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USE OF VIABILITY QPCR FOR QUANTIFICATION OF *SALMONELLA* TYPHIMURIUM AND *LISTERIA MONOCYTOGENES* IN FOOD SAFETY CHALLENGE STUDIES

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ABSTRACT

Validating food safety interventions can be expensive, time-intensive, and resource-intensive using culture-based plate and count methods (PAC). Viability Quantitative Polymerase Chain Reaction (qPCR) has the potential to increase speed, while also reducing costs and waste associated with quantifying pathogens in challenge studies. The purpose of this research was to develop an efficient viability qPCR protocol and compare its ability to quantify viable pathogens with that of PAC in two, small-scale challenge studies.

Listeria monocytogenes and *Salmonella enterica* serovar Typhimurium were chosen as pathogens of interest due to their association with many foodborne outbreaks, as well as the inherent physiological differences between the two organisms. Development of the viability qPCR protocol began with the selection and validation of qPCR primers, probes, and reagents. Standard curves measuring the qPCR reaction efficiency for *L. monocytogenes* yielded efficiencies of 96.9±2.1% (mean, σ, n=9). *S.* Typhimurium yielded standard curves with qPCR reaction efficiencies of 96.1±1.5% (mean, σ, n=9). R^2 values for all curves exceeded 0.99. These results were well within the acceptable range of 90% to 110% for efficiency and R^2 values above the 0.99. Four DNA extraction kits were then selected and tested for highest yield of pathogen target sequence. Of the four DNA extraction kits tested, Kit Q, the Qiagen DNeasy PowerFood Microbial Kit, demonstrated the greatest ability to extract the target sequences for qPCR analysis of *L. monocytogenes* and *S.* Typhimurium at multiple dilution levels ($p \leq 0.05$, n=8). To mitigate the PCR amplification of DNA originating from dead cells, the

performance of multiple DNA intercalating agents was evaluated. Of the six DNA intercalation treatments tested, treatment P using the product PMAxx, demonstrated the greatest reduction of dead cell DNA amplification across a variety of conditions, without reducing live cell signal ($p \leq 0.05$, n=9).

The optimized viability qPCR protocol was compared to PAC in challenge studies utilizing two different intervention steps: 60°C heat treatment of experimentally inoculated ground beef and a 6% lauric arginate (LAE) dip of experimentally inoculated meat. In the heat challenge study, qPCR and PAC yielded similar starting cell estimates for both organisms; however, qPCR overestimated final counts by 4.94 $\log_{10}$ CFU/mL for *L. monocytogenes* and 3.31 $\log_{10}$ CFU/mL for *S.* Typhimurium ($p \leq 0.05$, n=9). In the LAE experiments, starting estimates for qPCR and PAC were similar for *S.* Typhimurium; however, the qPCR underestimated the starting *L. monocytogenes* counts by 0.82 $\log_{10}$ CFU/mL ($p \leq 0.05$, n=12). qPCR underestimated live cells in LAE-treated *S.* Typhimurium samples by 2.59 $\log_{10}$ CFU/mL ($p \leq 0.05$, n=12). *L. monocytogenes* results trended similarly. These results highlight the potential use of viability qPCR for quantifying pathogens in challenge studies. However, more research is needed to address method limitations. While live cell qPCR estimates were accurate in 3 of the 4 conditions tested, intervention-treated live cells were both overestimated and underestimated, depending on the intervention used. If further development can overcome these challenges, qPCR holds promise for improving food safety and replacing costly, resource intensive, culture-based quantification methods for pathogens.

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Chapter 1

Literature Review

Salmonella spp.

History and Nomenclature

Salmonella spp. are among the most common and adaptable foodborne pathogens. The organism was first discovered in 1885 by Theobald Smith and Daniel Elmer Salmon (1). The bacterium is a Gram-negative, facultatively anaerobic bacilli of the family *Enterobacteriaceae*. *Salmonella* spp. were originally classified based on O (somatic) and H (flagellar) antigens using Kauffmann's serological identification scheme. Originally, each serotype was classified as a separate species. Given the vast number of serotypes and the high level of relatedness between many of the serotypes, people soon began to search for a different way to differentiate between species and serotypes (1).

The framework for the modern classification of *Salmonella* was laid in 1989 when MW Reeves and coauthors were able to demonstrate through DNA-DNA hybridization that all serotypes and subgenera of *Salmonella*, with the exception of *S. bongori*, belonged to a single species (2). After further debate and discrimination, the current nomenclature was decided upon for use by the U. S. Centers for Disease Control (CDC). In it, the genus *Salmonella* is divided into two species: *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies, each having a unique Roman numeral and name (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*). *S. bongeri* is a separate species with relatively few serovars and is given the Roman numeral classification of

type V (1). Of the more than 2,600 serovars of *Salmonella*, *S. enterica* subsp. *enterica* has the most numerous serovars; with over 1,500. In addition, these serovars are responsible for almost all infections in warm-blooded animals, including humans. The other serovars are largely limited to the environment and cold-blooded animals (1).

Characteristics

Salmonella spp. neither ferment lactose nor form spores. They are typically mesophilic with optimal growth occurring at 35 to 37°C, although growth can occur at temperatures ranging from 5 to 47°C. In addition, they can grow in a pH range of 4 to 9, with optimal growth at neutral pH. *Salmonella* spp. also can survive a water activity (A_w) of < 0.2; however, an A_w of 0.94 to 0.99 is required for growth (1).

Salmonella also can exhibit three major antigens: O (somatic), H (flagellar), and Vi (capsular). The O antigen is exhibited on the cell's outer membrane, with specificity determined by the exact pattern of sugars. The H antigen can occur in two forms, phase 1 or phase 2, and a single organism can often change between these two phases. The phase of H antigen present has important consequences for motility and immune invasion by the cell (3). Finally, the Vi antigen may overlie the O antigen; however, most serovars of *Salmonella* will not exhibit any Vi antigen (1).

The cell envelope of *Salmonella* contains a complex lipopolysaccharide (LPS) structure. It consists of three pieces: the outer O-polysaccharide coat, a middle "R" core, and an inner lipid A coat. The O-specific side chains vary widely and are used to distinguish between the various serovars of *S. enterica*. If *Salmonella* do not possess the full sequence of O-sugar repeats, they form "rough" colonies, as opposed to the smooth colonies of specimens equipped with the complete sequence. Typically, these "rough"

colonies have no, or attenuated virulence, as compared to their smooth counterparts (1). The R core of *Salmonella* LPS links the O-chain to the lipid A coat, is like that of many other Gram-negative bacteria, and may be targeted by antibodies (1, 4). The inner lipid A coat acts to anchor the R core and O chain to within the cell's membrane (4).

Salmonella spp. possess a wide range of virulence factors, many of which are linked to pathogenicity islands (SPIs). Over twenty SPIs have been associated with *Salmonella*. SPI-1 is crucial for the invasion of nonphagocytic cells since it encodes for a type III secretion system that moves bacterial proteins into the host cell cytosol. Once injected, these proteins cause conformational changes in the cytoskeleton that allow for *Salmonella* to enter via a membrane-bound vesicle. This role is postulated to be crucial in the invasion of intestinal epithelial cells by *Salmonella* (1). SPI-1 also includes the gene *invA*, the target for qPCR analysis of *Salmonella* in this thesis and other research (5).

Epidemiology and Disease

Salmonella is responsible for more confirmed foodborne disease outbreaks and illnesses than any other bacterium; second to only Norovirus in the number of single-etiology outbreaks and illnesses (6). In addition, the CDC reported that *Salmonella* was responsible for the most outbreak-associated hospitalizations (472, 66% of total hospitalizations) and deaths (14, 70% of total deaths) for 2017 (6). The number of confirmed outbreaks from *Salmonella* in 2017 was 113, resulting in 3,007 illnesses. These outbreaks were most commonly linked to raw chicken and fresh fruits, as well as turkey, vegetable row crops, pork, and raw or undercooked eggs. Serovars implicated in these outbreaks included Heidelberg, Javiana, Braenderup, Newport, Enteritidis, Paratyphi B, Montevideo, Infantis, and Typhimurium (6).

Non-typhoidal *Salmonella* (NTS) is rarely fatal in healthy, non-pregnant adults. Typically, these individuals experience self-limiting diarrhea. Young, pregnant, and immunocompromised individuals can experience more severe symptoms, chronic enteritis, secondary bacteremia, or abortion (1). The natural method for infection is typically through oral ingestion of contaminated foodstuffs. Organisms that survive the acidity of the stomach can colonize in the intestines and establish an infection. Unlike typhoidal *Salmonella*, NTS usually remain in the intestinal lumen, where they trigger a large immune response. This response involves a variety of cytokines and chemokines, as well as a Th17 immune response. In theory, this response should help localize and minimize the infection. In practice, *Salmonella* has evolved a wide variety of mechanisms that allow it to overcome or exploit the immune response and compete with the native microbiota of the gastrointestinal tract (1).

