-shaped structuring element, at a radius of 30 pixels (Blahuta et al., 2011). Then, *imtophat* function was applied to remove uneven background illumination from an image with a dark background (Ravindran, 2016). Images were then processed by median filtering (*medfilt2* function) in two dimensions: each output pixel equals the average of the median value in a 3 × 3 adjacent corresponding pixels in the input image. By applying the median filtering, the fluctuations of the signal during the acquisition process can be removed, without compromising the sharpness of the image (Altan et al., 2011). To enhance the contrast of the grayscale images,

adapthisteq function was then used to adjust images, followed by smoothing the images using a low-pass Wiener filter. The Wiener filter removes the constant additive noise (Gaussian white noise) to preserve edges or other high-frequency parts of an image (Altan et al., 2011).

4.2.9. Image analysis – image segmentation and quantification of morphological features.

The overall goal of image segmentation was to identify the boundaries of each *E. coli* cell and measure changes in cellular areas and shapes. Segmented images were then converted to binary images based on a global threshold. Binarization of images was a key step toward quantitative interpretation of image data from computer-aided machine vision, as opposed to human vision. To be more specific, *graythresh* function was applied to produce a global threshold. The *graythresh* function was based on Otsu's method which minimizes the intraclass variance between black and white pixels (Otsu, 1979). Then, the binary images were created based on the threshold generated from *graythresh* function. Basically, any point (x, y) in the input image which has the function of f (x, y) ≥T is going to be designated as a white pixel (object) while other points which have f (x, y) < T is going to be served as a black pixel (background). Generation of the binary image was created by *imbinarize* function (Altan et al., 2011). To further ease the process of image analysis, *imfill* function was applied to fill holes in the input binary image. The holes are defined as background pixels (black) that are surrounded by the object pixels (white) (Altan et al., 2011). In several cases, some small particles may show up as SYBR green I can occasionally stain non-specifically on Anodisc. The function *bwareaopen* was then applied to remove any particles that are less than 300 pixels (Nanduri et al., 2009).

After binary images were created, modified, and cleaned, quantification of morphological features was achieved using *regionprops* function. Bacteria did not undergo phage-induced lysis has a rod shape, with a confined DNA region. In comparison, bacteria that underwent phage lysis

appeared as round shape, with a diffuse distribution of released eDNA stained by SYBR Green I. To statistically and objectively compare the cellular morphology change and release of eDNA, “area” and “eccentricity” were chosen as two parameters representing the relative cellular morphology change. The property ‘area’ refers to the actual number of pixels in an object region which can be correlated with eDNA release (bigger area, more eDNA release) and the property “eccentricity” refers to the shape of an ellipse, and it measures the distance ratio between the foci of the ellipse and the length of its major axis. The ratio value of “eccentricity” is between 0 to 1 where 0 refers to a circle and 1 refers to a line segment. “Eccentricity” parameter is associated with the shape of bacteria where the rod shape bacteria have eccentricity closer to 1 while lysed round bacteria shape has eccentricity closer to 0.

The fluorescence intensity distribution was another morphological property chosen to detect the cellular shape change due to the release of the internal DNA which was represented by the full width at half maximum (FWHM) of the intensity distribution curve. For image analysis, all fluorescence images were analyzed by ImageJ. The ImageJ FWHM_Line plug-in was used to generate a two-dimensional graph of the intensities of pixels along a 100-pixel line that was drawn across the cross-section of individual cells. The intensity distribution data were fitted to the Gaussian distribution and the full width at half maximum was measured.

4.2.10. Statistical analysis.

All conditions that were subjected to area, eccentricity, and intensity distribution analysis were conducted in triplicate and from each replicate, 5-8 images per replicate were selected for analysis. A total number of 15-20 images were analyzed, containing total objects equal to or larger than 100 ($N \geq 100$). Area, eccentricity, and FWHM values were generated from each image and averaged. Mean value of area, eccentricity, and FWHM were statistically compared between

phage-lysed images and their corresponding negative controls, using SAS 9.4 (SAS Institute Inc., Cary, NC) by Tukey's honest significant difference (HSD) test to compare the means.

4.3. Results

4.3.1. E. coli cellular morphological change and eDNA release during LTCL.

The overall goal of this set of experiments was to develop an imaging-based framework for characterizing changes in cellular morphology and release of eDNA following the lysis of *E. coli* cells with phages during LTCL incubation conditions. As represented in Figure 4.2, the initial phage concentration was 10^2 PFU/ml and *E. coli* BL21 was at 10^3 CFU/ml in TSB. The phage-*E. coli* mixture was then incubated for 2 and 3 hours at 37 °C and images were captured as shown in Figures 4.2a and c, respectively. In comparison, the negative control without adding phages was also performed under the same conditions, as shown in Figures 4.2b and d, respectively. As illustrated in Figures 4.2a and b, co-incubating phage and bacteria for 2 hours did result in observed cellular morphology change or eDNA release. After 3 hours, cell lysis was observed, as shown in Figure 4.2c, with the transition from rod-shaped cells to round-shaped cells. The release of eDNA, as seen in Figure 4.2c, was also noticeable; appearing as enlarged cellular areas of fluorescent signal with a fuzzy boundary which increased the intensity distribution of the cell particles. Neither cell morphological changes nor the appearance of any eDNA signal was evident for the negative controls (Figure 4.2d).

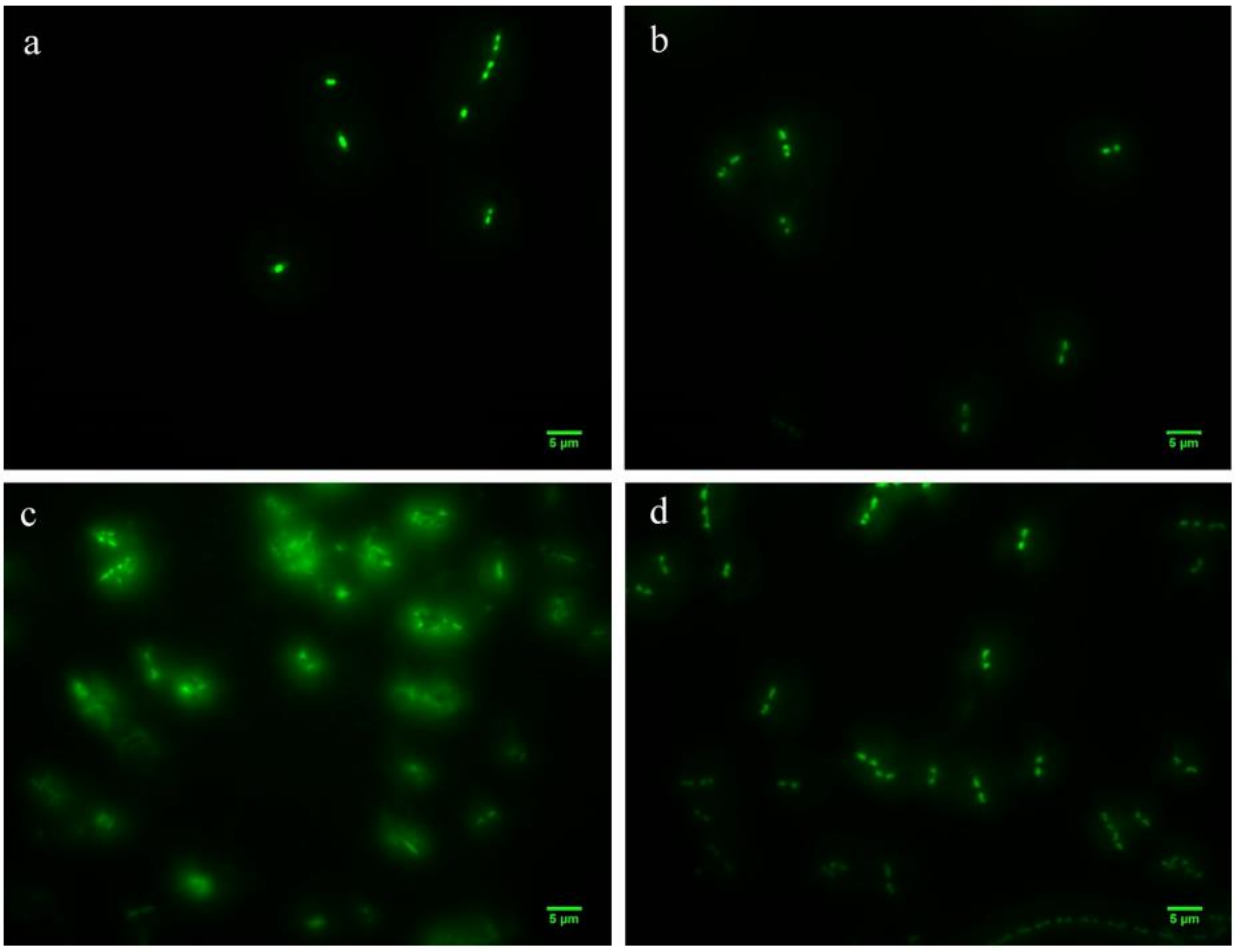

Figure 4.2. Bacteria morphology changes during phage-induced lysis while co-incubating *E. coli* and T7 phages for 2 and 3 hours. (a) cell morphology when co-incubating *E. coli* and T7 phages for 2 hours. (b) cell morphology when incubating just *E. coli* for 2 hours. (c) cell morphology when co-incubating *E. coli* and T7 phages for 3 hours. (d) cell morphology when incubating just *E. coli* for 3 hours.

4.3.2. Limit of detection of E. coli based on morphological change and eDNA release through LTCL.

In this approach, *E. coli* were co-incubated with a low phage titer to allow initial propagation of bacteria, followed by phage lysis. Area, eccentricity, and the FWHM parameter, taken together, were evaluated to distinguish "intact" *E. coli* cells from *E. coli* that are "lysed by phages". As indicated in Figure 4.3, *E. coli* at 10^3 CFU/ml can be detected based on changes in the selected morphological particles following incubation with 10^2 PFU/ml of T7 phages. Figures 4.3a and b illustrate the microscopic image and binary image of negative control (only *E. coli* was incubated for 3 hours). In comparison, Figures 4.3c and d illustrate microscopic images and binary pictures of *E. coli*-T7 phage for the LTCL incubation condition. Based on image analysis methods as described in the previous section, area and eccentricity values for *E. coli* lysed by T7 phages demonstrate significant differences compared to the control ($P < 0.05$). In summary, *E. coli* lysed by T7 phages revealed significant eDNA release and changes from a rod to circular morphology.

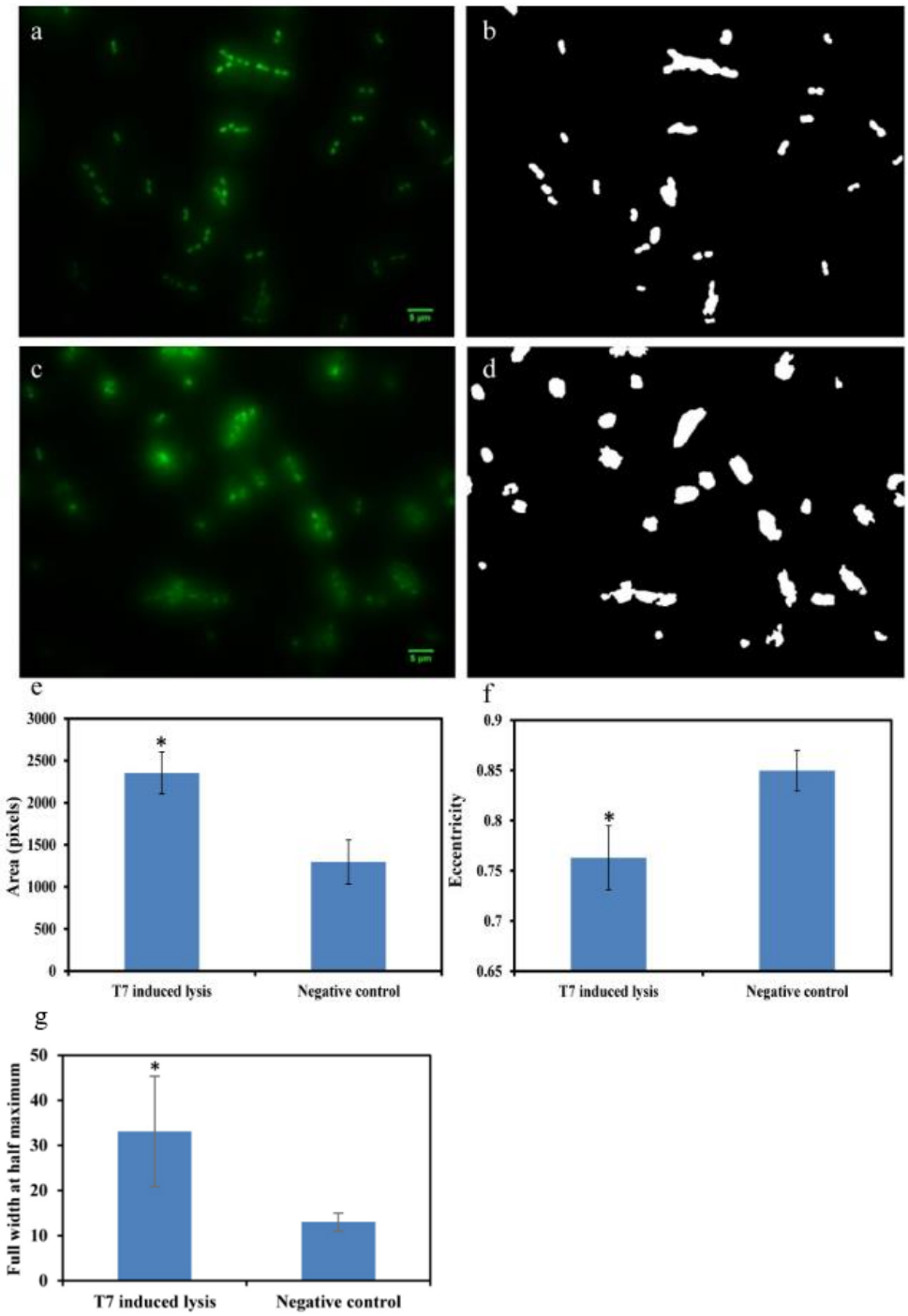

Figure 4.3. Detection of 10^3 CFU/ml *E. coli* through LTCL with 10^2 PFU/ml T7 phage. (a) negative control which contains only *E. coli* growing for 3 hours. (b) binary image of (a). (c) phage-induced lysis while co-incubating with *E. coli.* (d) binary image of (c). (e) comparison of area values extracted from (b) and (d). (f) comparison of eccentricity values extracted from (b) and

(d). (g) comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

Detection of *E. coli* at an initial concentration of 10^2 and 10^4 CFU/ml were also conducted similarly using the LTCL incubation conditions. Resultant images were shown, analyzed, and compared in Figure 4.4 and 4.5, respectively. As shown in Figure 4.4, no significant difference ($P > 0.05$) was observed for all parameters (area, eccentricity, and FWHM) between images from samples of *E. coli* incubated with the T7 phage and negative controls, indicating *E. coli* at an initial concentration of 10^2 CFU/ml cannot be detected using LTCL incubation conditions and image analysis, due to lack of significant cell lysis. In comparison, *E. coli* at an initial concentration of 10^4 CFU/ml can be detected through LTCL, image analysis and comparison to area, eccentricity and FWHM parameters (Figure 4.5).