One step in preventing *Salmonella* outbreaks related to meat and poultry is the use of carcass sampling, such as is done by the USDA-Food Safety and Inspection Service (USDA-FSIS) for the *Salmonella* performance standards/verification testing program (7). With the program, USDA-FSIS sets standards for the number of *Salmonella* positive samples allowed in a processing establishment over a 52 week period, based on the type of poultry and product being evaluated (7). Ready-to-Eat (RTE) foods have zero tolerance for *Salmonella* since the product may be consumed without further cooking and/or processing. Typically, processors/establishments must provide proof that product processing and/or interventions result in a 5-$\log_{10}$ reduction in pathogen load as required by the USDA-FSIS. It is important to note that certain products have different standards, such as a 7-$\log_{10}$ reduction required for cooked poultry products (8). Often, it is easiest

for small processors to adopt previously established lethality protocols rather than pay for a challenge study to validate their specific process. Perhaps the most widely used validation document in meat processing is Appendix A (8). This widely available documentation contains the time and temperature combinations (Ex. 145°F for 4 min to achieve 7-$\log_{10}$ reduction) required to meet lethality performance standards for many meat and poultry products (8). It originated from work exploring *Salmonella* lethality in beef and is commonly used by plants to verify that the heat treatment utilized satisfies the products lethality requirements (8).

Listeria monocytogenes

History and Nomenclature

Listeria monocytogenes had been described by 1923; however, it took nearly half a century (circa 1981) before its place as a foodborne pathogen in humans was widely accepted. The genus *Listeria* is currently split into four clades containing a total of 17 species. Only *L. monocytogenes* from clade (*1*) causes illness frequently enough to be considered a public health issue. *L. monocytogenes* was originally serotyped according to differences in teichoic acids and flagellar antigens, although the advent of PCR-based serology has largely made this methodology obsolete. Based on polymorphisms in various genes, the species has been divided into four lineages (I, II, III, IV). Each lineage is found in its own ecological niche, although these niches often have a degree of overlap. Human clinical strains are typically from lineages I and II; lineages III and IV are more prevalent in animals (9).

Characteristics

L. monocytogenes is a Gram-positive, rod-shaped (0.4–0.5μm×1–2μm) bacterium. It is facultatively anaerobic, and produces acids such as lactate from glucose fermentation, but not gas. *L. monocytogenes* is psychotropic and able to grow in conditions ranging from 0°C to 45°C although, as with most opportunistic pathogens, optimal growth occurs at 37°C. In addition, it can grow in up to 10% sodium chloride (NaCl), a pH range of 4.6 to 9.2, and $A_w > 0.92$ (9). It is also resistant to conditions such as freezing and drying. Its habitat includes a wide range of both natural and man-made environments and it is often categorized as being "ubiquitous." Common sources include plant matter, decaying vegetation, and soil. It is also thought to be carried asymptomatically by an unknown proportion of many animals and humans. *L. monocytogenes* has been isolated from a variety of common foodstuffs. Its "ubiquitous" nature allows it to enter the food chain via many different paths. From there, the ability to grow in conditions low in temperature and nutrients means that it can colonize and persist in food production facilities, creating an ongoing risk of product contamination. Once contaminated, the ability to tolerate refrigeration and high salt conditions allows the organism to continue growing during storage. This ability also poses an especially high risk for refrigerated RTE foods, as even low initial contamination can result in high numbers of the organism being ingested by consumers if enough time has passed (9).

Epidemiology and Disease

L. monocytogenes causes a small number of foodborne outbreaks, when compared to an organism like *Salmonella*, but its high fatality rate makes it a food safety priority. The number of cases in developed countries is typically below 0.5 cases per 100,000

individuals; but the overall fatality rate is from 3.8% to 21% (9). This fatality rate is 10-fold that of other common foodborne illness infections. Most outbreaks are associated with foods that would be considered RTE, such as cheese, retail sandwiches, frankfurter, and packaged salads. Often cases seem to be sporadic and no common source or outbreak is associated due to the long incubation period (9). The annual cost of treatment and deaths is estimated at $2 billion in the United States alone (9).

L. monocytogenes infections typically result from consumption of contaminated food, although animal contact and mother-to-fetus transfer are also possible routes. The infectious dose is highly dependent on the host immune function. Analysis of food from outbreaks usually yields 100 or more colony forming units (CFU/gram), although levels lower than 0.3 CFU/g were associated with a major outbreak in frankfurters. As a rule, levels below 100 CFU/g are considered to have a lower probability of causing disease (9). In healthy adults, this number is much higher and anything below 1000 CFU is considered unlikely to cause disease. The incubation period following ingestion can vary widely, with times from 24 hours to 91 days being reported (9). Humans with milder cases exhibit flu-like symptoms, with some experiencing vomiting and diarrhea. Gastroenteritis can result from ingesting exceptionally high doses ($>10^7$ CFU/g) but usually resolves in individuals with good immune function. More serious cases, referred to as listeriosis, can involve septicemia, spontaneous abortion, and meningitis (9).

Since *Listeria* spp. replicate within host cells, a cell-mediated immune response is crucial in clearing infection. This observation means that young, old, pregnant, or immunosuppressed (YOPI) individuals are more susceptible to listeriosis and can succumb to serious disease. YOPIs experience infection fatality rates of 13% to 34% and

may be several hundred times more susceptible to listeriosis than the general public (9). The ability of *L. monocytogenes* to cross the placenta and directly infect the fetus makes it of particular concern for pregnant individuals.

Control of *L. monocytogenes* can be quite difficult due to its ubiquity, salt tolerance, and ability to grow at refrigeration temperatures. Growth rates can be controlled significantly by keeping storage temperature as close to freezing as possible. In addition, a combination of growth hurdles such as low pH, low a_w, and low storage temperatures is usually more effective than any one hurdle. There also has been great interest in using the bacteriocins produced by various lactic acid bacteria to suppress the growth of *L. monocytogenes.* For example, nisin (derived from *Lactococcus lactis* ssp. *lactis*) can reduce the population and heat resistance of *L. monocytogenes* (9). Bacteriophages specific to *Listeria* spp. also have been approved and implemented as food processing aids during the production of various high-risk foodstuffs (9). Lauric arginate has also been shown to effectively reduce *Listeria monocytogenes* in high risk products, such as RTE frankfurters (10).

Since *L. monocytogenes* is classified as an adulterant in the United States, RTE foods that could support growth must contain less than 1 CFU/25g. According to FSIS 9 CFR 430, any RTE product containing *L. monocytogenes* or coming into contact with a food contact surface containing *L. monocytogenes* are considered contaminated under its "zero tolerance" policy (9,11). This intolerance has led to *L. monocytogenes* being responsible for a number of Class 1 recalls in the United States. Foods not supporting the growth of the bacterium can have up to 100 CFU/g before a recall is triggered (9).

Environmental testing for *Listeria* spp. is often used as an indicator for *L. monocytogenes* presence in food production facilities. In addition, testing specific to *L. monocytogenes* in final products is used to ensure that the product itself meets food safety standards (11).

Methods for isolating *L. monocytogenes* such as the U.S. Food and Drug Administration (FDA) method and International Organization for Standardization (ISO) methods follow the same basic procedure of enriching and selecting for *L. monocytogenes*. If it is suspected that the bacterium may be injured, a less selective enrichment might first be utilized prior to any selection. The first selective enrichment occurs in broth and uses lithium chloride and other selective agents to help control growth of other bacteria. This enrichment is incubated at 30° for 24 hrs then sub-cultured into a second, 48-hr enrichment in broth containing even higher concentrations of the selective agents. This second enrichment occurs at lower temperatures, from 30 to 37°C, depending on the exact protocol used. The final step involves culturing of the second enrichment on a selective-differential agar, traditionally Oxford or PALCAM agar. If specificity towards potential pathogens is desired, media which can detect virulence factors such as phospholipase can be used. Confirmatory assays of presumptive colonies can be biochemical, molecular, or immunological. The shift towards Polymerase Chain Reaction (PCR) and Whole Genome Sequencing (WGS) based analysis of pathogens will presumably continue to play an increasingly large role in confirmation and food outbreak traceback (9).

Since it is considered a “zero tolerance” organism in the United States, the presence of *L. monocytogenes* in a product or even contact of the product with a food

contact surface that tests positive renders the product adulterated, according to USDA-FSIS (11). Presence of *L. monocytogenes* is especially of concern in RTE meat and poultry products given its ability to grow during long-term refrigerated storage. There are three alternative options used by meat and poultry processors to control *L. monocytogenes* contamination post-lethality: the application of a post-lethality treatment (PLT) and an antimicrobial agent or process (AMAP); the application of a PLT or an AMAP; or the use of a sanitation program for control of *L. monocytogenes* (12). Sampling protocols vary depending on the alternative used. Another unique aspect of zero tolerance is that instead of showing a 5-$\log_{10}$ reduction, the main objective of challenge studies is often to demonstrate that an antimicrobial agent allows no more than 2 log of growth over the entire shelf life of the meat or poultry product (11).

DNA Extraction

Origins

Deoxyribonucleic Acid (DNA) was accidentally discovered in 1896 by Swiss physician Friedrich Miescher (13). His research aimed to determine the principles of life by analyzing the chemical composition of cells. To do this, he collected leucocytes from pus associated with surgical bandages and subjected it to various tests and procedures. He eventually was able to prove that proteins were the main constituent of a cell's cytoplasm; in the process, he noticed a separate compound precipitated from solution in the presence of acid, only to dissolve again if alkali was added. This precipitation was the first "extraction" of DNA. Miescher then refined the separation of DNA from proteins by trying to first separate the cells' nuclei before isolation but was unable to obtain enough DNA for further analysis. Later, he developed a method that could isolate larger

quantities of the precipitate, later named “nucleic acid” by his student, Richard Altman (13). Since this first, crude DNA extraction, researchers have continuously searched for methods to improve the purity, efficiency, and integrity of their extractions. The general steps for all extractions are: (a) disruption of membranes, (b) separation of DNA from lipids, proteins, and other nucleic acids, and (c) concentration and purification of DNA (14). How the various DNA extraction methods go about achieving this goal varies greatly; some of the more common approaches are as follows.