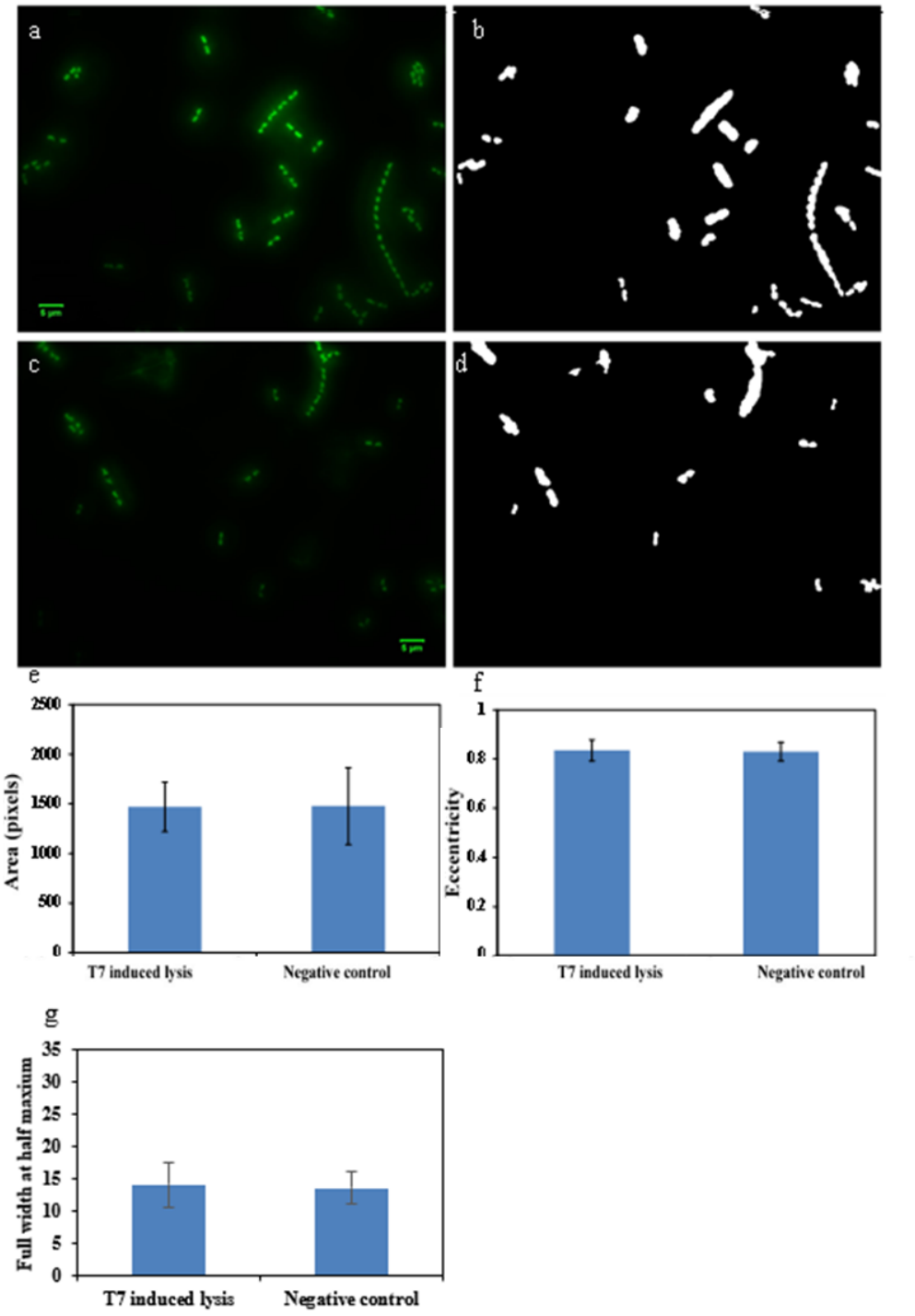

Figure 4.4. Detection of 10^2 CFU/ml *E. coli* through LTCL with 10^2 PFU/ml T7 phage for 4 hours. (a) negative control which contains only *E. coli* growing for 4 hours. (b) binary image of (a). (c) phage-induced lysis while co-incubating with *E. coli*. (d) binary image of (c). (e) comparison of

area values extracted from (b) and (d). (f) comparison of eccentricity values extracted from (b) and (d). (g) comparison of the full width at half maximum extracted from (a) and (c).

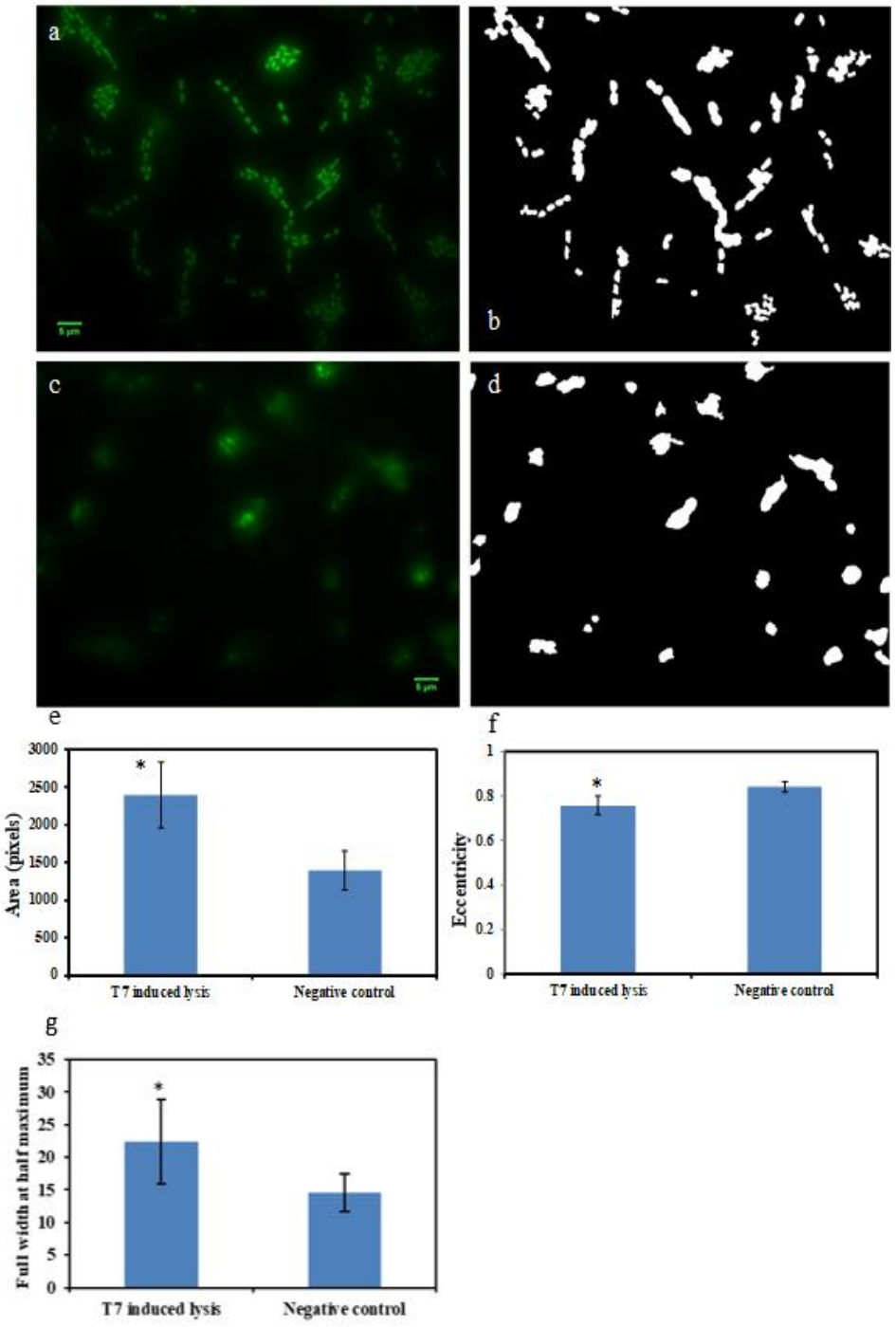

Figure 4.5. Detection of 104 CFU/ml *E. coli* through LTCL with 102 PFU/ml T7 phage for 2 hours. (a) negative control which contains only *E. coli* growing for 2 hours. (b) binary image of (a). (c) phage-induced lysis while co-incubating with *E. coli*. (d) binary image of (c). (e) comparison of area values extracted from (b) and (d). (f) comparison of eccentricity values

extracted from (b) and (d). (g) comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

4.3.3. Characterization of E. coli morphological change and eDNA release through HTTL.

E. coli at 10 CFU/ml can be detected by HTTL as shown in Figure 4.6. Figure 4.6a and b indicate the microscopic images and binary picture of negative control where *E. coli* was incubated for 5 hours. In comparison, Figures 4.6c and d represents the microscopic image and binary picture of *E. coli* lysed by T7 phage through HTTL. Quantitative image analysis comparisons between Figure 4.6b and d are presented in Figures 4.6e-g. To be specific, area difference was compared and summarized in Figure 4.6e where *E. coli* cells lysed by T7 phage demonstrated larger stained areas due to DNA leakage. Shape changes were also observed in Figure 4.6f where more circular morphology of *E. coli* was generated after T7 phage-induced lysis. In addition, diffused fluorescent signal intensity from T7 phage lysed *E. coli* cells were observed in Figure 4.6g based on quantification of FWHM parameters compared to control. In summary, all area, eccentricity, and FWHM parameters indicated a statistical difference between HTTL and negative controls ($P < 0.05$), indicating *E. coli* at an initial concentration of 10 CFU/ml can be detected through HTTL and image analysis.

Detection of *E. coli* at initial concentrations of 10^2 and 10^3 CFU/ml was also achieved through HTTL, image analysis, and comparison of area, eccentricity, and FWHM was presented in Figure. 4.5 and 4.6, respectively.

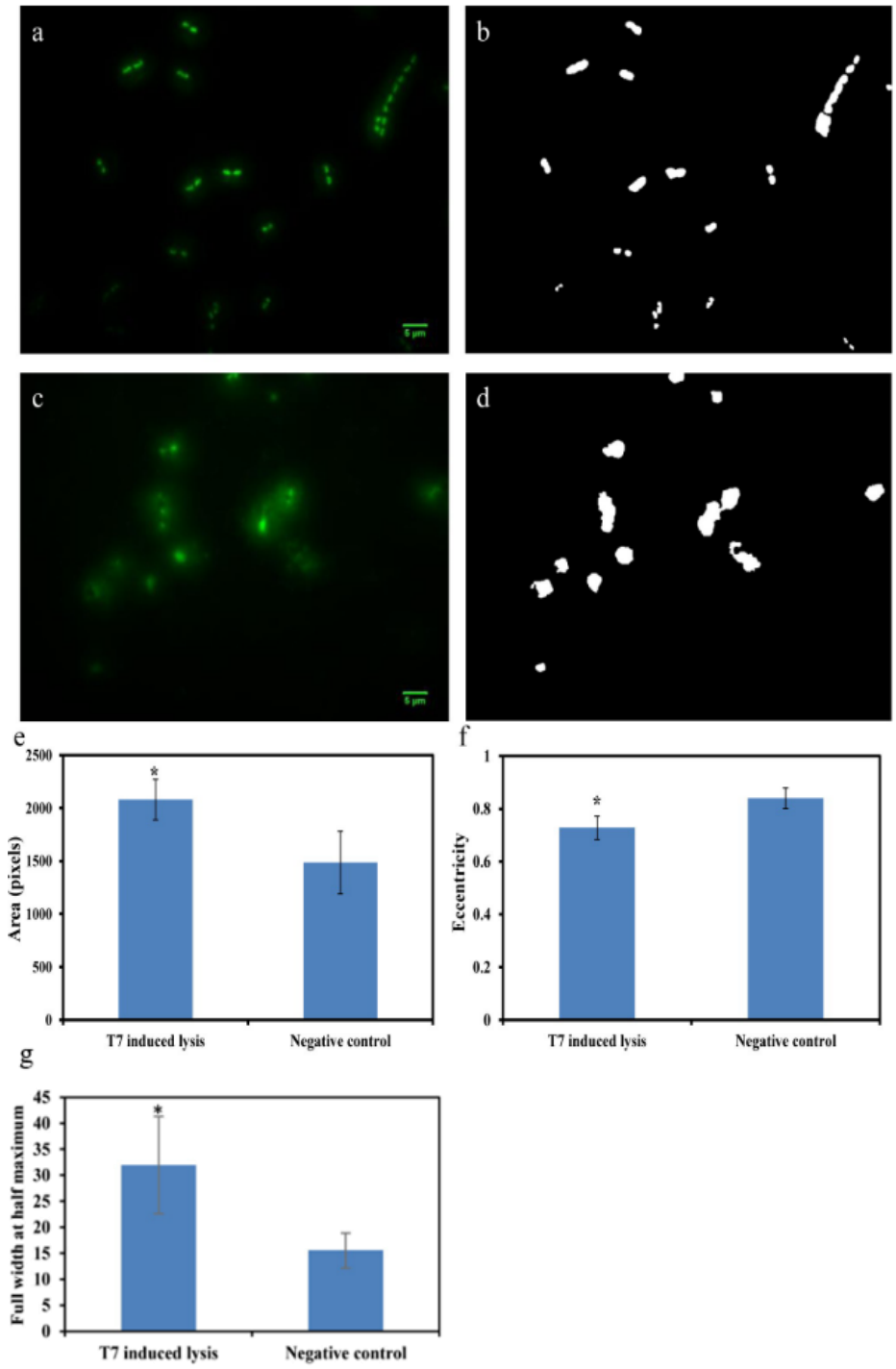

Figure 4.6. Detection of 10 CFU/ml *E. coli* through enrichment and HTTL (a) negative control which contains only *E. coli* growing for 5 hours. (b) binary image of (a). (c) phage-induced lysis after *E. coli* enrichment. (d) binary image of (c). (e) comparison of area values extracted from (b)

and (d). (f) comparison of eccentricity values extracted from (b) and (d). (g) comparison of the full width at half maximum extracted from (a) and (c). * indicated significant difference ($P < 0.05$).