Methods

Many early attempts at DNA extraction utilized chromatography to help separate the DNA from other cell components based on physical or chemical characteristics. Size-exclusion chromatography was developed in 1955 by Lathe and Ruthven and uses a column containing gel beads to divide molecules based on their size and shape (15). When an aqueous solution containing the molecules of interest is forced through the beads (typically composed of polyacrylamide, dextran, or agarose), small molecules, such as RNA and proteins, enter the pores within beads. The much larger DNA molecules are excluded from entering the beads. This procedure results in DNA traveling through the matrix at a higher rate, whereupon they can be collected off the column as a separate elution from the other, smaller molecules. DNA extraction also may utilize the properties of ion-exchange chromatography (16). In this method, a DNA anion-exchange resin column is used. The resin contains positively charged diethylaminoethyl cellulose groups which selectively bind the negatively-charged DNA. Other cell components such as proteins, lipids, carbohydrates, and RNA are eluted from the column using medium-salt buffers. A low pH or high-salt buffer is then used to recover the DNA from the column

(17). Ion-exchange chromatography has proven to be a relatively simple way to gather high-quality DNA (17).

Perhaps the most time consuming, costly, and material-intensive DNA extraction method is a cesium chloride (CsCl) gradient centrifugation method (18). CsCl is a heavy salt and very long periods of centrifugation at very high speeds cause components to collect at the bottom of the tube with a density gradient forming along the tube's length. This gradient means that cellular components of different densities will collect at different lengths throughout the tube, allowing components such as DNA to be separated off from other fractions within the column. This method can be further modified by including ethidium bromide (EtBr) which is able to intercalate DNA bases, causing uncoiling and forming a lower density complex than DNA alone. Closed, plasmid DNA has a lower EtBr saturation point than linear DNA. This means the saturated plasmid DNA will have a higher density than the saturated, linear DNA-EtBr complexes and form a separate band slightly lower in the tube. Despite these capabilities, the long time (overnight centrifugation is not uncommon), and personal and reagent intensity of this protocol, has made it "unfashionable" (18, 19).

Alkaline extraction is another method that can be used to extract plasmid DNA from bacteria. In it, sodium dodecyl sulfate (SDS) detergent is used to lyse cell membranes and denature proteins and sodium hydroxide (NaOH) denatures the DNA; cellular DNA fragments are linearized and irreversibly separate, the circular plasmid DNA loops remain topologically constrained. After the SDS/NaOH mixture is applied, the plasmid DNA is renatured, and the cellular DNA precipitated by using potassium acetate to neutralize the solution. Centrifugation is used to pellet the cellular DNA and

debris. The plasmid DNA can then be recovered from the supernatant via alcohol precipitation and centrifugation (19).

The use of silica matrices has become an increasingly popular form of DNA extraction as the method is simple, fast, cost efficient, and produces high quality DNA. Vogelstein and Gillespie were among the first to recognize that DNA had an affinity for silicates (20). The mechanism for this process is that the negatively charged DNA is attracted to a silica surface covered in positively-charged ion. When the DNA is securely bound to the silica matrix, various high salt washes can be used to remove other cellular components and purify the DNA. Finally, the DNA is eluted through use of a low ionic strength, neutral solution (21). To help reduce the costs of these systems, researchers have developed a method to allow for reuse of the silica column (21). The salting out method utilizes reagents to lyse cells, precipitate proteins, then precipitate the DNA. The first phase uses a lysis buffer containing reagents such as SDS and ethylenediaminetetraacetic acid (EDTA) to lyse cells and chelate metal ions thus inactivating DNases during overnight incubation. Protease K may also be added to help break down proteins, although this enzyme increases the complexity and cost of the process (22). Then, a saturated NaCl solution is added, and the sample is vortexed and centrifuged. The purpose of the high salt solution is to decrease protein solubility, allowing them to be precipitated from the solution. The DNA containing supernatant is then decanted and the DNA is precipitated from solution using ethanol (23). This method has the advantage of being relatively simple and not utilizing harmful solvents such as chloroform or phenol (23).

The phenol-chloroform method is widely used for DNA extractions, but it is time consuming and uses hazardous solvents. It begins by using a lysis and digestion buffer containing reagents such as SDS. A mixture of phenol and chloroform is then mixed in to denature the proteins. The mixed biphasic emulsion is then centrifuged, creating an upper aqueous layer containing the DNA as well as a lower organic phase containing proteins. The aqueous phase can then be transferred into a separate tube and the process is repeated until no protein is visible at the interface between the two layers. Since phenol and water are somewhat miscible, the aqueous phase is then mixed with an equal volume of chloroform and the extraction repeated to remove residual phenol. Once this process is achieved, the DNA can be precipitated from the aqueous layer using ethanol (19, 24).

The use of magnetic beads is another DNA extraction method. Benefits include yields equivalent or greater to that of conventional methods, quick procedure times, and a simple setup. As with most extractions, the first step is to lyse the cells using a lysis buffer, such as SDS detergent. Magnetic beads are then added to the solution. A combination of charge based and hydrophobic interactions cause DNA to bind to the beads (25). Silica and agarose are classic coatings, although the use of carboxyl-coated and naked beads have been explored more recently. The DNA-magnet complexes are then pelleted to the bottom of the tube using magnetic force. The cell component laden supernatant is then decanted off the pellet and the pellet washed with ethanol. The final step releases the DNA from the magnetic particles by resuspending the pellet in a buffer solution and incubating the particles at high temperatures (65°C) under agitation (26).

Cellulose-based (paper) DNA extraction is a relatively recent method that has become more common with the release of products such as Flinders Technology

Associates (FTA) cards. It is not necessarily able to yield high levels of pure DNA but has the redeeming feature of being incredibly simple and quick to use. In its simplest version there are only two steps: pipette the culture onto the card, then allow the card to air dry. The principle behind the method is that the DNA is attracted to and binds with the fibers in the card, while a chemical mixture lyses cells, denatures proteins, and "protects DNA from degradation." The dried sample can then be stored until needed or used right away. In some applications, the paper/DNA complex is used directly; in other applications, the DNA is eluted from the card into solution (27). Although this method has its drawbacks, its simplicity makes it attractive for fieldwork where proper nucleic acid preservation would be difficult (28).

Use in Food Matrices

Although DNA extraction has been around for decades, its use in food matrices and with emerging technologies such as qualitative PCR (qPCR) is rapidly evolving to meet the challenges posed by these systems. Many of the studies in this area measure the ability to extract total DNA from a food matrix to determine the composition of the food matrix as a check for adulteration or misidentification of the product. A number of these studies, their findings, and challenges they encountered are summarized in Table 1. One common issue across all studies was inhibition of the PCR reaction or higher cycle threshold (C_t) values resulting from DNA extraction of "complex" foods (29–31). The differentiating factor in these foods was usually a higher concentration of proteins, fats, or both. In addition, these complex matrices tended to yield better DNA extractions from silica column based kits, whereas a magnetic bead-based kit worked better for "simpler" matrices such as liquid milk and vegetable matter (32, 33). The studies also found that

high variability in DNA quality or yield could result from factors such as the tissue type used, level of processing, the exact species being used, packing liquid, and storage time (30, 34–37). Finally, it was found that commonly used metrics such as absorbance ratios (A_{260}/A_{280} provides insight of if DNA or RNA is present; A_{260}/A_{230} provides evidence if common contaminants are present) did not necessarily correlate well with PCR reaction success (34, 38).

Quantification of bacteria from food and other complex matrices may not encounter some of the complications found in these studies. For example, bacterial DNA would not undergo processing-related degradation if contamination occurred post-processing. In fact, degradation of dead bacterial cell DNA via processing may help reduce dead cell signal associated with qPCR. However, other issues that were found, such as inhibition of the qPCR reaction or binding of DNA by carbohydrates, proteins, and lipids, could interfere with qPCR quantification of bacteria. The degradation of dead cell DNA by processing could also cause issues if it were desired to retroactively estimate the level of contamination in a product or extract DNA for whole genome sequencing (WGS) when trying to determine the source of an outbreak. In addition, binding of bacteria to the food matrix can create interesting product sampling challenges, such as accounting for the concentration of *Brucella* at milkfat interfaces (40).

Table 2 provides a list of studies that investigated extraction of microbial DNA from complex matrices, such as processed foods, fresh milk, and fecal matter. Although not every matrix is a foodstuff, it would be expected that the broader trends discovered would be applicable in extraction of pathogen DNA from food systems. One issue was that high levels of protein and fat, were associated with poorer extraction outcomes (31).

Extraction efficiency also depended on the method of extraction used. Magnetic beads work well in some systems, but this issue did not seem to be consistent across studies (41–43). Silica column-based extractions were not always effective, but were generally among the most efficient at extraction across a variety of matrices (41, 42, 44–46). Sometimes, the simpler extractions, such as the cellulose-based FTA Elute, were able to yield good results (45, 47). It should be noted, that findings between studies did not always agree and one source found no major differences in qPCR suitability between methods such as magnetic beads, silica spin columns, and physical, chemical, or thermal lysis (48). Other researchers found that more than one method, such as silica columns and magnetic beads, had similar efficiencies (41, 42, 46). When shown that several extraction methods are equally effective for an application, it becomes important to account for other factors such as the time, labor intensity, and cost of an extraction. For example, methods using enzymatic lysis and phenol-chloroform extraction could be unfavorable, due to having a run time of nearly a day and utilizing a variety of toxic chemicals (42).

Several trends did seem to hold true throughout the studies. As mentioned before, high quality silica column extraction kits were effective across most matrices. Lysis method also seemed to play a large roll in extraction efficiency. Enzymatic and physical lysis were both found to be effective; but most effective of all, were protocols in which multiple lysis methods were combined. Useful lysis methods included bead beating, the use of one or more lysozyme type enzymes, ampicillin, heat, and various chemical solutions (42, 48–50). Cleanup steps such as the addition of proteinase K and RNase were also found to be helpful (46, 48). Finally, several papers found that traditional DNA quality measurements did not correlate with qPCR results (44, 48). One reason for this

discrepancy is that the extremely large quantity of host/matrix derived DNA inherent to these systems may mask the volume of bacterial DNA present. In addition, a highly efficient PCR reaction may yield useful results, despite the DNA extract having what would traditionally be considered unacceptable quality and quantity measurements.

Microbiological Challenge Testing and Viability qPCR

History and Elements

Microbial control of food to prevent disease and spoilage was first utilized thousands of years ago via fermentation of highly perishable products, like milk and juice, to produce more stable foodstuffs, such as cheese and wine (52). Although these first attempts to improve the food supply likely depended on the nature of bacteria to utilize nutrient supplies to kill off competition, humans recognized the value of these natural processes and have been searching ever since for new ways to control pathogens and spoilage organisms in food. Perhaps the most important advancement in modern microbiology towards achieving this goal was the implementation of agar plates by Robert Koch. The use of agar, suggested to Koch by Angelina Fanny Eilshemus, the wife of one of Koch's associates, greatly increased the ease with which pure cultures could be established and microbial samples could be enumerated (53). Once there was the ability to enumerate the number and type of bacteria present in foodstuffs, it became possible to quantify how effective a given process was at killing or inhibiting the growth of a bacterial species.

This desire to quantify the efficacy of a food safety process led to what is known as a "Microbiological Challenge Testing." The food safety process tested may rely on a single "intervention step" or critical control point (CCP) to control pathogens, or on the

combined effect of many interventions in what is known as “hurdle technology.” Hurdle technology utilizes the fact that while a single intervention may not produce the desired effect, the synergistic action of multiple interventions may result in sufficient microbial control (54). This concept is useful, as the degree of application needed for a single intervention to work can often have detrimental effects on the quality of the product. Fermented sausages are a classic example where a combination of reduced pH, low A_w, and other interventions (ex. packaging, heat treatments, etc.) are utilized to create a product that is safe without being unpalatably dry or acidic, which might result if only one such intervention was utilized. It is possible that the use of certain A_w, pH, processing technology, or a combination thereof, may have been previously established as resulting in sufficient pathogen control. In this case, pertinent products may not require challenge testing (55). If it has been determined that a product will need challenge testing, multiple factors must be considered in the design of the study. To accomplish this approach, it is necessary to find individuals with the expertise to design and facilities to conduct the experiments needed.

Challenge studies can be further divided into three broad categories: pathogen growth inhibition, pathogen inactivation, and combined growth and inactivation studies (55). Growth inhibition studies are used when it is desired to know if a specific product will inhibit growth of relevant pathogens under set time and temperature conditions. Inactivation studies evaluate the ability of a formulation, process, or combination thereof, to inactivate the relevant pathogens over the life of the product. Combined growth and inactivation studies combine elements of both previous types of studies.

The product used in a challenge study should mimic the commercial product as closely as possible; it should replicate any characteristics such as A_w and pH that may impact microbial control. Processing variation is also used in determining the worst-case scenario for microbial contamination (ex. high levels) and survival to ensure that even the worst of the product meets food safety standards. The microbiota of the product must also mimic that of the commercial product as it can both increase or, more commonly, decrease the ability of pathogens to attach to and survive in the product (55).

Selection of pathogens is an important part of the study design. The organisms used should reflect the pathogens that present the highest risk in that product, based on past outbreaks in similar products (56). For example, when evaluating challenge studies in a fresh/raw beef product, researchers should consider the use of enterohemorrhagic *E. coli* and beef-specific *Salmonella* spp., while using *L. monocytogenes* for a RTE beef product. Similarly, a challenge study with a fresh/raw poultry product may utilize *Campylobacter* spp. and poultry-specific *Salmonella* spp. and *L. monocytogenes* for RTE poultry products. For some bacterial species, use in challenge testing is rare, since contamination risks overlaps with that of other, hardier organisms. For example, *Shigella* spp. are not commonly used in challenge studies when *Salmonella* spp. are commonly found in the same sources and exhibit higher rates of growth and survival than *Shigella* spp., especially under adverse conditions (56). Since different strains of a species may exhibit different susceptibility to the process, it is recommended that a cocktail of three to five strains be used (55). These recommendations are selected based on an association with previous outbreaks in similar products or known resistance to the process/intervention being used. Any genetic differences between strains may also be

useful in determining which strains were most successful over the course of the study. If the relevant pathogens cannot be used due to a lack of facilities or unreasonably high risk, it may be possible to use surrogate strains. These strains should be nonpathogenic, grow and be inactivated in a manner similar to the pathogen of interest, and not persist as "spoilage" organisms in production facilities after the trial is completed (56). Some pathogen surrogate pairs such as *Clostridium sporogenes* PA3679 and *C. botulinum* or *L. innocua* and *L. monocytogenes* are well established; use of novel surrogates would entail preliminary validation of the pair (55). Finally, strains should be tested in advance for antagonism between strains and the manner in which the cultures are prepared should be standardized (56).

Inoculation preparation and microbial levels play a large part in the success of challenge testing. If the goal of the study is to monitor growth, it is necessary to use high enough levels of the organisms to mimic a heavily contaminated product, but levels should not be so high as to artificially overwhelm the antimicrobial factors in use (55). Levels of 2 to 3 $\log_{10}$ CFU/g typically allows for achievement of both objectives, although this level can vary depending on the product being used. In contrast, inactivation studies almost always require much higher inoculation levels by their very nature. Depending on the exact Code of Federal Regulations (CFR) and regulating agency (FDA or USDA-FSIS) involved, inactivation testing typically requires proof of a 4 to 7 $\log_{10}$ (4 to 7 D) reduction. To demonstrate this reduction, a starting inoculation level of at least 1 $\log_{10}$ higher than the required reduction is desired (56). The inoculum must be prepared in a consistent and suitable manner. One element of this process is trying to preserve the genetics of the pathogen as much as possible. This approach

requires that a culture have as few passages from the original culture to the challenge testing inoculum as possible; typically less than five passages is preferred (55). The cells to be used in challenge testing are generally prepared via growth for 24 hours on nonselective media at optimal conditions, washing of the resulting culture to remove metabolites, and resuspension in as little carrying media as possible. This last step is crucial to avoiding alteration of key properties such as A_w and pH in the foodstuff. In certain conditions, it may be appropriate to precondition the cells with acid, cold temperatures, or some other variable so their level of stress and resistance more closely mimics that expected of cells naturally found in the product (55). Immediately after inoculation, the product should be tested to ensure that sufficient levels of inoculation were achieved. This approach can prove especially challenging when fast-acting antimicrobials are involved, since they may begin to reduce the level of recoverable microbial cells (55).

The conditions and duration of product storage after inoculation are dependent on intended use. Variables such as temperature and humidity should represent the "worst-case scenario" that would reasonably be expected for the product. This approach may dictate unfavorable static conditions or the use of variable conditions to replicate the various steps in the product's supply chain (55). The duration of the study should encompass, at minimum, the shelf life of the product. However, it is typically encouraged, and perhaps even required for the study to extend past at least one third of the product's intended shelf life (56). This approach is done under the assumption that consumers will often retain and consume products beyond the intended date and well-designed testing will account for this variation. The numbers of samples taking during the

study varies, depending on factors such as length of shelf life; however, five to seven samplings is generally the minimum desired (56). This method could result in the product being tested anywhere from every few hours to less than once a week. Typically, sampling occurs most frequently at the start of the study with frequency decreasing as the storage stage of monitoring proceeds.

Sampling and enumeration are largely carried out according to previously established guidelines and protocols. Usually, samples are taken in triplicate and a minimum of two, time-independent trials are conducted (55). Sampling procedures and culturing methods should be designed based on previously established guidelines, often involve the use of selective media when multiple species are used, and enrichment if cells are expected to be injured. Physical factors such as A_w and pH are also often tracked throughout the course of the study, as they are often crucial to microbial suppression and inactivation (55).

If a process is successful in suppressing growth, inactivating the required population (log_{10}), or preventing toxin production, the data can be used to support the use of the process in safe manufacturing. If the process fails to achieve its goals, the process may be adjusted until it is successful (56).