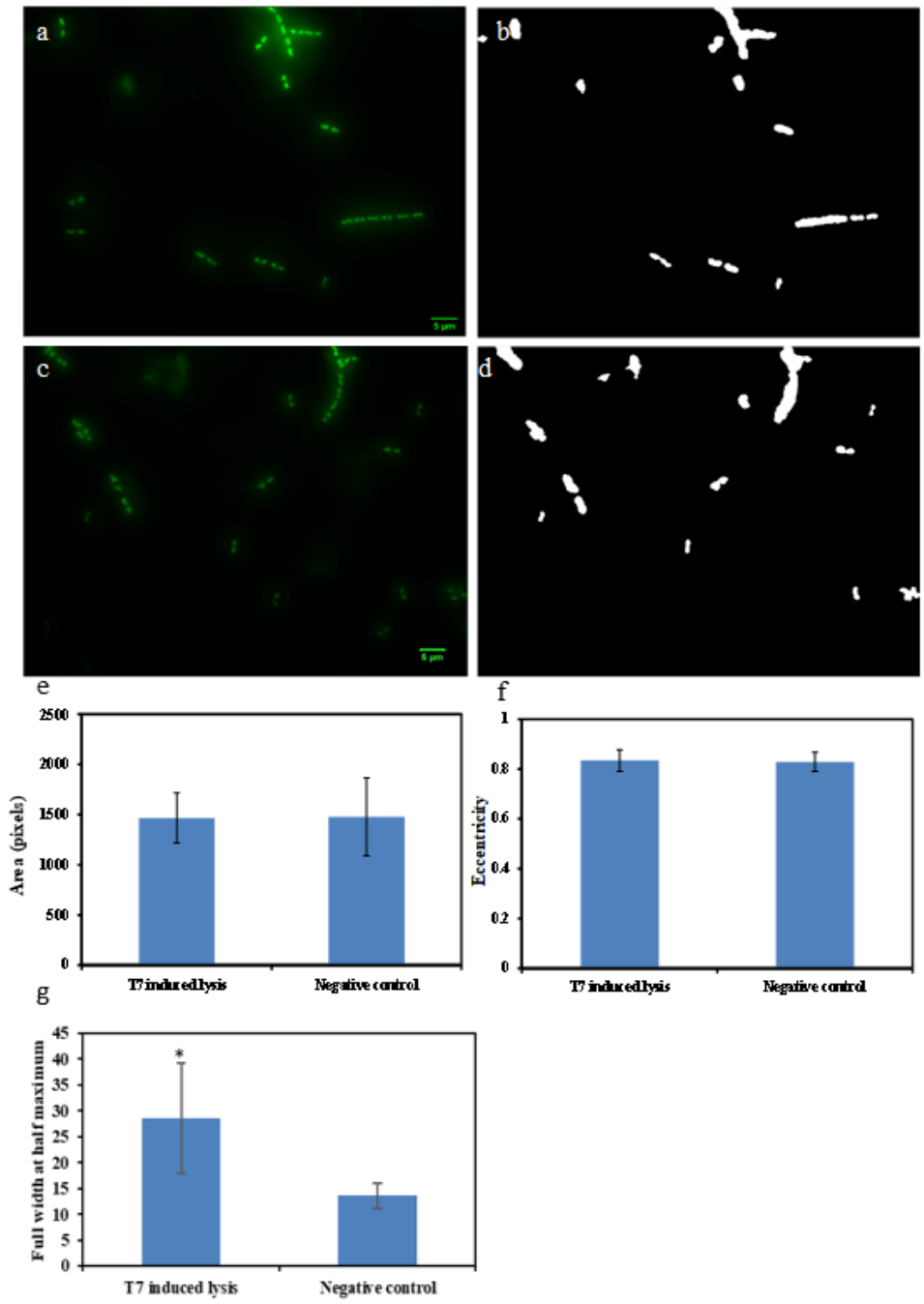

Figure 4.7. Detection of 10^2 CFU/ml *E. coli* through 4 hours enrichment and HTTL. (a) negative control which contains only *E. coli* growing for 4 hours. (b) binary image of (a). (c) phage-induced lysis after *E. coli* enrichment. (d) binary image of (c). (e) comparison of area values extracted from

(b) and (d). (f) comparison of eccentricity values extracted from (b) and (d). (g) comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

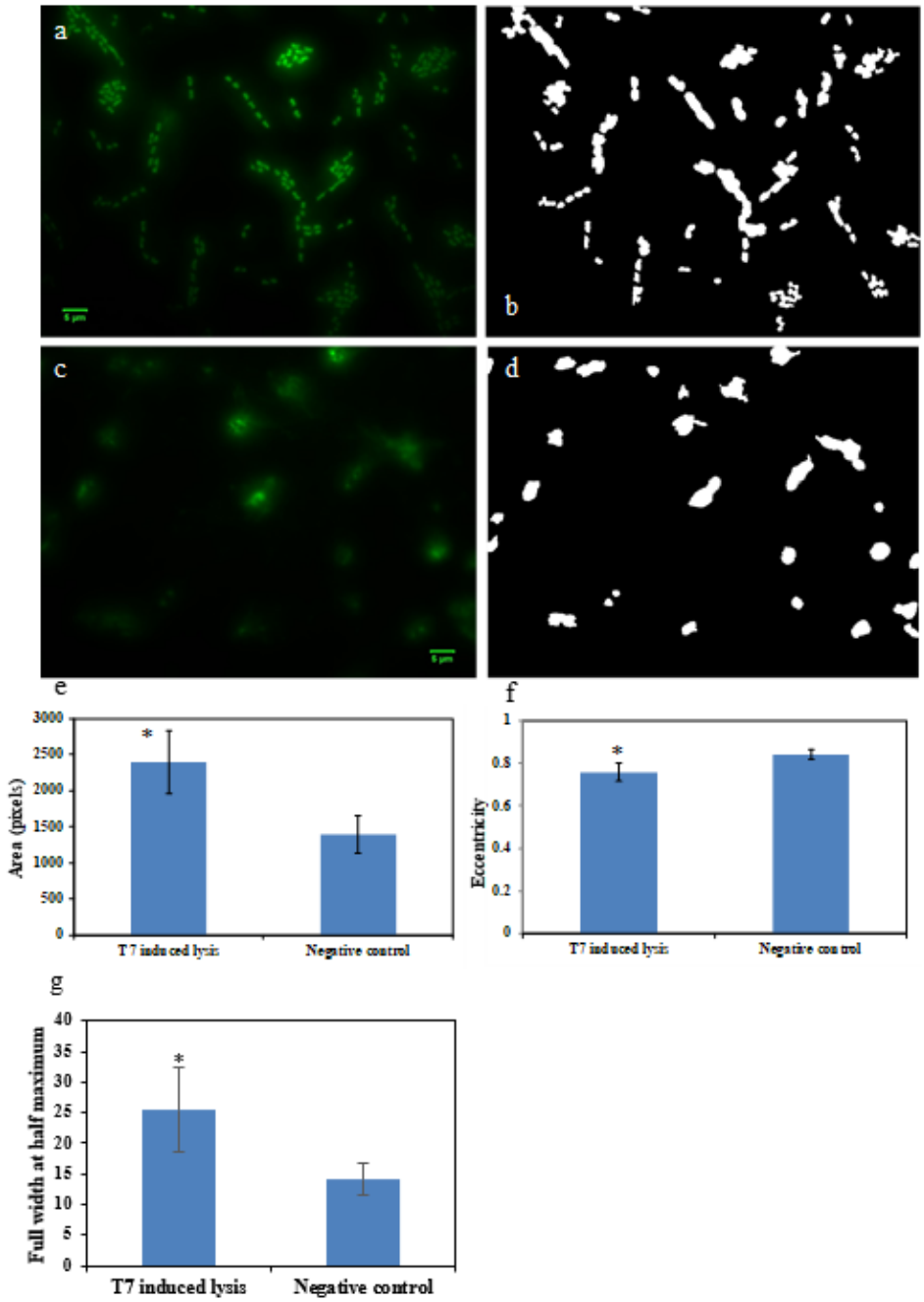

Figure 4.8. Detection of 10^3 CFU/ml *E. coli* through 2 hours enrichment and HTTL (a) negative control which contains only *E. coli* growing for 2 hours. (b) binary image of (a). (c) phage-induced lysis after *E. coli* enrichment. (d) binary image of (c). (e) comparison of area values extracted from

(b) and (d). (f) comparison of eccentricity values extracted from (b) and (d). (g) comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

4.3.4. Detection of E. coli at 10 CFU/ml in artificial wash water and coconut water.

HTTL was selected as a preferred lysis approach for rapid detection of *E. coli* in realistic food samples, as HTTL demonstrates a lower detection limit (10 CFU/ml) compared to LTCL (10^3 CFU/ml) as previously described based on area, eccentricity and FWHM parameters.

As shown in Figure 4.9, *E. coli* at 10 CFU/ml can be detected through HTTL coupled with image acquisition and analysis. Figures 4.9a and b indicate the microscopic image and binary picture of negative control where *E. coli* was enriched in artificial wash water-TSB for 5 hours. In comparison, Figure 4.9c and d represents the microscopic image and binary picture of *E. coli* lysed by T7 phage through HTTL after enrichment. Area, eccentricity, and FWHM parameters were extracted and statistically compared in Figures 4.9e, f, and g. As mentioned in the previous section, larger staining area, smaller eccentricity (more circular morphology), and larger FWHM (diffuse fluorescence signal) values indicated the lysis of *E. coli* cells upon incubation with T7 phages. In summary, based on significant differences in quantitative image analysis parameters, results indicated *E. coli* at 10 CFU/ml can be detected through HTTL and image analysis in simulated wash water samples.

Similar experiments were conducted in coconut water and the resultant images, binary pictures, and parameter analysis are shown in Figure 4.10. *E. coli* at 10 CFU/ml can be detected through HTTL and image analysis in coconut water. The proposed novel detection method is also validated in the spinach wash water, detailed data analysis and results are included in the supporting information

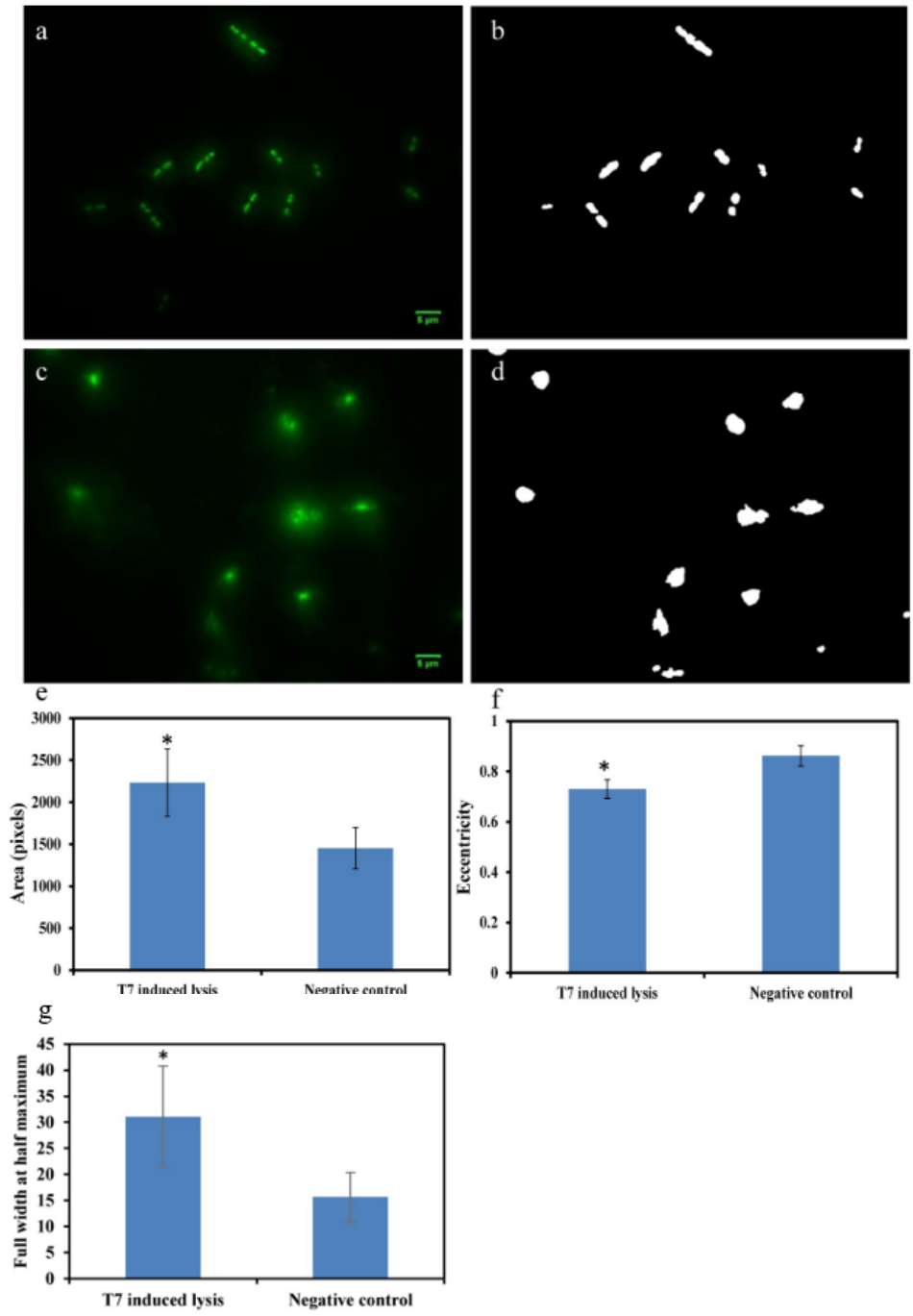

Figure 4.9. Detection of 10 CFU/ml *E. coli* through enrichment and HTTL in the artificial wash water. (a) negative control which contains only *E. coli* growing for 5 hours. (b) binary image of (a). (c) phage-induced lysis after *E. coli* enrichment. (d) binary image of (c). (e) comparison of area values extracted from (b) and (d). (f) comparison of eccentricity values extracted from (b) and

(d). (g) comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

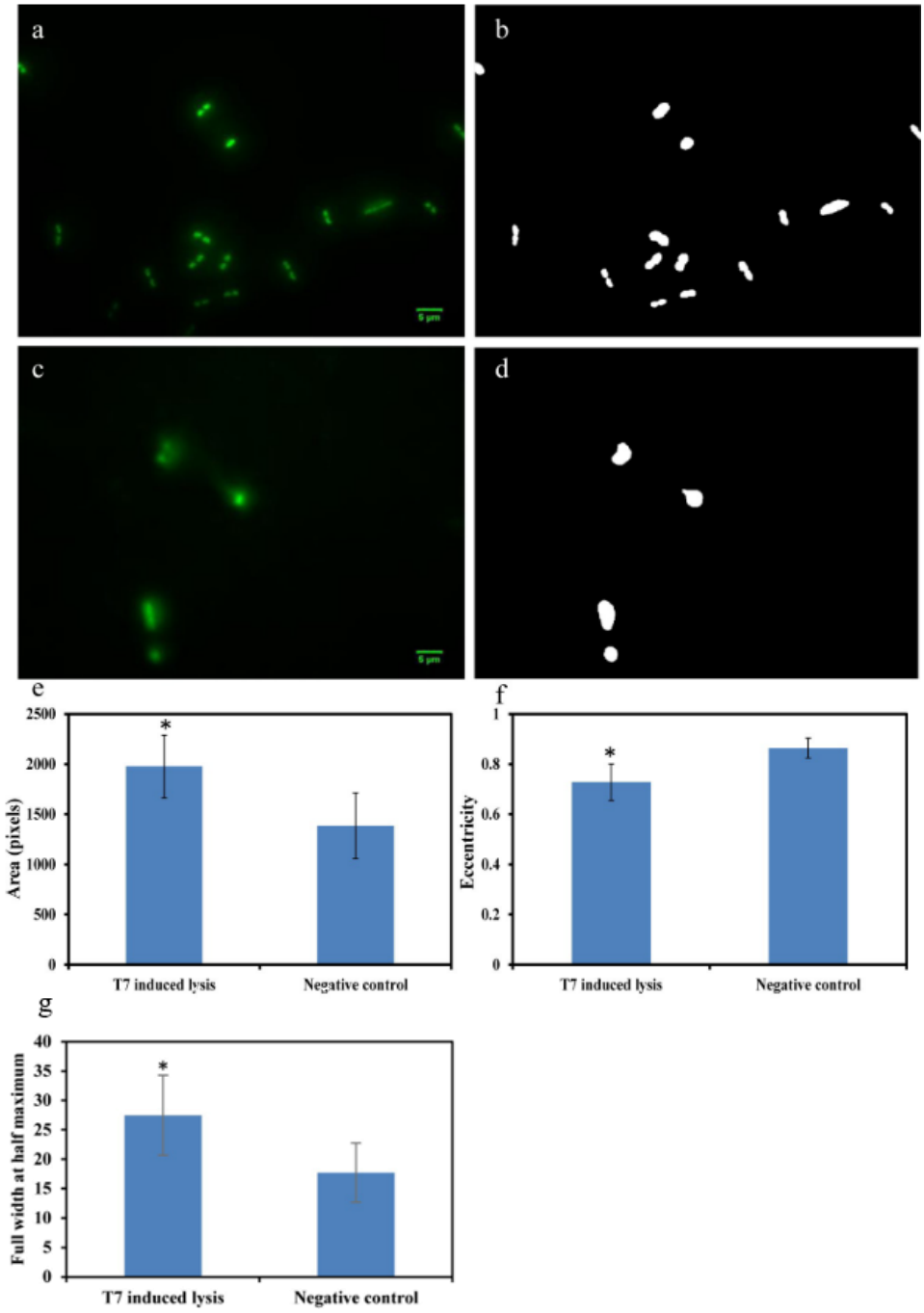

Figure 4.10. Detection of 10 CFU/ml *E. coli* through enrichment and HTTL in coconut water. (a) negative control which contains only *E. coli* growing for 5 hours. (b) binary image of (a). (c) phage-induced lysis after *E. coli* enrichment. (d) binary image of (c). (e) comparison of area values extracted from (b) and (d). (f) comparison of eccentricity values extracted from (b) and (d). (g)

comparison of the full width at half maximum extracted from (a) and (c). *indicated significant difference ($P < 0.05$).