Quantitative PCR (qPCR)

Much credit should be given to the traditional “plate and count” method (PAC) for pathogen enumeration since it has improved food safety; however, it has numerous drawbacks. The number of plates, spreaders, and dilution tubes needed to challenge test a process may run in the thousands (57). Often, this equipment is disposable, and an enormous amount of waste is generated, which can end up in public landfills. PAC

methods are also very time intensive in terms of both human and absolute hours. Even a proficient lab technician requires a great deal of time to plate agar/petri dishes required over various time points and to conduct dilutions of the samples. In addition, the minimum culturing time for many plates is 24 hrs, with 48 hrs often being typical (58). In medical applications, a genus such as *Mycobacterium* often requires three weeks before detection, and confirmation may take even longer (59). The combination of time and material intensity makes these studies very expensive. The exact cost depends on factors such as duration, pathogens used, interventions employed, and number of trials. Realistically, $10,000 is a minimum, estimated cost with potential costs much higher (60). This laborious process, time, and cost are onerous burdens for most food processors (especially small and very small) and may discourage the development and/or validation of novel food safety processes.

Traditional PAC methods also are unable to detect the presence of viable-but-non-culturable organisms (VBNC). VBNC cells are unique in that they cannot be recovered by traditional culturing methods, yet remain metabolically active (61). The main concern of VBNC cells is the presence in food after the application of an antimicrobial or intervention, yet remain undetected by PAC procedures. The original work on VBNC was done with *E. coli* and *V. cholerae* (61). Since that time, other food pathogens, including *C. coli*, *C. jejuni*, Enterohemorrhagic *E. coli*, *L. monocytogenes*, and *S. enterica* have been known to enter the VBNC state (62–65). Approximately 80% of *Campylobacter* cells can become VBNC after three days of unfavorable conditions (65). Whether VBNC pathogens can cause infection of the host has been debated and may be specific to certain species or conditions. Several reviews of studies conducted with

various pathogens have reached a conclusion that while most VBNC cells are not necessarily infectious in the VBNC state, they retain virulence and can cause infection upon revival (64, 66). Not only do plate and count methods fail to detect VBNC members of many common pathogen species, researchers have yet to develop any type of PAC methods for the majority of bacterial species (67).

The use of quantitative polymerase chain reaction (qPCR) may overcome the cost and culturability issues associated with PAC methods. Quantitative PCR is much less material-intensive than PAC. While the basic resources needed to process the food are the same, the only media used is in the stomaching and/or homogenization of samples. Instead of consuming hundreds, if not thousands of dilution tubes and petri dishes, the entire DNA intercalation and extraction process for a sampling point can be carried out in a few dozen 2 mL tubes, and a single 96 well qPCR plate. In addition, samples can be quantified in a matter of hours, not days. The combination of these factors has the potential to reduce processing costs, equipment requirements, and improve sample turnaround. Rather than being limited by the ability to culture an organism, quantification should be possible for any organism that has been successfully sequenced. This approach also would greatly increase the number of quantifiable species (67). It is also possible to create primers specific to genes only present in certain serotypes, allowing for increased discrimination in testing, something that is difficult or impossible with PAC methods. For example, an assay has been designed that is highly specific to *E. coli* O157:H7, yet has little to no cross reactivity with serotypes such as O104:H4, O103, and O121 (68).

Quantitative PCR has already been utilized with success for detection or quantification of bacteria. Reischl *et al.* found that qPCR was suitable for detecting the

presence or absence of *S. aureus* and an antibiotic resistance gene (69). Nogva *et al.* were able to quantify *L. monocytogenes* with limited success in various milk samples, although they found that the limit of detection varied greatly, depending on the DNA extraction and matrix (70). It has also been demonstrated that qPCR may be suitable for the rapid analysis of *L. monocytogenes*-containing biofilms (71). Ricchi *et al.* did further work comparing culture, qPCR, and digital Polymerase Chain Reaction (dPCR) quantification and found that the PCR methods yielded similar results to PAC methods for *L. monocytogenes* in broth culture although, a roughly one $\log_{10}$ difference in quantification could be found between PCR methods and culturing for other organisms (72). Acharya *et al.* found that qPCR demonstrated a higher degree of agreement with and more overall positive samples than culturing for detection of *M. avium* ssp. *paratuberculosis* in cattle herds (73). Sensitivity of the qPCR also was significantly increased by diluting the DNA extract to help reduce the level of inhibitors present in the sample. Walker *et al.* (2017) found that a qPCR assay for *E. coli* was both more inclusive for *E. coli* and exclusive towards related genera such as *Shigella,* than the culture-based assay, although some of the positive environmental samples may have been due to the presence of non-viable cells (74). Detection and quantification of *Campylobacter* spp. may be an area where qPCR is especially advantageous over culturing. *C. jejuni* is a fastidious microbe and even processing steps not typically associated with microbial lethality, such as freezing and atmospheric oxygen levels, may cause cell injury or induction of the VBNC state (75, 76). The use of qPCR would allow detection of the non-culturable cells and has been shown to detect *C. jejuni* at higher rates than culturing (77). It should be noted that the dilution of samples and DNA can have a major effect on qPCR limit of detection and

could lead to qPCR having lower quantification and detection abilities than PAC methods (78). An in-depth comparison of qPCR, traditional culture or PAC methods, and compact dry (a type of commercially available, preprepared petri dish) cultures for *Salmonella* spp., *E. coli*, and *S. aureus* in UHT milk, sterile ground beef, and sterile oyster meat found that while qPCR tended to have one $\log_{10}$ lower quantification than the traditional culture method, it was never significantly lower, and in one case, was significantly higher (79). There was only one case in which the compact dry culturing method did not yield significantly higher quantities than both qPCR and traditional culture. In addition, the researchers found that limit of detection was typically around 1 $\log_{10}$ of gene copies. The use of qPCR has also been tested for fungal pathogens and it was found that qPCR allowed for identification and relative quantification of two fungal pathogens in samples, whereas traditional methods only identified a single pathogen (80). Researchers also found that very high relative abundances (roughly 1000:1 or greater) of competing pathogen or host DNA resulted in underestimation or variation of quantification ability of the less prevalent DNA. Investigation of epidemics and foodborne outbreaks may also benefit from qPCR analysis. qPCR has already been used in a longitudinal study of porcine epidemic diarrhea virus to track viral load and prevalence in several pig farms (81).

Droplet digital PCR (ddPCR) also has been explored for use in identification of pathogens. In ddPCR, thousands of sample droplets are formed, and PCR amplification is carried out in each one. Each droplet is then measured for the presence of the target and Poisson statistics are used to determine starting target concentrations (82). Some studies suggest it may have higher sensitivity than qPCR, although it is still a relatively new

technology and more studies will be required to reach any definite conclusions (72, 83, 84).

Several conclusions about qPCR can be made from the culminated evidence of the studies mentioned. First, it is evident that qPCR likely has a place in the detection of microbes. It has the advantages of being rapid, having extremely high specificity, being able to accurately discern between strains of an organism or look for relevant genes, and detect organisms that are traditionally unculturable, due to injury, VBNC state, or a lack of sufficient advances in media design. However, the current ability of qPCR to quantify with high levels of accuracy or at low levels of pathogen is likely not to the point where it can fully replace PAC methods. Additional research and advances will hopefully overcome these issues. A further drawback of conventional qPCR for use in detecting and quantifying pathogens is that the amplification process is specific to the target DNA sequence but does not distinguish if that sequence was derived from a living or dead cell. An important caveat for all the qPCR detection and quantification studies previously mentioned is that it is possible at least some of the amplification signal was coming from cells that were not just VBNC, but dead. In applications where the number of dead cells is expected to be low or detection of both live and dead cells is desired, this observation is not an issue. However, the inability to detect live and dead cells with qPCR poses a problem for challenge testing and foodborne outbreaks where only live cell counts are of relevance (85).

Viability qPCR and Intercalating Dyes

The most promising approach to distinguish DNA from live and dead cells is the use of DNA intercalating agents, which can be used to remove or inactivate DNA from

dead cells before extraction and amplification of live cell DNA, in what is known as "viability qPCR." DNA intercalating agents are compounds that can diffuse into dead cells, but are passively blocked from diffusing into living cells (86). Once in the dead cell, the intercalating compound forms a complex with the DNA. One molecule per four to five nucleotides seems to be the upper limit for intercalation with ethidium bromide, less is known for other agents (87). The agent then can be photoactivated using a proper light source. This activation causes the compound to form covalent linkages that damage the DNA. Hixon *et al.* (83) have suggested that the linking of the agent to DNA causes damage, which inhibits amplification. Soejima *et al.* (84) suggest that the combination of the agent and light causes direct cleavage of double-stranded DNA. In contrast, Nocker *et al.* (85) believe that the reduction in amplification of dead cell DNA is due to removal of the DNA during the DNA extraction process (88). These researchers presume that the dead cell DNA is cross linked to other cell components during photoactivation, although the precise mechanism is unknown. Insolubility of the resulting DNA-intercalating agent complex, leading to its precipitation, is another possibility. Photoactivation also serves the dual purpose of causing any unbound intercalating to react with water, forming a hydroxylamine molecule (89). The hydroxylamine will not intercalate and inactivate further DNA molecules, allowing for the live cell DNA to be successfully extracted and amplified.

Although there are many agents that demonstrate some degree of DNA intercalating ability, relatively few compounds have demonstrated the ability to effectively intercalate dead cells, have low suppression of signal from living cells, and be photoactivated in a manner that irreversibly decreases amplification of the target DNA.

Ethidium monoazide (EMA) and propidium monoazide (PMA) are the most promising agents thus far. The two molecules are largely similar, with the notable difference that PMA has two positive charges, as compared to the one positive charge in EMA. The major disadvantage of EMA is that it can cross the membrane of live cells and cause significant live cell DNA loss under select conditions. Research suggests that EMA can inactivate the DNA of non-viable cells, while additional studies have shown that while the suppression is most dramatic for dead cell DNA, EMA also can non-selectively reduce the amplification signal of viable cells (86, 88, 90). This method is an unacceptable result in most food safety applications as it could lead to the underestimation of pathogens and unsafe products being declared safe, a much more dire mistake than the overestimation of pathogens. Conversely, PMA does not seem to cause significant reductions in live cell population and has been successfully used for viability qPCR across several food pathogens and matrices. Nocker *et al.* (88) compared PMA with EMA for use in viability qPCR. They demonstrated that inhibition of DNA extract correlated with increased light and PMA exposure and higher ratios of PMA treated dead:live cells resulted in lower qPCR signal (higher C_t values). In addition, it was shown that PMA did not affect live cell DNA yield for the species tested, whereas EMA could penetrate live cells and reduced the live dead DNA yield for many species, including *S. aureus, L. monocytogenes,* and *M. luteus*, although some species such as *S.* Typhimurium and *P. syringae* appeared more resistant to EMA uptake. The inability for the live cells to efficiently export the EMA was suggested as a likely cause for the loss of live cell DNA. This study provided strong evidence for the preference of PMA to EMA as a DNA

intercalating agent in viability qPCR. Below is a review of additional viability qPCR studies and their findings for various species.

Campylobacter:

Rudi *et al.* (81) found that the use of EMA-based viability qPCR was unaffected by background flora and could measure up to 4 $\log_{10}$ CFU/mL of kill of spiked *C. jejuni* and that exclusion of EMA seemed to be a passive process. EMA viability qPCR has also been used to demonstrate cell membrane damage by zinc nanoparticles (91). PMA qPCR has also proved useful in quantifying *C. jejuni* from chicken skins. The addition of PMA was found to completely repress up to 10^6 CFU/mL of nonviable, heat treated cells and a range of 10^2 to 10^7 was quantifiable (92). Furthermore, PMA qPCR showed a high rate of correlation with the culture-based enumeration, but non-PMA qPCR also showed a high correlation suggesting that the presence of dead or VBNC cells in the carcasses were low.

E. coli O157:H7:

Essential oil treatment inactivation of cells combined with PMA qPCR demonstrated complete suppression of inactive cells at 10^4 CFU/mL, although at 10^6 CFU/mL of inactive cells, the PMA appeared to be overwhelmed and was only able to cause a 2 to 3 $\log_{10}$ reduction in signal (93). If the system could not suppress more than 10^4 CFU/mL of dead contaminants and has a level of detection (LOD) of 10^2 CFU/mL (not determined but estimated based on other studies), there is a relatively narrow window in which the quantification process would be useful. Research comparing the use of various intercalation protocols found that the combination of sodium deoxycholate (SD) and PMA was most effective at reducing dead cell signal and SD-PMA-qPCR

estimates correlated well with plate counts of *E. coli* O157:H7. The limit of detection was also still low (10^2 CFU/mL), supporting the use of SD to increase the action of PMA in cells with mild inactivation treatments, such as the 63°C for 2 minutes (94). A similar study using SD and PMA qPCR also found that *E. coli* O157:H7 quantification yielded similar results to plate counting with a detection limit of 10^2 CFU/mL in milk, even with a background level of 10^6 CFU/mL of nontarget bacteria (95). Li *et al.* found that PMA had virtually no effect on live cell detection, that 8×10^7 CFU/g of dead cell signal could be repressed past their 35 cycle limit, and the addition of an 8-hour enrichment allowed them to detect 8×10^1 CFU/g of *E. coli* O157:H7 cells in ground beef (96). Similar research found that 10^5 CFU/g of live cells could be detected in ground beef, but the addition of an 8-hour enrichment allowed detection of even 1 CFU/g of *E. coli* O157:H7 (97). However, viable cells were slightly overestimated in PMA qPCR when there was $\leq 10^3$ CFU/g of live cells plus 10^6 CFU/g of dead cells, although there was still no signal solely from the 10^6 dead cells. A combination of qPCR, EMA qPCR, and culturing suggests that a chemical treatment may result in most cells in a biofilm becoming VBNC, resulting in a several $\log_{10}$ difference between the amount of culturable cells and the number of cells considered viable by EMA qPCR (98). More research is needed to determine if the difference is due to overestimation by viability qPCR or underestimation by culturing; but this approach could prove a powerful tool in estimating the prevalence of VBNC cells in samples. Interestingly, inactivation method seems to play a large role in the ability of PMA to reduce dead cell signal as viability loss could not be tracked if ultraviolet (UV) light was used, presumably due to a lack of membrane damage by the treatment (99). It was also confirmed that the UV-treated cells did not experience

increased permeability to PMA over time, unless exposed to heat. Mild heat treatments cause similar problems with cells injured or killed by temperatures from 52°C to 72°C, showing little or no DNA inactivation by PMA; whereas, PMA inactivation of cells killed by temperatures ≥80° C was highly effective (100). The addition of 0.5% to 1% sodium deoxycholate was able to overcome the lack of DNA activation in cells killed at 52°C, although suppression was still reduced when live cells were under 1% of total cell makeup. In contrast to UV and mild heat treatment, ultrasonic inactivation of *E. coli* O157:H7 likely causes significant membrane disruption. This observation is supported by work showing that PMA qPCR quantification of a 4.4 $\log_{10}$ reduction in cells from ultrasonic inactivation matched quantification by plate counting, although the 20 CFU/mL limit of detection for their PMA qPCR assay prevented detection at very low levels of pathogen (101). Another study demonstrated that ultrahigh pressure, ultrasound, and high-pulsed electric field inactivation of *E. coli* O157:H7 resulted in significant cell membrane disruption and demonstrated high degrees of correlation between plate count and PMA qPCR quantification of viable cell reduction. However, PMA qPCR does appear to overestimate the number of viable cells for the high-pulsed field treatment (102).

L. monocytogenes:

As with other organisms, PMA was shown to be a superior viability PCR intercalation agent for *L. monocytogenes* since it did not decrease live cell viability. Conversely, EMA demonstrated roughly 1 to 4 cycles of suppression for viable cell amplification; suppression correlated positively with increase in intercalation incubation temperature (103). The drawback of PMA was that it demonstrated 1-2 cycles lower

suppression of heat-killed dead cells than EMA; however, overestimation is preferable to underestimation in the context of food safety. The PMA qPCR assay was used to accurately quantify the number of cells in peroxide-treated biofilms under certain conditions but showed reduced suppression of dead cell DNA at dead to live cells ratios $\geq 10^4$ and viable cell concentrations below 10^3 CFU/mL This lack of suppression is in line with the findings of previously mentioned papers. Multiplex analysis of *L. monocytogenes* and *V. parahaemolyticus* in spiked shrimp samples found that PMA qPCR quantification of both species was similar to culture quantification (104). The optimal PMA concentration was 100 µM and could suppress 10^8 dead cells past the 40-cycle limit while having a LOD between 10^1 and 10^2 CFU/g and causing less than a one cycle reduction in live cell signal. Benzalkonium inactivation of *L. monocytogenes* also demonstrates high correlation between PMA qPCR and culture based quantification (99). This method is likely due to it relying on membrane damage for inactivation and supports the idea that PMA intercalation is most effective when membranes have been sufficiently compromised. Work by Rudi *et al.* (103) found that a viable to dead cell fraction of 0.5% could be accurately quantified by EMA qPCR, but a combination of growth and qPCR was required to quantify levels of bacteria below 10^2 CFU/g. It was not determined if the intercalation protocol suppressed very large populations ($>10^6$ CFU/g) of the organism, which would have been valuable, given it is a known side effect of EMA in other organisms.

Salmonella:

Viability qPCR may prove useful in monitoring levels of *Salmonella* in environments of concern and has been used to monitor serovars of interest over time

(105). Viability qPCR was able to detect loads as low as 10^2 in soil and allowed tracking of viable and non-viable populations when both qPCR and PMAxx qPCR were used. The researchers stated that viability qPCR was more sensitive than culturing; although the performance of viability qPCR to identify VBNC cells or dead cells with intact membranes can be debated. Another study demonstrated that an increasingly high hypochlorite treatment correlated with greater reductions in a qPCR signal, but culturability dropped off at much faster rates, suggesting that intercalation suppression was outpaced by death rate. It is possible the discrepancy was due to VBNC cells (99). The addition of sodium deoxycholate may help PMA permeate the membranes of dead but intact *Salmonella* cells and has been used for PMA qPCR detection of pathogens in milk. However, additional experiments and controls are needed to determine the limitations of this system (95). The addition of 0.2% sarkosyl is also being explored for increasing PMA efficacy (106). Researchers concluded that 0.2% sarkosyl, 30 µM of PMA, and a 20-minute dark incubation followed by a 10-minute light crosslinking period was the optimal combination of efficacy and efficiency and could be used to quickly detect *Salmonella* Typhimurium, *Legionella pneumophila*, and *S. aureus* in water samples.