4.4. Discussion

The study illustrates a straightforward, rapid, and cost-effective biosensing strategy to detect *E. coli* in simulated wash water, coconut water, and spinach wash water. This concept can be potentially expanded to detect other bacteria of interest in food safety using host-specific lytic phages. The principle of the proposed biosensing strategy is rather straightforward: it focuses on morphological changes in host cells and the release of eDNA upon host cell lysis. As far as we are aware, this is the first study to describe combining morphological changes, eDNA release, and image analysis to achieve rapid detection of *E. coli* in realistic food matrices. The application of imaging and image analysis as a detection approach has also been applied in BARDOT – a bacterial rapid detection using optical scattering technology. In BARDOT, a laser beam would usually pass through the bacterial colony to analyze 3D morphological and optical characteristics to generate an optical "fingerprint". Our proposed biosensing strategy using a similar optical imaging and image analysis approach enables significantly rapid detection compared with colony-forming assays used for BARDOT. For instance, it took at least 6-8 hours or 24-36 hours to achieve detectable *Bacillus* and *Listeria* colonies, respectively (Bae et al., 2007; Banada et al., 2007; Kim et al., 2014). In comparison, the rapidity of our proposed biosensing strategy is indicated in Table 1 and as shown in Table 1, the total amount of time for this proposed biosensing strategy is less than 8 hours. This is a time frame that fits within a typical industrial shift packaging perishable produce products. It could be argued that this approach has not eliminated the initial enrichment process as it adds significant time to the overall procedure but using the current imaging analysis approach was not possible without compromising the detection limit. Several other rapid detection methods based on PCR, phage and immunoassay still use enrichment as a pre-detection step to enhance the performance of the methods (Mukhopadhyay and K.

Mukhopadhyay, 2007; Chen et al., 2011; Laube et al., 2014; Sharp et al., 2015; Li et al., 2019; Wisuthiphaet et al., 2019). There is another acceptable reason to consider enrichment. It has been noted that bacterial cells that are naturally in the food/environment display an injured phenotype due to various stresses, which may reduce the sensitivity of bacteria to phages during both infection and propagation step. The enrichment step could resuscitate injured cells thereby making them susceptible to phage infection and therefore, detectable by the proposed method.

The biosensing strategy developed in this study is also cost-effective. Table 1 summarizes the cost of the detection method, which is around $5.50 per sample. The major expense of the biosensing strategy is Anodisc ($4.40), which could potentially be replaced by another lower-cost material that yields similar performance with low fluorescence background upon staining with SYBR Green I.

Table 4.1. Total time and cost analysis for liquid food matrix (25 ml) using proposed biosensing strategy to detect *E. coli*. All prices were quoted from the suppliers listed in the material and method section.

Steps	**Time**	**Supplies Used**	**Cost**
Enrichment	5 hours	Food Sample, TSB broth	$0.20
Lysis	30 min	Phage	N/A
Filtration	30 min	Anodisc	$4.83
Staining	20 min	SYBR green	$0.03
Imaging	30 min	Microscopic slide, antifading reagent, coverslip	$0.44
Image Analysis	10 min	Matlab Software	N/A
Total	**7 hours**		**$5.50**

Some of the potential challenges in translating the results of this study to field application may include: precise control on phage activity and presence of other bacteria with circular morphology in a food matrix. Based on our observations, phage activity needs to be controlled precisely to achieve timely detection as an older batch of phage due to reduced phage titer or weaker infectious potency, may take a longer time for lysis. To partially resolve the phage activity inconsistency, a new batch of phages was prepared every two weeks. Another potential limitation of the biosensing strategy lies in the background microflora. The presence of coccoid bacteria, such as *Staphylococcus* spp., may influence the eccentricity value during image analysis. The presence of bacteria that are significantly larger than *E. coli*, such as some of *Pseudomonas* spp., could also influence the calculation of area. The potential challenge from background microflora was partially addressed in the manuscript by simultaneously investigating changes in both area and eccentricity parameters, and validation in realistic spinach wash water samples to conclude the presence/absence of *E. coli*. 'Intensity distribution' which is the fluorescence intensity as a function of the distance of the line cross-sectioned through the centroid of a bacterium cell is another parameter that is able to detect bacterial cell lysis. The lysis of the cell membrane results in diffused distribution of the DNA. This diffuse distribution increases the width at half maximum while un-lysed cells showed focused DNA staining and reduced full width at half maximum.

The manuscript also describes two modes of lysis, LTCL, and HTTL. Both lysis pathways have advantages and disadvantages. LTCL provides easier sample preparation as phages and *E. coli* were co-incubated together, without additional step before filtration. This mode of sample preparation for the detection of target bacteria using phages has also been documented in several studies (Anany et al., 2018; Tilton et al., 2019). However, major drawbacks for LTCL were

noticed as the relative insensitive detection limit at10^3 CFU/ml. In comparison, HTTL provides a better detection limit in our current study at 10 CFU/ml, even though sample preparation requires both enrichment and phage addition at two different steps. To conclude, HTTL lysis mode is a better approach in our biosensing strategy.

4.5. Conclusion

In conclusion, this study demonstrates a straightforward, rapid, and cost-effective biosensing strategy to be developed which focuses on host cell morphology change and eDNA release, followed by automated imaging acquisition and analysis. The proposed method has been tested to rapidly detect *E. coli* at 10 CFU/ml within 8 hours of the entire process. The method has also been validated in three different realistic matrices -- artificial fresh produce wash water, coconut water, and spinach wash water. Future research may be conducted to include a variety of background microflora and to test the robustness of the proposed biosensing strategy in a greater variety of food matrices.

4.6. Potential future works

4.6.1. Artificial intelligence (AI)-detection platform

In order to develop an automatic detection platform based on the morphology change of bacterial cells upon T7 phage lysis, the AI approach can be applied to automatically detect lysed cells from the imaging data. A convolutional neural network (CNN) is an AI algorithm that has been used for image classification. CNN algorithm automatically extracts features of images and performs image recognition with high accuracy and this approach has been employed in diverse applications (Kusumoto and Yuasa, 2019).

Fluorescence images of bacterial cells after phage-induced lysis and intact bacterial cells will be manually annotated and used as the ground truth data for training the CNN model for a binary classification between lysed and non-lysed cells. The models will be evaluated by detecting lysed cells in the fluoresce images of the mixture of *E. coli* with other non-target bacteria containing both lysis and non-lysis cells. The accuracy of the model will be obtained and further optimized to achieve higher accuracy and sensitivity. The optimized model will be used to detect *E. coli* in samples with complex matrices such as fresh produce or agricultural water. The outcome of this study will demonstrate the flexibility and robustness of this detection platform.

The present-day smartphone technology with a high-resolution camera allows fluorescence imaging at a microscopic level with additional microscopic lens installation (Koydemir et al., 2014; Agbana et al., 2018; Müller et al., 2018; Chung et al., 2021). The smartphone's high computing power and network connectivity will enable on-site detection using this AI-assisted detection approach.

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CHAPTER 5:

Quantitative Imaging of Bacteriophage Amplification for Rapid Detection of Bacteria in Model Foods[4]

Abstract

Rapid detection of bacteria in water and food samples is a critical need. The current molecular methods like RT-PCR can provide rapid detection after initial enrichment. However, these methods require significant preparation steps, specialized facilities to reduce contamination, and relatively expensive reagents. This study evaluates a novel approach for detecting bacteria based on imaging of bacteriophage amplification upon infection of the target host bacteria to mitigate some of these constraints and improve the specificity of discriminating live vs. dead bacteria. Thus, this research leverages the natural ability of lytic bacteriophages to rapidly amplify their genetic material and generate progeny phages upon infecting the host bacterium. This study uses a nucleic acid staining dye, a conventional fluorescence microscope, and quantitative image analysis for imaging the amplification of bacteriophages. The sensitivity and assay time for imaging-based quantification of phage amplification for detecting *E. coli* were compared with RT-PCR and the standard plaque-forming assay for detection phage amplification in model systems, including coconut water and spinach wash water. The results demonstrate that the imaging approach matches both the sensitivity and speed for detecting *E. coli* using the RT-PCR method

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without requiring isolation of nucleic acids, expensive reagents, and specialized facilities. The quantitative imaging results demonstrate the detection of 10 CFU/ml of *E. coli* in coconut water and simulated spinach wash water with a COD of 3000 ppm within 8 hours, including initial enrichment of the bacteria. In summary, the results of this study illustrate a novel phage amplification-based approach for detecting target bacteria in complex food and water samples using simple sample preparation methods and low-cost reagents.

Keywords: Bacteriophage amplification, *Escherichia coli*, Pathogen detection, Fluorescence imaging, qPCR

5.1. Introduction

Detection of pathogens and indicator bacteria in food and water systems is one of the essential tools to manage the risk of foodborne outbreaks. Common pathogenic bacteria, including *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp., have been reported as the major causes of bacterial foodborne illness outbreaks (Bintsis, 2017). Current methods of detecting these bacteria include the traditional plate counting assay or more rapid molecular methods such as PCR or enzyme-linked immunoassay (ELISA) (Gracias and McKillip, 2004). Among diverse bacteria associated with foodborne illnesses, *Escherichia coli* is a Gram-negative bacterium typically found in the enteric tract of humans and warm-blooded animals (Kaper et al., 2004). Even though most *E. coli* strains are not pathogenic, *E. coli* presence is an indicator of fecal contamination and, therefore, their presence indicates the potential contamination of water and food supplies by other pathogenic bacteria (Krumperman, 1983; Molina *et al.*, 2015; Yang *et al.*, 2017). Thus, *E. coli* is one of the well-established indicator microorganisms for ensuring the hygiene and safety of food products (Choi *et al.*, 2018). In addition, there are specific strains of *E. coli* that are pathogenic to humans, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Yang et al., 2017). Thus, improving the detection of *E. coli* can enable monitoring of general sanitation and hygiene in food environments and enable the detection of specific pathogens in food and water systems.

For detecting diverse pathogenic and indicator bacteria in food and water systems, culture-based methods with selective media are the most established approaches used routinely by diverse sections of the food and agriculture industries (De Boer and Beumer, 1999; Feng et al., 2011). However, this detection approach is labor-intensive and often requires several days to obtain the

results (Wang and Salazar, 2016). Complementary to the culture-based assays, molecular assays based on nucleic acid-based amplification and detection, such as polymerase chain reaction (PCR), are commonly used for the detection of both indicator and pathogenic bacteria, including *E. coli* (Feng *et al.*, 2011; Molina *et al.*, 2015; Choi *et al.*, 2018). Even though PCR offers rapid and specific detection, it has limited sensitivity to differentiate between viable and non-viable cells (Yuan et al., 2018). Among immunological-based rapid detection approaches, enzyme-linked immunosorbent assay (ELISA) is one of the most widely used methods for bacterial detection. ELISA utilizes the specific antigen-antibody interaction and the catalytic properties of an enzyme to provide a sensitive detection (Gracias and McKillip, 2004; Pang et al., 2018). ELISA can be automated and analyze a large sample number simultaneously (Ivnitski et al., 1999). For example, Vidas (bioMérieux) is a fully automated system developed to detect bacteria such as *Listeria monocytogenes* using an enzyme-linked fluorescent assay technology (Gangar et al., 2000). However, like PCR, it cannot distinguish between live and dead cells (De Boer & Beumer, 1999). Detecting live/viable bacteria is of interest to food producers to eliminate false positives due to residual dead microbes or molecules of microbial origin left behind following food processing and sanitation. In addition, these aforementioned rapid detection approaches share some drawbacks such as limited sensitivity when the samples contain a low number of target bacteria, interference from the food components and significant sample preparation steps in the case of PCR methods, and limited stability of immunoreagents for immunoassays (Jaykus, 2003; Hornbeck, 2015). In addition, many molecular approaches such as PCR methods also rely on the enrichment of bacteria before detection. In many cases, these enrichment processes can range from 6 to 24 hours before sample preparation steps, such as isolation and purification of nucleic acids for PCR assay (Choi et al., 2018; Manage et al., 2019). Therefore, there is a need to develop molecular-specific

detection methods using low cost, stable reagent, simple sample preparation steps, detection within 6-8 hours of sample collection, and reduce the need for specialized laboratory environments.

Among various options, bacteriophages or phages can enable low-cost detection of target bacteria with simple sample preparation methods. For *E. coli* detection, the T7 phage is a well-established model system due to its ability to infect a wide range of *E. coli* hosts (Studier, 1969). With genetically modified T7 phages, previous studies have illustrated the detection of 10-100 CFU/mL of target bacteria in water and beverage samples within 6-8 hours without isolation of nucleic acids or extensive sample preparation steps (Chen et al., 2015, 2017; Wisuthiphaet et al., 2019, 2021). However, the application of this approach requires genetic modification of phages. It limits the broad applicability of phage-based detection methods, especially given the enormous diversity of phages in the environment. In addition, these genetically modified phage assays require isolation of the enzyme after its expression in target bacteria to increase the sensitivity of detection (Hinkley et al., 2018; Tilton et al., 2019). Furthermore, in certain instances, background levels of enzymatic activity in the non-targeted and targeted bacteria and the food products can interfere with the detection approach (Talbert *et al*., 2016; Meile *et al.*, 2020).

We have evaluated the potential of morphological changes induced by phage lysis to detect bacteria for addressing some of these constraints. This approach could detect 10 CFU/mL of *E. coli* in 8 hours (Yang et al., 2020). Similar to the concept of morphological changes in the bacteria, the lytic phage infection of the host cell also generates significant and rapid amplification of the progeny phage particles. For example, infection of an *E. coli* cell with a T7 phage may generate more than 100 progeny phages within 25-30 minutes. Thus, phage infection of target bacteria can result in significant amplification of phage particles. The presence of bacteria in media and food matrices can be detected based on the isolation of DNA from amplified phages and its subsequent

amplification using a real-time qPCR technique (Tolba et al., 2008; Anany et al., 2017). Although PCR is considered a rapid and sensitive technique for measuring bacteriophage amplification assay, it requires DNA isolation and several sample-preparation steps to prolong the detection process. In addition, the presence of inhibitors from food matrices can influence nucleic acid amplification efficiency. Phage amplification can also be detected using a conventional culture-based method like plaque assay, but it requires an extended incubation time to allow the growth of bacterial lawn to enumerate phage particles (Anderson et al., 2011).

This study aims to develop an approach for phage amplification detection using fluorescence imaging and quantitative image analysis to address these limitations. After phage lysis of the target bacterial host, the amplified phages were enriched by removing the bacterial debris using a simple centrifugation step. The phage enriched solution was stained with a nucleic acid stain (SYBR green I), and the stained phage particles were enumerated based on quantitative imaging measurement. Based on this approach, this study demonstrates both visualization and quantification of the amplification of T7 phages upon infecting *E. coli*. Sensitivity and the total assay time for this novel approach were compared with the qPCR detection of phage DNA amplification and the plaque assay. The developed imaging-based detection method was then applied to detect *E. coli* in model food systems. Coconut water was selected as a sample representing beverage matrices containing sugar, lipids, and minerals (Prades et al., 2012). Simulated spinach wash water was chosen to represent a complex matrix related to fresh produce production since several *E. coli* outbreaks are related to the consumption of leafy greens. Postharvest cross-contamination is one of the key risk factors associated with these outbreaks (hydro cooling or washing) (Gil et al., 2009).