Use of EMA qPCR has detected as low as 10^1 CFU/10g of *Salmonella* Typhimurium in coalho cheese, although the limit of differentiation was 2 $\log_{10}$ higher, which compared favorably to results by culturing (107). The same study found that EMA significantly reduced live cell signal in the other organism tested, such as *S. aureus*. The use of enrichment in combination with PMA qPCR shows promise for detection of microbes in contaminated food. The limit of detection in lettuce contaminated with

Salmonella was 10^3 CFU/g without enrichment, but dropped to 10^1 CFU/g with a 12 hour enrichment, providing a relatively rapid way of testing for pathogens in fresh produce (108). Work by Barbau-Piednoir *et al.* (108) on the limitations of PMA found that prolonging PMA incubation from 5 minutes to 60 minutes or increasing the concentration from 75 µM to 150 µM did not significantly reduce or suppress dead cell signal. While the researchers found that PMA does not significantly affect live cell signal at high cell concentrations (~10^7 and 10^9 CFU/mL), it was demonstrated that lower (~10^4 and 10^5 CFU/mL) concentrations of living cells experienced significant signal reduction with PMA treatment (1 to 2.5 cycles). This observation led the authors to recommend use of an enrichment to avoid missing detection of low levels of pathogen in foodstuffs. Finally, the researchers demonstrated that there was not necessarily a correlation between the percentage of cells with compromised membranes and ability of PMA to decrease cell signal. Kanamycin treatment, which left 80% of the cells with intact membranes, demonstrated a higher reduction in C_t value with intercalation than isopropanol or freezing, which left 13% and 2% of cells with intact membranes, respectively. Only heat treatment yielded a higher C_t reduction. These results are not in line with previous beliefs about PMA and raise questions on the exact mechanism of PMA signal suppression. The use of PEMAX dye, double tube change, and double photoactivation in viability qPCR demonstrates good suppression of dead cells without live cell suppression (109). The double photoactivation gave an additional 2 cycles of dead cell suppression within the experiment and a higher incubation temperature may have increased suppression. However, studies comparing this methodology to other PMA qPCR protocols are needed to determine if there is significant advantage.

Amplicon size is also a very important factor in viability qPCR success. Longer amplicons are typically less efficient than shorter ones (1045 bp vs. 605 bp) (105). However, intercalation suppression is more efficient in longer amplicons and a comparison between 95, 285, and 417 bp amplicons found that only the 417 bp amplicon allowed suppression of 10^8 CFU/g of dead cells and a detection limit of 10^3 live cells with the optimal PMA concentration of 50 µM (110). TaqMan qPCR chemistry yielded a higher efficiency than SYBR Green. A separate study found that an amplicon length of 130 bp was the optimal length to allow for efficient amplification while still being long enough to allow for high (17 cycle difference) suppression of dead cells and minimal (0.5 cycle difference) suppression of live cells (68). Using their assay, 30 CFU/g of live *Salmonella* could be detected after a 4-hour enrichment of spiked samples.

Other organisms:

PMA qPCR may be useful for removing dead cell DNA from environmental samples, although success rate varied depending on material source and the temperature at which cells were stressed (111). PMA qPCR has shown promise in quantification of human adenovirus-2 and mengovirus. If the system could be applied to other viruses, this method could prove to be very useful in foodborne norovirus and hepatitis A outbreaks (112). On the other side of the microbial spectrum, EMA qPCR of *Zygosaccharomyces bailii* has been shown to suppress amplification of dead cells and show good correlation with culture-based quantification, suggesting opportunities in quantifying spoilage yeasts (113). Controlled kill and PMA treatment of *Legionella* cells exhibited amplification reduction; although when tested in biofilms and environmental samples, PMA qPCR gave much more variable results, due to differing matrix properties, differing inactivation

methods, varying proportions of VBNC cells, or interference from some factor specific to *Legionella* physiology (114, 115). Additionally, viability qPCR is less efficient at suppression when there are very high concentrations of dead cells in a complex matrix. Having a very long amplicon (2,451 bp) also has been shown to cause complete suppression of 10^7 $\log_{10}$ CFU/mL of dead coliforms using EMA qPCR (116). The study suggests that viability qPCR could be used for quantification of low levels of broad microbial classes such as "coliforms" in products such as milk, although EMA treatment reduced viable cell amplification by 3 to 10 cycles, depending on concentration used. *Arcobacter*, an emerging pathogen, can be detected with PMA qPCR and early results suggest that this method may be more sensitive than culture-based approaches for their detection (117). Based on this information, viability qPCR may be useful for the quantification of emerging pathogens lacking in well-established culturing protocols.

Conclusions

There are several conclusions that can be drawn based on the referenced studies. PMA seems to be a more suitable dye than EMA. EMA tends to permeate not just dead cells, but also living cells to such a degree that living cell amplification signal is reduced (103, 107, 116, 118). The caveat of using a less aggressive dye is that it will be difficult to penetrate a cell that has died, but in which the membrane is not yet compromised (99). PMA also struggles to achieve complete suppression of very high dead cell counts and may cause slight live cell signal reduction (68, 93, 97, 103, 119). The ability of dyes to intercalate dead cell DNA may be increased through process modifications, such as the use of multiple intercalations, sodium deoxycholate, higher incubation temperatures, and sarkosyl (94, 95, 100, 106, 109). It has also been suggested that the use of a cold-shock

treatment can increase uptake of viability dyes, perhaps allowing for more accurate identification of dead cells (120). One advantage of viability qPCR is that it will detect injured or VBNC cells that cannot be picked up using culture-based quantification. Further research is needed to determine if increasing dead cell membrane permeability would inadvertently increase intercalation and suppression of signal from VBNC cells. This finding should also be noted as a caveat to many of the studies mentioned. Any discrepancy between viability qPCR results could have resulted from over- or underestimation by the viability qPCR or they could have resulted from the ability of culturing to detect highly injured or VBNC cells. The exact line between highly injured, VBNC, and recently dead cells can also be very hard to determine with current technology, making the issue even harder to resolve. Amplicon length also has a major effect on the ability of PMA to reduce dead cell signals; long amplicons show better suppression of the dead cell signal, but this suppression comes with the cost of reduced amplification efficiency (68, 105, 110, 116). The way cells are inactivated also plays a large role in the ability to eliminate dead cell signal. Methods that heavily compromise the cell membrane such as ultrasound and high heat treatment usually see very good signal reductions, whereas methods that do little to compromise membrane integrity like mild heat, UV light, and certain disinfectants, result in limited dead cell suppression (99–102, 119). The exact mechanism and parameters of intercalation are still being researched and it is possible variables beyond membrane integrity may play a large role in viability dye efficacy (119). The limit of detection of viability qPCR is also higher than desired due to logistical limitations in extraction and reaction size, and tends to be in the 10^2 to 10^3 range (93–95, 99, 107, 108, 110, 121). Although this approach may be sensitive

enough for some applications, the use of a 4 to 12 hour enrichment (depending on the species and conditions of interest) can greatly decrease the limit of detection in cases where quantification is less important than detection (68, 96, 97, 108, 119).

Statement of the problem

To help ensure a safe food supply, it is necessary to conduct food safety challenge studies. Currently, PAC methods are the only accepted method for quantification of pathogens in these studies. The replacement of PAC methods with viability qPCR would allow for faster and potentially more accurate detection and quantification of pathogens. However, there is a distinct lack of published research that addresses the use of qPCR in challenge studies for quantification of pathogens in food matrices that does not involve the spiking of live and pre-killed dead cells into the food matrix, as was utilized in most of the studies referenced previously. Further research exploring the optimization of DNA extraction and intercalation for viability qPCR and its use for quantification in challenge studies is necessary. The first challenge in this process is ensuring the high levels of the target DNA can be extracted from even complex food matrices. Next, further characterization and selection of DNA intercalating agents is necessary to remove signal from dead cells. Finally, the application of viability qPCR for quantification of pathogens in different food matrices after various food safety interventions is needed.

Statement of objectives

The objective of this research is to develop a highly efficient viability qPCR protocol and compare its ability to quantify pathogens with that of PAC in challenge study applications. Two common food-borne pathogens, *L. monocytogenes* and *S.* Typhimurium, will be used. Development will require validation of the qPCR efficiency,

selection of a highly efficient DNA extraction kit, and development of an optimized DNA intercalation protocol. The method developed will then be tested in two small scale challenge studies. One of the studies will utilize ground beef as a matrix and heat as the intervention; the other will utilize beef rose meat as a matrix and lauric arginate (LAE) as the intervention.

Research presented herein, as well as forthcoming studies by other researchers in the area, will continue to resolve current viability qPCR issues and help validate its use as a fast and accurate tool for improving the reliability of interventions used by processors to ensure the safety of the food supply.

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Tables and Figures

Table 1: Summary of research on DNA extraction in food matrices.