The key advantages of this approach include the use of non-genetically modified phages to detect the low concentrations of bacteria in liquid food samples. In addition, the imaging method developed in this study for the detection of amplified phage particles following infection of the target bacteria provides an alternative approach to detecting bacteria using qPCR methods for the detection of amplified phages (Stanley et al., 2007; Kutin et al., 2009; Anany et al., 2018; Garrido-Maestu et al., 2019; Malagon et al., 2020). Furthermore, the approach in this study does not require immobilization of phages on substrates or paper strips to reduce the background signal from parent phages as observed in the previous study (Anany et al., 2018).

In summary, this novel image-assisted quantitative phage amplification detection approach can provide a robust yet straightforward strategy to address some of the critical unmet needs for detecting target bacteria in food and environmental samples using low cost, stable reagents, and limited sample preparation steps.

5.2. Material and Methods

5.2.1. Bacteriophage and bacterial strain

T7 phages were purchased from American type culture collection (#BAA-1025-B2) and propagated by infecting the log-phase *E. coli* BL21 for 15 minutes at 37°C in an incubator-shaker. Then, the phage-bacteria mixture was centrifuged at 16,100 × *g* to harvest infected bacterial cells. The cells were then resuspended in sterile tryptic soy broth (TSB, Sigma-Aldridge, St. Louis, MO, USA) and incubated at 37°C with 200 rpm constant shaking for complete lysis for 3 hours or until no visible turbidity was observed. Chloroform was then added to the mixture to a final concentration of 20% (vol/vol) and vortexed vigorously for homogeneity. The mixture was incubated on ice for at least 10 minutes before centrifugation at 5,000 × *g* for 10 minutes to

precipitate the cell debris. The upper phase of TSB containing phage particles was collected and washed three times by resuspending in a sterile phosphate buffer solution (PBS, Fair Lawn, NJ, USA) and centrifugation at 16,100 × *g* for 10 minutes. After washing, the phage particles were resuspended in PBS, and the phage titer was enumerated as 10^8 PFU/mL. The phage stock was stored at 4°C until used.

Escherichia coli BL21 (ATCC BAA-1025) obtained from American type culture collection was used as the host for the T7 phages. The bacterial culture was stored in TSB containing 15% (vol/vol) glycerol at -80°C. For short-term storage, the glycerol stock of bacteria was aerobically grown overnight in TSB at 37°C with constant shaking at 200 rpm. The culture was streaked onto tryptic soy agar (Sigma-Aldridge, St. Louis, MO, USA) plates and incubated overnight at 37°C. The agar plates were stored at 4°C for further experiment

5.2.2. Amplification of T7 using various concentrations of E. coli host cells for further enumeration

A single colony of *E. coli* BL21 was inoculated in TSB and incubated aerobically at 37°C with constant shaking at 200 rpm for 16 hours to obtain a 10^9 CFU/mL concentration. The bacterial culture was washed twice and resuspended in a sterile PBS before serial dilution. Then, the *E. coli* BL21 suspension was serially diluted in 10 mL TSB to obtain solutions with 10-10^3 CFU/mL of bacterial cell concentration. These solutions were then individually enriched by incubating at 37°C with constant shaking at 200 rpm for 4 hours.

T7 phages were added to the 4-hour enriched *E. coli* with constant shaking at 37°C for 0-4 hours. Chloroform (20% vol/vol) was added to the mixture, followed by incubation on ice for 5 minutes before centrifugation at 16,100 × *g* for 10 minutes. The upper liquid phase containing

bacteriophage particles was collected for analysis. The samples were analyzed using the standard plaque counting assay, RT-PCR, and quantitative imaging.

5.2.3. Fluorescence imaging of bacteriophage particles

Figure 5.1 illustrates the overall procedure for detecting bacteria using a combination of phage and an imaging approach. After T7 phage amplification, 50 µL of the phage solution was filtered through a 0.02-µm pore size Whatman® Anodisc inorganic filter membrane with a 13-mm diameter (GE Healthcare, Buckinghamshire, UK) and air-dried for 1-2 minutes. The Anodisc filter was then put back-side-down onto 20 µL of SYBR green I (25×) spotted on the glass slide and kept in the dark for 5 minutes. After staining, the excess dye was removed by Kimwipe, followed by adding 20 µL of 1% of *p*-phenylenediamine on top of the filter as an antifading reagent (Patel et al., 2007). The Anodisc filter was covered with a coverslip and observed under the Olympus IX-71 inverted fluorescence microscope with a 100× (1.25 NA) objective lens. An average of 10-12 images was acquired for each Anodisc sample using the Metamorph imaging software (version 7.7.2.0, Universal Imaging Corporation). The fluorescence excitation/emission wavelength of SYBR Green I stain was 480 ± 30 and 535 ± 40 nm, respectively.

T7 phage stock solution was serially diluted in PBS to have concentrations of 10^4-10^8 PFU/mL before fluorescence imaging for evaluating the limit of detection of T7 concentration by this imaging method.

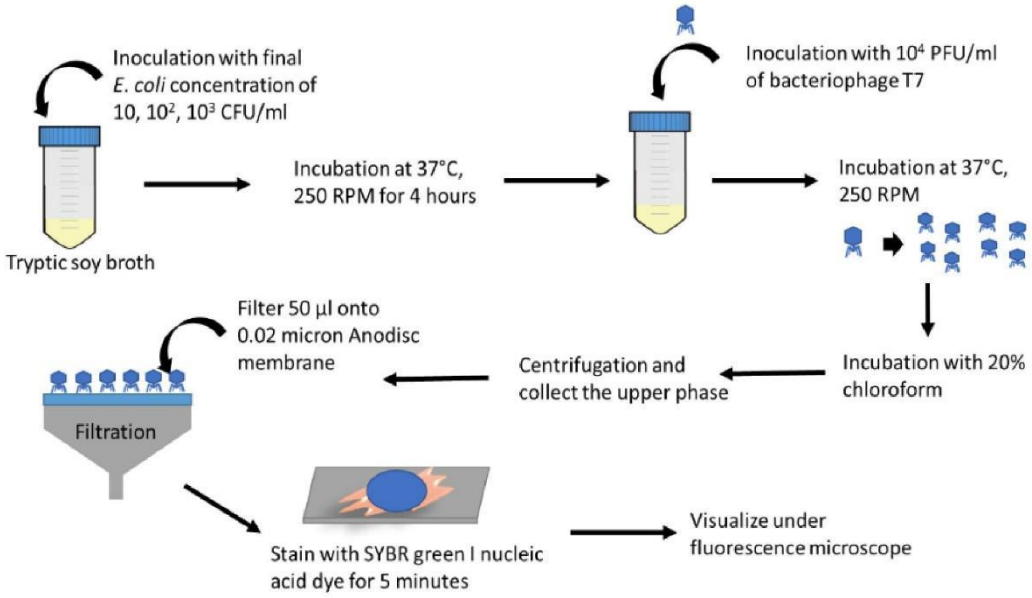

Figure 5.1. Schematic diagram illustrating the steps for the bioassay based on imaging of progeny phages after infecting target bacteria

5.2.4. Image analysis for bacteriophage amplification enumeration

The acquired fluorescence images were analyzed using a custom MATLAB code (Section 1 in the supplementary information section). To segment the phage particles in the fluorescence images, the *strel* function was used to define the shape of the phage particles, which were disk-shaped elements with a radius smaller than 30 pixels. Subsequently, the function *imtophat* was used to remove uneven background, followed by *medfilt2* function for reducing background noise. The function *adapthisteq* was used to enhance the contrast of the grayscale image and improve the visibility. The function *wiener2* was used to remove fine noise where each output pixel contains the median value in the 3-by-3 neighborhood around the corresponding pixel in the input image. The segmented images were then converted to binary images using the *imbinarize* function based on the intensity threshold computed by the *adaptthresh* function. For enumerating the number of particles, the function *bwlabel* was used to label bacteriophage particles in a binary image and count the number of particles present in the images as an output.

5.2.5. Quantification of bacteriophage by real-time qPCR

The amplified phage particles from section 2.2 were also quantified by real-time qPCR. The phage solution was heated at 100°C for 15 minutes for denaturation of the phage particles and release of the phage DNA. The real-time qPCR assay was performed using the forward (5'-CCT CTT GGG AGG AAG AGA TTT G-3') and reverse (5'-TAC GGG TCT CGT AGG ACT TAA T-3') primers (Peng et al., 2018) designed from the partial genome sequence of T7 phage. The PCR reaction was conducted with a total volume of 10 µL containing 5 µL of PowerUp SYBR Green Master mix 2× (Life Technologies, Grand Island, USA), 1 µL of 100 µM primers, 2 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen Life Science Technologies), and 2 µL of the denatured T7 phage DNA. The thermocycler condition were 2 minutes at 50°C, 2 minutes

at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The melt curve setting was 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. The Ct value of the samples was recorded. The concentration of the phage DNA was calculated based on the standard curve prepared by using purified T7 phage DNA (39.9 kbp, Boca Scientific) and reported in picogram units (pg).

5.2.6. Plaque assay

The plaque assay of the amplified phage solutions obtained from section 2.2 was performed using a soft agar prepared by supplementing the TSB broth with 0.7% agar before sterilization. A 10 mL of the molten soft agar was aliquoted to a sterile 15-mL centrifuge tube and cooled down to less than 50°C. As described in section 2.2, the amplified phage solution was ten-fold serial diluted in a sterile PBS. A 100 µL of each dilution of the phage solution was mixed with the aliquoted soft agar along with 100 µL of *E. coli* BL21 overnight culture. The mixture was then poured into a sterile Petri dish (10 cm diameter). The plates were gently rocked to distribute the soft agar on the Petri dish surface homogeneously. After the soft agar was solidified, the plates were inverted and incubated overnight at 37°C. The titer of the phage particles was calculated based on the number of plaques formed on the agar and reported in PFU/ml.

5.2.7. Detection of E. coli in coconut water and simulated spinach wash water

Baby spinach was purchased from a local grocery store. Spinach of 50 g was combined with 500 mL sterile water before blending two times using a sanitized blender for 30 seconds at a maximum speed. The blended solution was then centrifuged for 10 minutes at 11,000 × *g*. The supernatant was collected and centrifuged again at the same speed. The supernatant was collected as the simulated spinach wash water. The spinach wash water's chemical oxygen demand (COD)

was measured to be approximately 3,000 mg/L. Coconut water (Vita coco 100% coconut water) was purchased from a local grocery store.

For bacterial enrichment, 5 mL of coconut water (Vita coco 100% coconut water) and 5mL of simulated spinach wash water were mixed with 5 mL double concentrated TSB before inoculation with 10 CFU/mL of *E. coli* and incubation at 37°C with constant shaking at 200 rpm for 4 hours. The enriched mixture was inoculated with T7 phage for infection of the target bacteria. The lysate was isolated for quantifying phage amplification using the image-based, PCR, and plaque assay methods described above.

5.2.8. Statistical Methods

The phage particle number and phage DNA concentration obtained from both the imaging and qPCR measurements, respectively, at each infection time, were converted to a log scale value (y) and normalized by dividing by the log value of the maximum phage count assessed based on these measurements (y_0). The normalized values (y/y0) of phage level quantified by both methods were fitted to equation 1 using the curve fitting toolbox in MATLAB. The parameter k (sec^{-1}), indicating the rates of bacteriophage amplification quantified by the imaging and PCR methods, were obtained. The linear correlation between both imaging-plaque assay and PCR-plaque assay was obtained using OriginPro 9.1 software.

Equation 1: $\frac{y}{y0} = 1 - e^{-kt}$

5.3. Results and Discussion

5.3.1. Identification of initial phage inoculum concentration

The overall goal of this experiment was to determine the threshold concentration of phages that can be detected using a simple imaging approach. This threshold concentration will establish the initial inoculum concentration of phages for infecting bacteria while generating minimal background signal in the imaging measurement.

Phage particles were visualized by fluorescence imaging after staining with a nucleic acid binding dye SYBR green I. The method used in this study was modified from the earlier work by Patel et al. (2007), where this fluorescence imaging was used for investigating virus-like particles from the marine environmental samples (Patel *et al.*, 2007). By filtration, bacteriophages were captured on a 13-mm diameter Anodisc filter with a 0.02-micron pore size. T7 phages with a concentration range between 10^4 to 10^8 PFU/mL were imaged using this approach. Figure 5.2 shows fluorescence images of phage particles with an initial concentration of 10^8, 10^7, and 10^6 PFU/mL. The phage particles appeared as small dots homogeneously distributed on an Anodisc filter. Phages with a concentration lower than10^5 PFU/ml were not detectable using the imaging settings and configuration selected for this study. This measurement establishes that the initial phage inoculum levels at 10^4 or 10^5 PFU/mL may not generate significant background staining. The results also illustrate that amplifying the phage titer to at least 10^6 PFU/mL after infection of the target bacteria enables the detection of phage particles using conventional fluorescence microscopy. Since amplification of T7 phage results from phage infection and lysis of their bacterial host *E. coli* cells, detection of T7 amplification after phage inoculation and incubation with samples will indicate the presence of *E. coli* contamination. T7 phage has a short infection and lysis time, and it releases more than 100 copies of its progeny phages after 25 minutes of the

initial infection (Qimron et al., 2010), 10^4 PFU/mL of phage concentration was selected as an initial inoculum for this study. An increase in phage concentration at or above 10^6 PFU/mL upon infection will enable specific detection of the presence of the target bacteria.

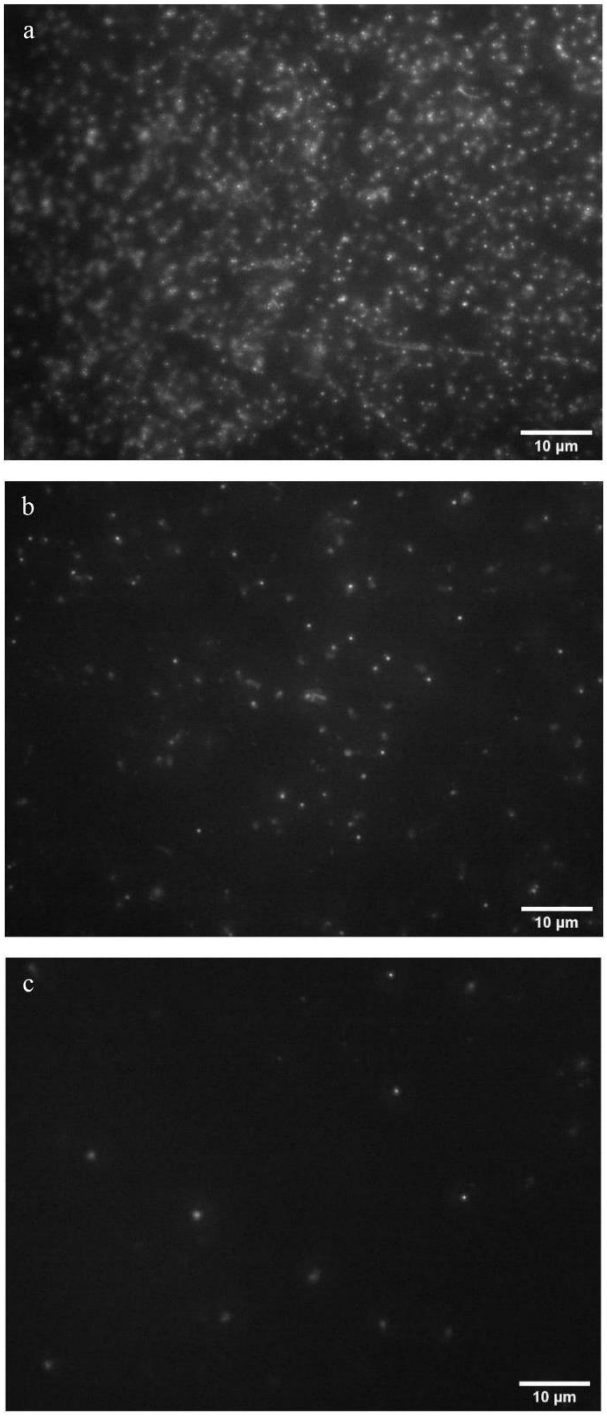

Figure 5.2. Fluorescence images of the bacteriophage particles at concentration levels of (a)108 PFU/mL, (b) 107 PFU/mL, and (c) 106 PFU/mL.

5.3.2. Detection of target bacteria based on bacteriophage amplification and imaging

The overall goal of this sub-task was to determine the influence of infection time on the levels of amplified phages for a range of initial bacterial concentrations and to correlate the imaging-based phage amplification measurements with the conventional plaque assay.

Before T7 phage infection, *E. coli* BL21 with an initial concentration between the range of 10-10^3 CFU/mL was enriched in TSB for 4 hours. This step mimics the typical enrichment process used for both the culture-based and rapid detection methods, including the qPCR methods to detect bacteria in food and water systems. After enrichment and infection with T7 phage, the samples were collected at 30-minutes intervals for the subsequent 4-hour period. After collection, the samples were filtered, labeled, and imaged, as illustrated in Figure 5.1.

The examples of fluorescence images of phage particles are shown in Figures 5.2-5.4. The images were obtained when the initial bacteria concentration was 10-10^3 CFU/mL, respectively, with 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours of phage infection.

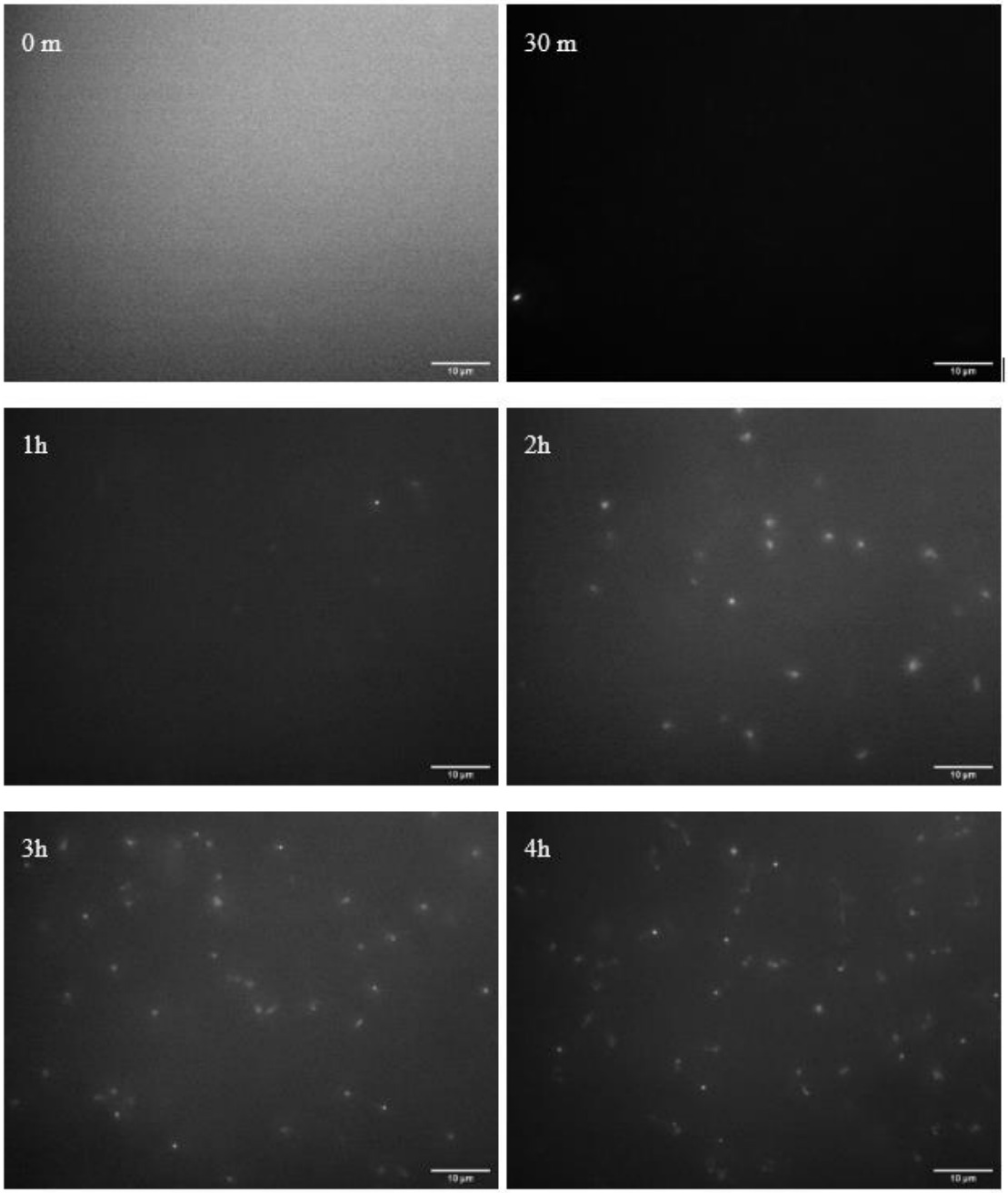

Figure 5.3. Fluorescence images of the bacteriophage particles after 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours of infection of *E. coli* BL21 (10 CFU/ml initial concentration) with 104 PFU/ml of T7 phages. *E. coli* cells were enriched for 4 hours prior to infection with phages.

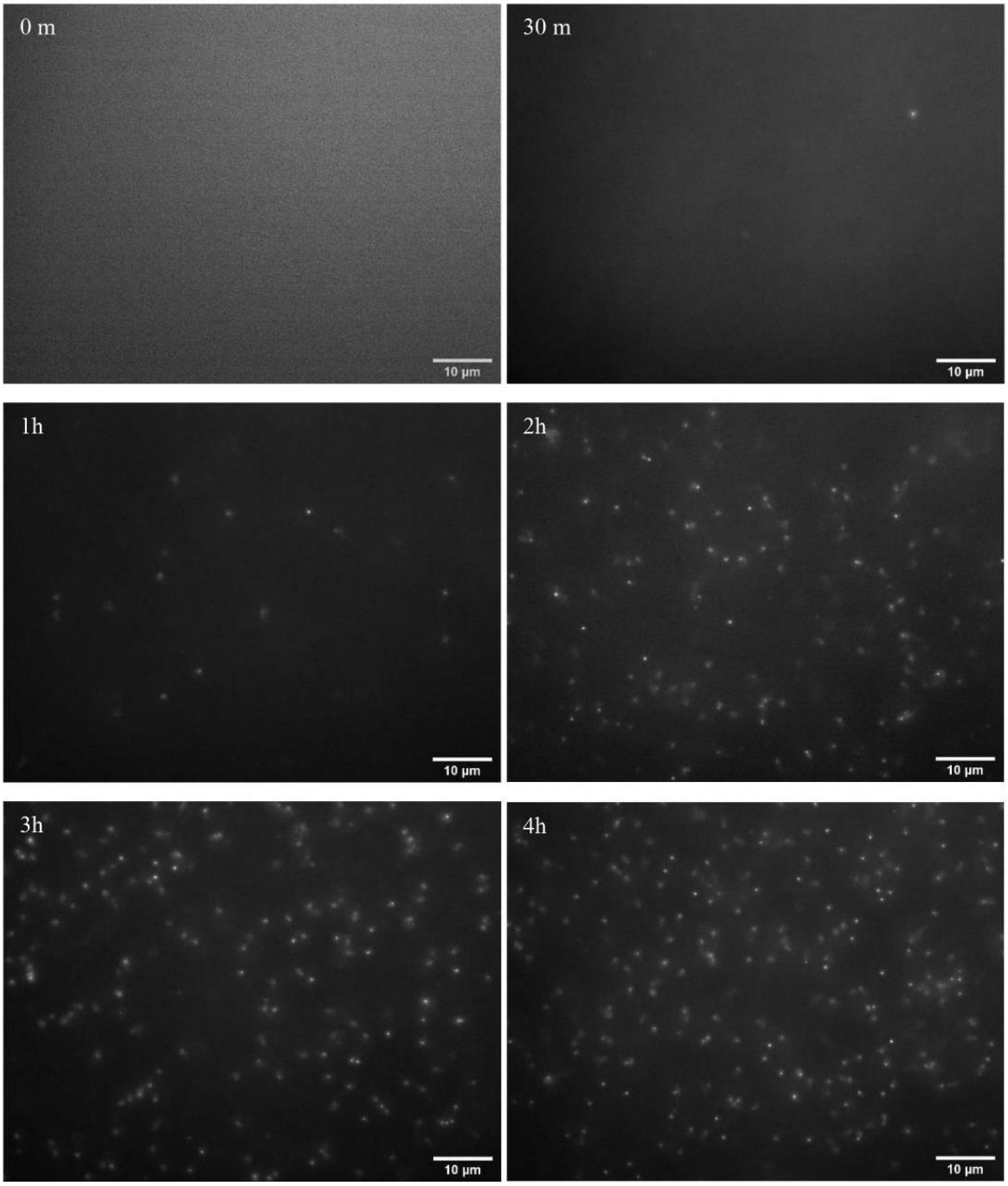

Figure 5.4. Fluorescence images of the bacteriophage particles after 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours of infection of *E. coli* BL21 (10^2 CFU/mL initial concentration) with 10^4 PFU/mL of T7 phages. *E. coli* cells were enriched for 4 hours prior to infection with phages.

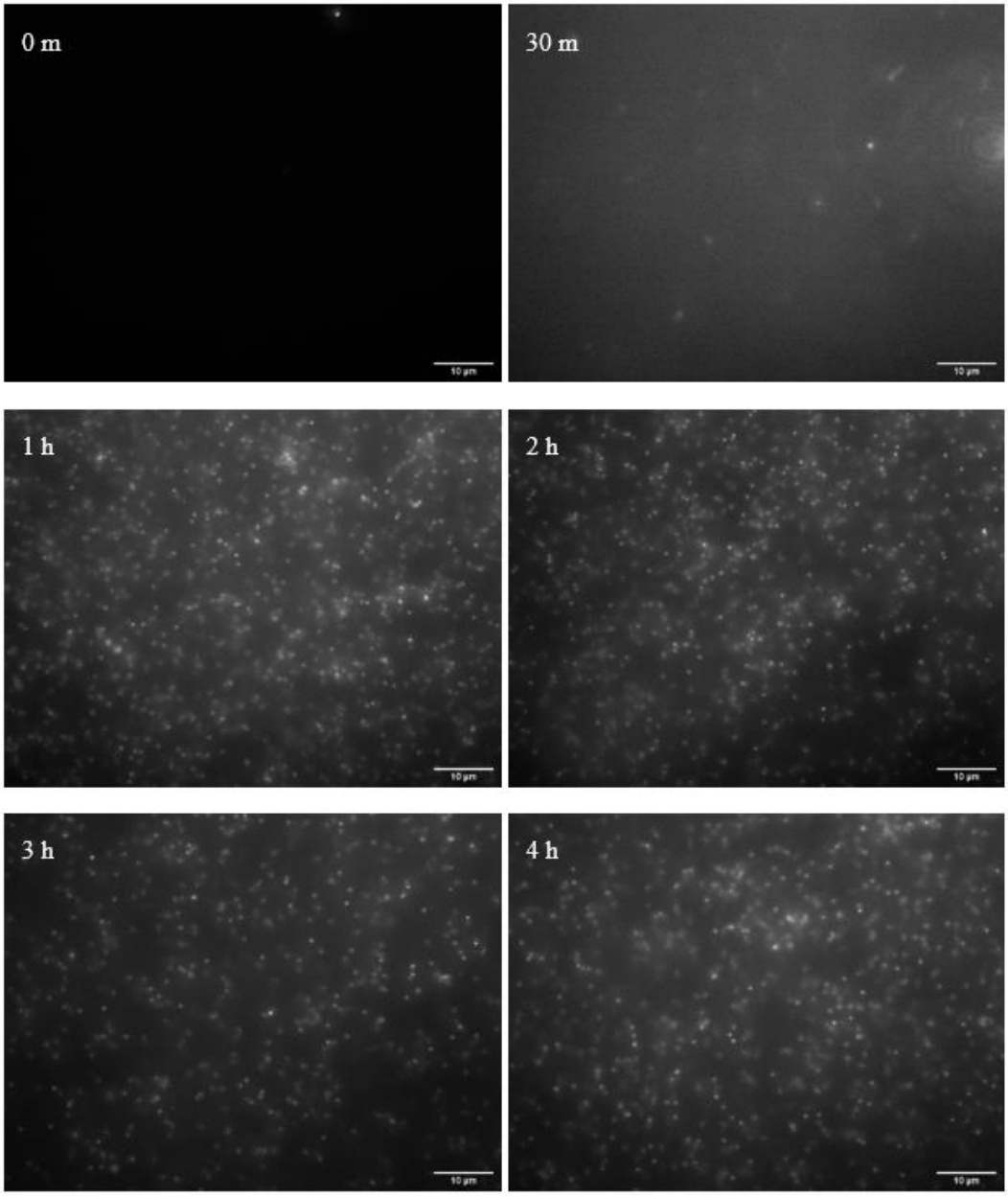

Figure 5.5 Fluorescence images of the bacteriophage particles after 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours of infection of *E. coli* BL21 (10^3 CFU/ml initial concentration) with 10^4 PFU/ml of T7 phages. *E. coli* cells were enriched for 4 hours prior to infection with phages.

Figure 5.6a shows the number of phage particles enumerated based on image analysis of the fluorescence images acquired using 10, 10^2, and 10^3 CFU/mL of the initial concentration of bacterial inoculum. For each initial concentration level of bacteria, 10-12 images were acquired and analyzed for phage particles quantification. The initial bacteria concentration was enriched for 4 hours and infected with T7 phages at 10^4 PFU/mL. Immediately after introducing the T7 phage at 10^4 PFU/mL to the enriched samples, no phage particles were observed in the fluorescence images as the fluorescence signal was below the detection limit. When the initial concentration of *E. coli* was 10 CFU/mL, after 4 hours of enrichment and 1-hour infection, fewer than ten phage particles/image were observed, where each image represents an area of 6×10^3 μm^2. The number of phage particles increased to 30-50 particles/image after 2 hours of infection, and around 100 particles/image was observed within 4-hours of infection. With an increase in the initial *E. coli* concentration to 10^2 CFU/mL, after 4-hour enrichment and 30 minutes of infection, fewer than five bacteriophages/image particles were visualized. The number of phage particles in the images increased to around 10-20 particles/image after 1-hour infection and to more than 100 phage particles/image after 2 hours of infection. The number/image remained constant after 3 and 4 hours of phage infection of the target bacteria. For the initial *E. coli* concentration of 10^3 CFU/mL, the number of particles increases more rapidly to around ten phage particles/image within the first 30 minutes of infection and subsequently increased to 300-400 phage particles/image after a 1-hour incubation. The number of phage particles remained constant with a longer infection time for 10^3 CFU/mL initial inoculum. According to the results, *E. coli* at 10 and 10^2 CFU/mL can be detected using the phage amplification-based imaging method after 4 hours of enrichment followed by 1 hour of phage infection. A 4-hour enrichment and 30-minute phage infection time were required to detect 10^3 CFU/mL of *E. coli*.

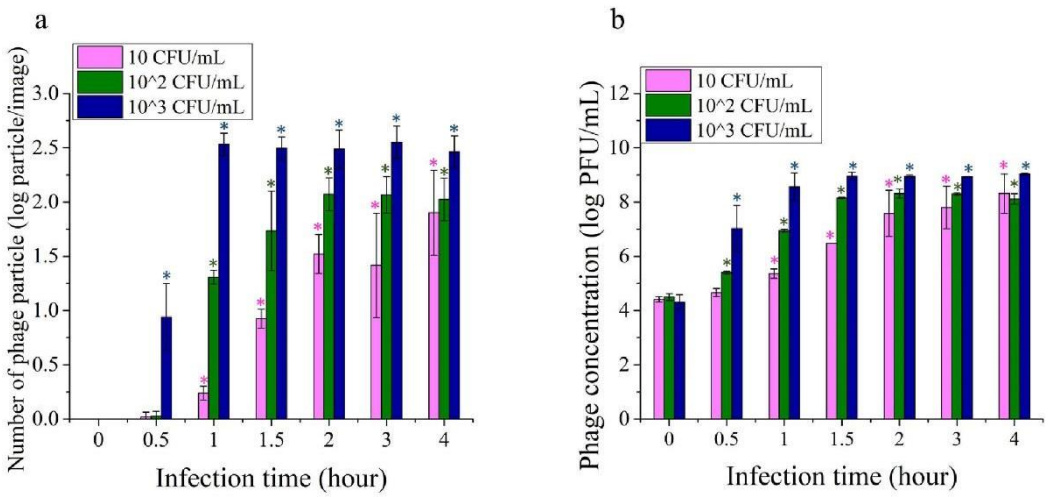

Figure 5.6. (a) Imaging-based quantification of the increase in the number of phage particles upon infection of *E. coli* BL21 as a function of infection time (0-4 hours). (b) Quantification of plaques generated based on infection of *E. coli* BL21 using the standard plaque counting assay. For both of these assays, *E. coli* cells with initial concentration levels of 10, 10^2, and 10^3 CFU/mL were enriched for 4 hours prior to infection with phages. Treatments with '*' are significantly different ($p < 0.05$) from control at 0 min infection. Error bars indicate ±standard deviation of means.

The increase in the numbers of phage particles in the fluorescence images was comparable with the titer of bacteriophage enumerated by the plaque assay. Figure 5.6b illustrates the titer of phage obtained by the standard plaque assay after infection of a 4-hour enriched *E. coli* with T7 phages at 10^4 PFU/mL. When the initial concentration of *E. coli* was 10 CFU/mL, it required approximately 1.5 h of infection time for the phage titer to reach the level of 10^6 PFU/mL. The concentration of 10^6 PFU/mL was the lowest level of phage count that can be detected by fluorescence imaging, as illustrated in Figure 5.1. When the initial concentration of *E. coli* was 10^2 and 10^3 CFU/mL, 10^6 PFU/mL of T7phages can be achieved after 1h and 30 min of infection time, respectively.

The results also show that the amplification rate of phage depends on the concentration of bacteria. This trend was in agreement with a previous study that described the phage bacterial binding process as dependent on both the concentration of free phages and the concentration of bacteria (Ellis and Delbrück, 1939; Sinha et al., 2018). Thus, a higher initial *E. coli* concentration results in a higher phage amplification rate, and thus a relatively higher titer of bacteria (initial concentration of 10^3 or 10^4 CFU/mL) after enrichment can be detected with a short infection time. In addition, due to a rapid lytic cycle of the T7 phage, a significant amplification of the phages with each lytic cycle and sensitivity of the imaging approach, even multifold lower levels of bacteria, for example, 10 CFU/mL could also be detected within 6 hours. This highlights the overall advantage of this approach that enables rapid and sensitive detection of target bacteria at levels as low as 10 CFU/mL within 6 hours for enrichment and infection. This result also highlights that the combination of phage amplification and sensitivity of an imaging approach can reduce the influence of multifold differences in the bacterial titer, e.g., a 100-fold difference between 10 CFU/mL and 10^3 CFU/mL after enrichment.

5.3.3. Comparison of phage quantification using imaging and qPCR methods

For comparing the sensitivity of the imaging and qPCR approaches, amplified phages following infection were detected using both quantitative imaging and qPCR. Amplification of phages has been previously quantified using quantitative-PCR methods (qPCR) (Edelman and Barletta, 2003). In addition, the detection of bacteria based on measuring phage amplification using qPCR has been reported in previous studies (Stanley et al., 2007; Kutin et al., 2009; Park et al., 2013; Garrido-Maestu et al., 2019; Malagon et al., 2020). To validate and benchmark this novel imaging-based method, bacteriophage concentration at each time point was also quantified by qPCR. The normalized values (y/y0) of phage level quantified by the imaging and PCR respectively at each infection time point are shown in Figure 5.7 a-c, when the initial bacteria concentrations were 10-10^3 CFU/mL, respectively.

The phage amplification rates (k) obtained from the imaging and qPCR method were presented in Table 1. With 10 CFU/mL initial *E. coli* concentration, the k value from both methods was comparable at around 0.7 sec^{-1}. When the initial *E. coli* concentration increased to 10^2 CFU/mL, the k value from the imaging method increased to 0.835 sec^{-1}, while the k value from qPCR was 1.112 sec^{-1}. When the initial *E. coli* concentration was 10^3 CFU/mL, the k value from the imaging method (1.879 sec^{-1}) was higher than that obtained from qPCR (1.190 sec^{-1}).

The curve fitting results (Figure 5.7) show that phage quantification by imaging and qPCR approaches follows a similar trend. The amplification rate (k) from imaging and qPCR methods increased with an increase in the concentration of the initial inoculum of bacteria, which suggested that the relatively higher titer of the bacterial host speeded up the amplification of the phages. The qPCR method gave a slightly higher k value when bacteria concentration was 10 CFU/mL and 10^2 CFU/ml, with the ratio of ki to kp equal to 0.938 and 0.751, respectively. For the bacteria

concentration of 10^3 CFU/mL, the amplification rate detected by imaging was significantly higher with the ki/kp of 1.578. With the initial *E. coli* concentration of 10 CFU/mL, the increase in phage level was detected after 1 hour of infection using both imaging and PCR methods. However, the increase in phage level quantified by imaging was observed after the first 30 minutes of the infection with the initial bacterial concentration of 10^2 CFU/mL and 10^3 CFU/mL. In the case of qPCR, the increase in phage DNA level was detected after one hour of infection.

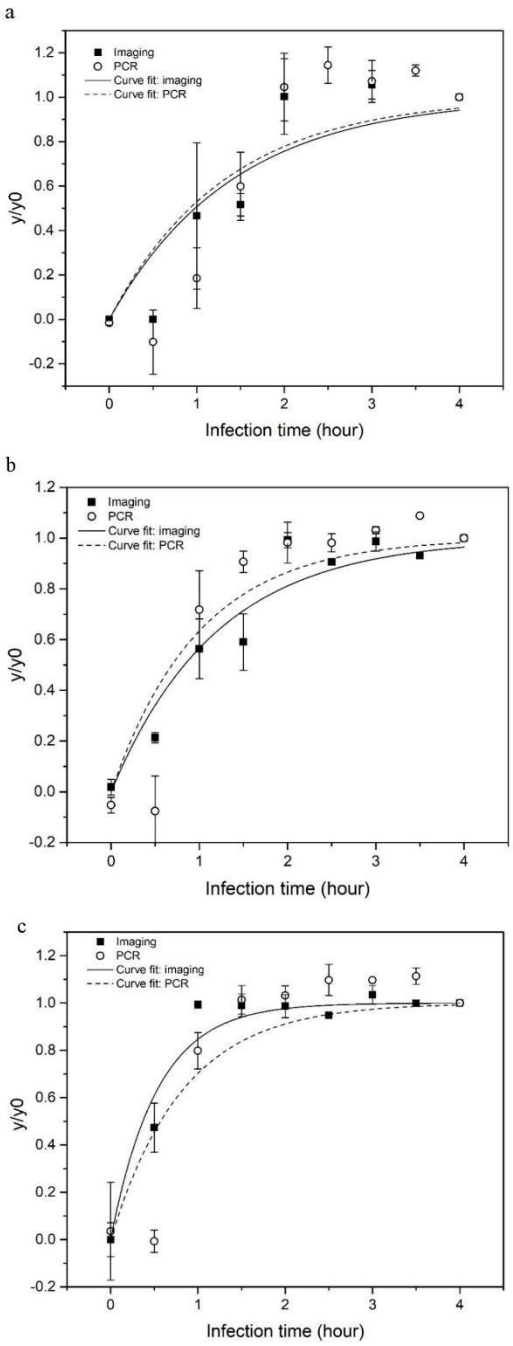

Figure 5.7. Normalized concentration of phage particles and phage DNA obtained based on imaging (log particle/image) and qPCR (log pg/mL) respectively as a function of infection time. *E. coli* BL21 was enriched for 4 hours prior to infection. The initial concentration of *E. coli* was (a) 10 CFU/mL, (b) 10^2 CFU/mL, and (c) 10^3 CFU/mL

5.3.4. Quantification of amplified phages using imaging and qPCR

Plaque assay is the gold standard for the determination of phage titer. The method involves phage infection on immobilized target bacteria in a molten soft agar overlayed on the nutrient agar. After several hours of incubation, progeny phages generated from an initial infection will infect and lyse nearby bacteria and create a clearance zone called a 'plaque' on the opaque lawn of inoculated bacteria. The plaque assay provides an assessment of viable plaque-forming units to represent the concentration of viable phages in a solution, and therefore, it is considered the gold standard for the determination of bacteriophage titer (Anderson et al., 2011)

The data sets obtained with PCR and imaging assays were correlated with the plaque assay. Figure 5.8 depicts the correlation between the imaging method (Figure 5.8a) and qPCR (Figure 5.8b) with the plaque assay. The linear relationship of log phage particle/image against log PFU/mL was $Y= 0.548X-2.385$, $R^2 = 0.958$ (Figure 5.8a). The linear relationship of log DNA concentration in pg/mL against log PFU/mL was $Y= 0.981X-4.289$, $R^2 = 0.959$ (Figure 5.8b). In both cases, R^2 values were around 0.96, illustrating a good linear correlation between both the imaging and qPCR methods with the standard plaque assay. Even though plague assay is considered the gold standard for phage enumeration, it requires extended incubation time (between 16-20 hours). Therefore, more rapid detection methods like qPCR and imaging methods are preferable in foodborne pathogen detection applications.

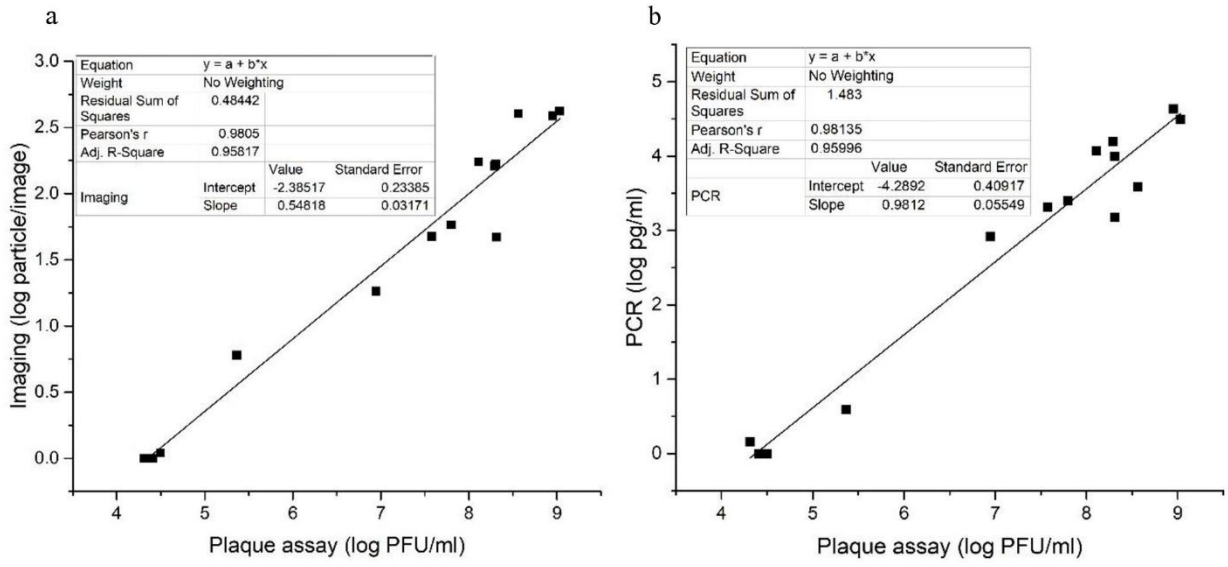

Figure 5.8. Correlations between (a) bacteriophage particle numbers obtained based on imaging measurements and (b) bacteriophage DNA concentrations obtained based on qPCR measurements with the phage plaque count measured based on the standard plaque-forming assay.

In addition to the detection of amplified phages following infection of target bacteria using imaging and RT-PCR, there have been efforts made to detect phages in water (Rajnovic et al., 2019, Charm Sciences Inc, 2022). These methods have been motivated by the use of coliphage as an indicator of fecal contamination. Since the objective of these methods is to detect phages, these methods typically require the addition of a large concentration of host bacteria to the phage sample. Thus, analytically these approaches are designed to enable the detection of low titer of phages using a high concentration of host bacteria, while the method developed in this study is aimed at the detection of low concentration of target bacteria using phages. The method to detect phages has also used optical methods such as changes in optical density based on interactions of phages with high titer of host bacteria. However, these methods have only been tested with samples without the presence of food particles or other interferences that may limit the use of these optical approaches.

Overall, the developed bacterial detection method based on phage amplification and fluorescence imaging provides rapid and sensitive results as it enables the detection of *E. coli* at 10 CFU/mL within 6 hours.

5.3.5. Detection of bacteria in food samples

Two model food systems, i.e., coconut water and simulated spinach wash water, were selected to evaluate the effectiveness of the phage amplification-based detection of contaminating bacteria in food samples. These samples were selected based on the unmet needs in the beverage and fresh produce industries to provide rapid detection of bacteria contaminants and our prior experience with these model systems (Tilton et al., 2019; Yang et al., 2020). A 10 CFU/mL of *E. coli* BL21 was inoculated in two selected food samples. After initial enrichment and infection with T7 phages, the amplified phages were detected using the imaging, qPCR, and the standard plaque

assay. Figure 5.9a. shows the average number of phage particles per image following amplification of the phages after incubation with the *E. coli* contaminated coconut and spinach samples. The plot shows changes in the number of phage particles after 0, 30 minutes, 1 hour, 1 hour 30 minutes, 2 hours, 3hours, and 4 hours of infection. At the infection time of 0 minutes, representing the initial T7 inoculum, around 10 and 15 particles were observed on the images/field of coconut and spinach wash water samples, respectively. The particles observed at the initial inoculation time might result from some fluorescent residues from the coconut water and spinach wash water that are auto-fluorescent or can be stained by SYBR green. For coconut water, 2 hours of infection was required to significantly increase ($P < 0.05$) the number of phage particles from the initial background level. The particles increase to around 300 with an infection time of 3 hours. For the spinach wash water samples, there was a significant increase ($P < 0.05$) in the number of phage particles within 1 hour of infection, and the phage particles increased to around 300 particles after 2 hours. No significant increase in the phage particles per image was observed with an extended incubation time greater than 2 hours for the spinach wash water samples. For both coconut water and spinach wash water samples without *E. coli* inoculation, there was no significant increase in the number of phage particles after 4 hours of infection. These results demonstrate that the imaging method can detect amplification of phages in coconut water and spinach wash water, and 10 CFU/mL of *E. coli* in coconut water and spinach wash water can be detected with an infection period of 2 hours and 1 hour, respectively, using the imaging approach.

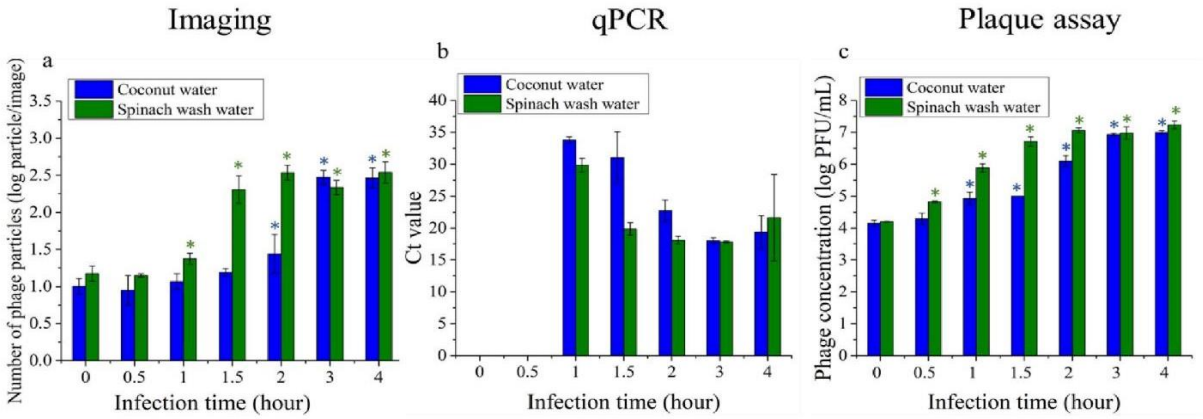

Figure 5.9. Quantifications of T7 phages amplification after 0 minutes, 30 minutes, 1 hour, 1 hour 30 minutes, 2 hours, 3hours, and 4 hours infection of 10 CFU/ml concentration of *E. coli* BL21 cells with T7 phages at a concentration level of 104 PFU/mL. *E. coli* BL21 cells were enriched for 4 hours prior to infection with T7 phage. The phage amplification was measured based on (a) imaging, (b) qPCR and (c) plaque assay. Treatments with '*' are significantly different ($p < 0.05$) from control at 0 min infection. Error bars indicate ±standard deviation of means.

A qPCR was also performed to detect the increase in the concentration of T7 phage DNA which represents phage amplification. The level of phage DNA was expressed in terms of the cycle threshold value (Ct value), which is defined as the number of reaction cycles required for the fluorescence signal to exceed the threshold. The samples with lower Ct values contain a higher T7 phage concentration. Figure 5.9b shows the average Ct values for coconut water and spinach wash water samples with *E. coli* inoculation. The phage DNA level in the samples with the infection time of 0 and 30 mins were not detectable as the fluorescence signals were lower than the threshold. With a 1-hour infection, the average Ct value of the coconut sample was 33.79, while the average Ct value of the spinach samples was 29.88. After 1 hour of infection, there was an increase in the level of T7 phage DNA indicating T7 phage amplification. Increasing the infection time to 2 hours decreased the Ct value of the coconut water and spinach wash water samples to 31 and 19.8, respectively. The lower Ct values indicate the higher level of the target DNA; therefore, the amplification T7 phage rate was relatively higher in the spinach wash water than those in the coconut water.

The concentration of T7 phage in both types of food samples was also quantified using the plaque counting assay. A significant increase ($P < 0.05$) in T7 phage concentration was observed when the infection time was 30 minutes and 1 hour for spinach wash water and coconut water samples, respectively. The amplification rate of T7 phage was higher in the spinach wash water samples, which is consistent with the results of phage quantification performed using the imaging and qPCR methods.

The results suggest that the imaging-based method has the sensitivity to detect 10 CFU/mL of *E. coli* in the selected food samples, coconut water, and spinach wash water. In complex food samples, the background signal from the food components is considered a constraint for the

detection of pathogens since it reduces the detection sensitivity. For the imaging-based detection approach, the centrifugal step removed some of the large debris from food products along with the bacterial debris. Despite this step, small fragments of the food particles did contribute to a small detectable background in the case of samples imaged at an infection time of 0 hours. Despite this background, the imaging approach was able to detect 10 CFU/ml within 6-7 hours of the total assay time, including enrichment of the inoculated bacterial samples.

The presence of food matrices may either affect the growth of bacteria during enrichment or the infection and amplification of T7 phages. Therefore, total detection times are presented as a function of selected food matrixes for different detection methods in Table 2. The phage amplification rate was lower than using PCR and plaque assay in a coconut water sample for the imaging method. Thus, a longer infection time was required to detect bacteria in coconut water using an imaging approach. It is possible that components of coconut water may slow the growth of *E. coli* which is in contrast with the study of Awua et al., (2012) that fresh coconut water is favorable for the survival and growth of *E. coli* (Awua, 2012). Another possibility is that the components in coconut water decelerate the infection and amplification of the phages. In the case of spinach water samples, the infection time was shortest for the plaque assay (0.5 hours), while both the PCR and imaging assay required 1 hour of infection time.

Even though the imaging method requires a longer infection time to observe phage amplification for the coconut water sample, it saves one hour of total operation time than those required for the qPCR method. The imaging method requires 7 and 6 hours to detect 10 CFU/mL of *E. coli* in coconut water and spinach wash water. The qPCR method requires 7 hours to detect the same concentration of *E. coli* in both samples. The longer time required for the qPCR method

is a result of the fact that a DNA extraction step was necessary, and a 40-cycle amplification during PCR takes almost 1 hour 30 minutes.

On the other hand, the imaging approach has the necessary sensitivity to detect phage particles with a simple method based on filtration and imaging. Thus, it saves both time and resources required for the extraction of DNA and molecular laboratory environment requirements. Moreover, the protocol of the imaging method is relatively more straightforward and does not require well-trained personnel for the operation, which can make it more cost-efficient. Another key advantage of the imaging-based method is the automation of imaging procedures, such as automated scanning of the slides is now routine in many commercial imaging systems (Conze et al., 2010; Lu et al., 2019). These advantages of the imaging method also enable the possibility to develop a portable detection tool that can be operated in the field.

A possible limitation of the imaging method is that the filtration setup we were using may not be suitable to test multiple samples simultaneously. This constraint may be mitigated by applying a control pump and flow regulators to have multiple channels for the simultaneous processing of samples. A high-throughput filtration system has been developed as a screening tool for viruses. This setup is a 96-well plate format, allowing multiple samples to be filtered simultaneously (Tang *et al.*, 2020). The requirement for the fluorescence microscope might be considered another constraint of this method. However, smartphone-based fluorescence microscope developments with high magnification allow low-cost and convenient fluorescence imaging of viral particles (Cho *et al.*, 2016; Müller *et al.*, 2018; Chung *et al.*, 2021). These technologies may further develop this detection method to have a more straightforward setup and be applicable for on-site bacterial pathogen detection.

The success of the image analysis-assisted enumeration of bacteriophage for detecting *E. coli* can lead to rapid, cost-effective, and easy-to-use bacteria detection methods applicable in food and agricultural industries. The detection approach developed in this study does not require complicated sample preparations. Using wild-type bacteriophage T7 without any genetic modification opens opportunities to develop other phage-based assays using environmentally isolated phages.

5.4. Conclusion

T7 phage particles can be visualized using SYBR Green I nucleic acid stain and fluorescence microscope. The number of the phage particles in the fluorescence images can be enumerated using image processing software, which allows the rapid enumeration of phage amplification upon infection of the target bacteria. The rate of phage amplification detected by the image analysis-assisted enumeration is comparable with the qPCR method. The phage quantification results obtained from the imaging method shows high correlation with the plaque assay method. With this approach, *E. coli* 10 and 10^2 CFU/mL in TSB can be detected after 4-hour enrichment and 1 hour of infection, and 10^3 CFU/mL of *E. coli* can be detected after 4-hour enrichment and 30 minutes of infection. The imaging detection method can detect 10 CFU/mL of *E. coli* in selected food matrices, coconut water, and spinach wash water, within 7 and 6 hours, respectively. The sensitivity of the imaging approach also eliminates the need for extensive sample preparation and secondary amplification of the target phage DNA using RT-PCR or other amplification methods. This image analysis-based rapid bacteria detection approach can be further developed to be applicable for pathogen detection in agricultural industries and other foodborne pathogens.

5.5. Potential future works

5.5.1. Automated imaging system and AI-based image analysis

The advantages of fluorescence imaging include that the imaging system can be developed as an automated system allowing several images to be acquired automatically and reducing the hurdle of manually acquiring images. Consequently, for the phage particle enumeration step, a large number of images can be processed simultaneously allowing high throughput analysis of images. In addition, with the availability of smartphone-based fluorescence microscopes and machine learning technology (Koydemir et al., 2014; Zeinhom et al., 2018), this detection approach can be further developed as a portable bacteria detection device that can be employed for on-site bacteria detection.

5.5.2. Detection of phage amplification using CRISPR/cas12a

The Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins technology or CRISPR/Cas was first discovered as a bacterial immune system against the invasion of bacteriophages and other genetic materials (Hille et al., 2018). The system consists of a short-repeated DNA called guided RNA (gRNA) which its sequence is complementary to the target DNA. It is used to guide the Cas nuclease protein to the target site and then cleaves the specific sequence of the target DNA (Yao and Liu, 2018). Among various types of the Cas nuclease protein, cas12a not only cleave the target DNA at the specific site but also possess a collateral cleavage activity that after a ternary complex of Cas12/gRNA/target DNA is formed, the cas12a nuclease will also non-specifically cleave any collateral single-stranded DNA (Li et al., 2018, 2019). CRISPR/cas12a system has been applied to detect microbial pathogens when coupled with DNA amplification techniques such as PCR, Loop-Mediated

Isothermal Amplification (LAMP), or Recombinase Polymerase Amplification (RPA) (Li et al., 2010, 2021; Chen et al., 2018; Ma et al., 2021; Zhou et al., 2022). The CRISPR/cas12a system can, therefore, be applied to detect T7 phage amplification by targeting the increase in T7 DNA upon T7 infection on *E. coli*. With this approach, the isothermal amplification of *E. coli* DNA is not required, which enables a relatively simpler CRISPR/Cas based *E. coli* detection approach.

The detection of T7 DNA using the CRISPR/Cas12a system can be conducted using guided RNAs that are specific to T7 phage DNA sequences. After the formation of the gRNA and cas12a protein complex, the samples containing T7 DNA will be added along with single-stranded DNA fluorescence probes. After the gRNA and cas12a protein complexes bind with the target T7 DNA, the collateral effect of the cas12a protein will cleave not only the T7 DNA but also the single stranded DNA fluorescence probe generation fluorescence signal. Detection of the fluorescence signal can be done by using a simple fluorometer. The reaction condition such as temperature and the gRNA:Cas12a protein ratio can be optimized to improve the detection sensitivity. This detection approach can be validated by detection of *E. coli* in food complex samples such as beverages and agricultural water.

5.5.3. Detection of bacterial lysis and phage amplification using the excitation-emission matrix of fluorescence spectroscopy

Another method that can be used for the detection of bacteriophage amplification is fluorescence excitation-emission matrix (EEM) spectroscopy which is the analytical method that provides the multidimensional information (Li et al., 2020). This detection technique is being used to characterized and quantify organic materials as it provides insight into samples' structure, properties, and composition through the characteristic of its a three-dimensional map (Ramsay et al., 2018). EEM spectroscopy has also been applied to detect bacterial cells with a combination

with multivariate analysis (Sohn et al., 2009; Nakar et al., 2020). Therefore, this technique can be applied to detect the amplification of phage and the lysis of bacterial host cells in complex samples.

Machine learning algorithms for classification such as Support Vector Machine (SVM) can be applied to identify the presence or absence of *E. coli* in some selected food samples. SVM is a classification algorithm that doesn't require a large dataset for model training and is not affected by the outliers (Burges, 1998). Since the combination of using phages, EEM spectroscopy, and machine learning for the detection of bacteria in food has never been implemented before, this seems to be a promising plan for future study.

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CHAPTER 6:

Conclusions

In this research, rapid bacteriophage-based bacterial detection methods were developed in order to enable simple and specific detection of bacteria in complex food matrices. This research investigated bacteriophage T7 and engineered bacteriophage T7-ALP as tools for *E. coli* detection. For the T7-ALP phage-based detection, both enzymatic colorimetric and fluorescence substrates were used for enzymatic reaction with the overexpressed alkaline phosphatase and to generate precipitated products which can be observed by the naked eye and fluorescence imaging. By using wildtype T7 phages, bacterial cell lysis and phage amplification can be detected by fluorescence imaging. In addition, quantitative image analysis was applied to facilitate the detection of *E. coli* in food and beverage samples.

Chapter 2 investigated the application of engineered T7-ALP phage to detect *E. coli* in model beverages. By using an alkaline phosphatase substrate (ELF-97) that generates precipitated fluorescence products, the phage-infected *E. coli* cells can be visualized using fluorescence microscopy. The acquired images were analyzed using ImageJ software to quantify the fluorescence intensity which successfully indicates the presence of *E. coli* 100 CFU in 1 ml of coconut water and apple juice within less than 6 hours.

Chapter 3 demonstrated the detection of *E. coli* in coconut water and on baby spinach leaves by using the engineered T7-ALP phages and the alkaline phosphatase substrate that yields visible dark purple precipitates (NBT/BCIP). The formation of the enzymatic product can be visualized by the naked eye enabling a simple readout with no complicated bacteria isolation steps required. The color formation was also detected by measuring the color values using the colorimeter allowing quantitative detection of the color change. This approach successfully

detected 10 CFU of *E. coli* in 1 ml of coconut water and 100 CFU of *E. coli* on 1 g of baby spinach leaves within 8 hours.

Chapter 4 investigated the detection of *E. coli* based on the change in the cellular morphology after infection with wildtype T7 phages. With fluorescence staining, the change in the cellular morphology can be observed under a fluorescence microscope. A custom MATLAB code was used to analyze the morphological parameters of the cell particles in the fluorescence images. In addition, the concentration of T7 phage used for the infection and the infection time were investigated. The results indicated that this approach can be applied to detect *E. coli* 10 CFU/ml within 8 hours in laboratory medium, simulated fresh produce wash water, coconut water, and spinach wash water.

Chapter 5 demonstrated an *E. coli* detection method based on the detection of T7 phage amplification. The results suggested that T7 phage particles can be visualized using fluorescence imaging, and the number of phage particles can be enumerated using the custom MATLAB code. Phage quantification using the imaging method was compared with other conventional phage quantification methods. The results revealed the comparable phage quantification efficiency between using the fluorescence imaging method and the qPCR method. With this imaging-based detection approach, 10 CFU/ml of *E. coli* in coconut water and simulated spinach wash water can be detected within 8 hours.

Overall, the phage-based bacteria detection methods developed in this research demonstrated the potential of applying phages in combination with imaging technology as a tool for rapid and specific bacteria detection. The methods can be applied to detect target bacteria in complex food samples while having simple protocols since bacterial isolation steps are not required. The success in developing these detection approaches may lead to further study to

improve the efficiency of foodborne bacterial detection. For example, developing portable biosensing devices that can be utilized for on-site detection, applying AI-based techniques to increase the detection sensitivity and allow automatic quantitative analysis, and applying these detection approaches with multiple phages to broaden the target bacterial strains.