Matrix, target organism	**Findings and challenges**	**Citation**
Vegetable Oil, Oil Seed	Polysaccharides, phenolics, and other compounds can persist throughout the extraction and inhibit DNA amplification.	(29)
Tuna	Any heat treatment prior to extraction tended to reduce the concentration of DNA extracted. DNA concentrations did not always correlate to PCR success.	(37)
Various fish	Despite all being fish, DNA extraction yields varied between species, different species yielded different levels of DNA depending on extraction method. General extraction trends usually held across species, but significance levels varied. DNA still was suitable for PCR amplification despite lower that ideal A_{260}/A_{280} in a number of samples. Poor A_{260}/A_{280} readings likely stemmed from protein and reagent contamination.	(34)
Light tuna	The best method of DNA extraction varied depending on whether the tuna was packed in brine, oil, or vinegar and tomato sauce.	(35)
Cattle, pig, lamb, goat, chicken, turkey, and duck	The more highly processed the sample, the less DNA could be extracted. Complex, high fat tissues yielded lower and more variable levels of DNA.	(30)
Roughly divided into high vegetable content such as maize flour, and complex or processed such as horse meat and cherry marmalade. DNA inherent to the matrix was measured.	The magnetic DNA (Promega Wizard) method was more effective in the high polysaccharide, high polyphenolic vegetable matrix. The silica column method (DNeasy Tissue) was more effective in complex and highly processed matrixes.	(32)
Cow, goat, and sheep milk	The use of a “milk clearing solution” to help remove PCR inhibitors combined with a	(39)

	commercial DNA extraction kit helped yield samples suitable for amplification.	
Whole, semi-skimmed, and UHT milk, yoghurt, cream, butter, and Emmental cheese. Total DNA and bovine DNA C_t values from the samples were measured.	The magnetic kit (Promega Wizard) was most effective in extracting DNA from liquid milk matrices. CTAB extraction worked well for all methods. High lipid products showed higher C_t values. Commercial kits yielded lower standard errors, but also had higher C_t values than the non-commercial extraction methods.	(33)
Olive oil, Amplified Fragments Length Polymorphisms	A decrease in the quality of DNA extracted from olive oil would be expected if more than a month has passed since milling of the oil.	(36)
Beef	The modified salt, CTAB, Qiagen DNeasy Blood & Tissue Kit, and Wizard Genomic DNA Purification kits yielded the lowest C_t values. This did not necessarily correlate with gel electrophoresis or absorbance results. For example, the urea method yielded high numbers for gel and absorbance but ultimately did not perform very well in qPCR.	(38)

Table 2: Summary of research on DNA extraction of pathogens from matrices.

Matrix, target organism	**Findings and challenges**	**Citation**
Ham, salami, chicken salad, four different cheeses; *L. monocytogenes*	More complex food matrixes resulted in greater inhibition of PCR. Proteinase activity in cheese may have a negative effect of PCR. High levels of oil, salt, carbohydrate and amino acid were tolerated by PCR but PCR reactions containing large amounts of protein were inhibited. High fat products seem to have more amplification issues. High amounts of culturing media could interfere with amplification.	(31)
Milk, *Brucella* spp.	*Brucella* cells have a high affinity for the milk fat phase and adhere to the interface. Milk proteins can lower pellet solubility and inhibit PCR.	(40)

Sliced bread, ground beef, bagged salad greens, salad dressing; *E. coli* O157:H7	Of the four kits tested, one magnetic kit (Bugs'n Beads) was significantly less sensitive than the other (Wizard Magnetic DNA Purification System for Food). There were not significant differences between any other kits (NucleoSpin food kit (spin column), Prepman Ultra (solution)).	(41)
Bovine tissue, *Mycobacterium bovis*	The DNeasy Blood and Tissue Kit (silica spin column) showed the highest performance and sensitivity. Genomic DNA Mini Kit (paper description conflicts with current kit) and FTA Elute Micro Card (cellulose) plus enzymatic digestion also yielded good results.	(45)
Meconium (fecal); 16s rRNA, spiked *Streptococcus agalactiae*	The MoBio MagAttract PowerMicrobiome kit yielded higher levels of DNA than the three silica spin column kits. It also showed the lowest spiked DNA recovery efficiency, dilution resolved this. Different kits selected for different phyla of bacteria. The removal of host DNA in a kit may have also resulted in a loss of bacterial DNA. Low levels of contaminating bacterial DNA were present in all four kits.	(43)
Broth culture, eleven bacterial species common to the human body	Protocols including bead beating and/or mutanolysin were better at representing the true bacterial community than methods without. A cocktail of lytic enzymes was significantly more effective than any single enzyme for 3 of 6 species.	(50)
Autoclaved cattle manure, spiked with *E. coli*	The three silica column kits yielded amplifiable DNA whereas the phenol-chloroform-isoamylalcohol method did not. Addition of PVP and CTAB to the most efficient kit reduced efficiency. There was no evidence that A260/280 ratios below 1.7 or above 2.0 were responsible for qPCR inhibition.	(44)
Brain Heart Infusion broth; *S. aureus* subsp. *aureus* ATCC 25923	The FTA Elute kit, QIAamp DNA Mini Kit, and a boiling based DNA extraction procedure were compared. For *E. coli*, extraction efficiencies were FTA 76.9%,	(47)

or *E. coli* ATCC 11775	boiling 43.7%, and Mini 7.7%. For *S. aureus*, efficiencies were FTA 108.9%, Mini 97.7%, and boiling 9.0%. FTA Elute was the only method with extract control for *E. coli* that did not show an inhibitory effect compared to the water control; no treatment showed inhibition in the *S. aureus* assay.	
Blood; spiked *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*	Larger blood sample volumes resulted in lower C_t values. The non-enzymatic Polaris method had a higher detection rate and was more reproducible than the enzymatic MoIYsis method or the Triton-Tris-EDTA EasyMAG method. The Gram-negative *P. aeruginosa* had a lower detection rate than the Gram-positive *S. aureus.*	(51)
Ovine blood; total DNA was measured spectrophotometrically and with Qubit, ovine PRNP and *Campylobacter coli* glyA genes were targeted for qPCR.	Of the eleven DNA extractions tested, only four yielded satisfactory results. These included three silica-based test kits (Modified Nucleospin Blood, Modified Nucleospin Tissue, and Modified Nucleospin Dx) and one in-house magnetic bead protocol. The successful modified protocols used buffy coat, increased volumes of lysis buffer and proteinase K, an increased proteinase K incubation, and a chloroform wash step.	(46)
Isolation from milk then culturing in MRS broth, *Lactobacillus* spp.	A simple DNA extraction using ampicillin to weaken cell walls then a lysozyme-based treatment for extraction yielded high levels of good purity DNA. The use of ampicillin is a simple and novel way of helping overcome challenges posed by the resilient Gram-positive cell wall.	(49)
Feces; total bacterial levels, select groups, and *E. coli*	A variety of kits using principles including magnetic beads, bead beating, silica spin column, heat lysis, and chemical lysis were used. The qPCR results did not show any kit having a distinct efficiency advantage, despite several of the kits resulting in higher DNA yields and bacterial diversity profiles. A lack of mechanical lysis may	(48)

	have led to reduced DNA yields for one kit. The use of an optional RNase treatment also improved spectrophotometric purity readings for that kit (QIAmp DNA Stool Mini Kit).	
Raw milk, raw milk cheese; *L. monocytogenes, S.* Typhimurium	Of the seven kits used to extracted DNA, the Powerfood Microbial DNA Isolation kit and in-house Lytic methods most consistently extracted high concentrations of pure DNA for qPCR. The lytic method involves a 20 hr run time and hazardous reagents (phenol/chloroform), thus the Powerfood kit was preferred. This solid phase/column extraction kit was chosen over two other solid phase/column extraction kits, two mobile phase/magnetic bead extractions, and two liquid-liquid extractions. The magnetic kits worked well in cheese, but not in liquid milk. Modifications such as heat treatment, step exclusion, ethanol precipitation. or lysozyme+mutanolysin lysis were evaluated as ways to improve yields for the PowerFood kit. Of these, the highest heat treatment (70°C for 10 min followed by 10 min of vortexing) resulted in the largest yield increase.	(42)

Chapter 2

Selection and Validation of qPCR Components, DNA Extraction Kits, and DNA Intercalating Agents

Abstract

Food safety challenge studies play a crucial role in creating a safe food supply chain by ensuring that interventions or critical control points (CCPs) can reduce high levels of pathogens that may be present in the product. Traditionally, monitoring of pathogen levels is performed by culture-based plate and count (PAC) methodologies. The rise of methods that use quantitative polymerase chain reaction (qPCR) may offer a faster and more versatile method of pathogen quantification for food safety challenge studies. The goals of these experiments were to develop a methodology that works for both Gram-positive and Gram-negative pathogens, uses highly efficient qPCR primers and reagents, employs a highly effective and sensitive DNA extraction, and utilizes a DNA intercalating protocol that can effectively reduce amplification of high levels of dead cell DNA without interfering with live cell signal. These results demonstrate that a combination of primers, reagents, DNA extraction kit, and DNA intercalating protocols were able to differentiate between viable and dead cell DNA for both *L. monocytogenes* and *S.* Typhimurium in a model ground beef system.

Introduction

Salmonella spp. and *Listeria monocytogenes* are two of the most ubiquitous and harmful bacterial food pathogens in the United States. *Salmonella* spp. can survive across a broad range of conditions and possess a wide range of virulence factors, allowing for the invasion of multiple cells types and production of a cytolethal distending toxin (1–3). These factors contribute to its ability to cause serious foodborne infections. *Salmonella* spp. were responsible for 66% of total hospitalizations and 70% of deaths from foodborne disease outbreaks in 2017 (4). The ubiquitous nature of *L. monocytogenes* in the environment, combined with its ability to grow despite high salt and low temperatures, makes it especially problematic in ready-to-eat foods and subsequent outbreaks associated with the pathogen (5). Although *L. monocytogenes* rarely causes disease in healthy individuals, young, old, pregnant, and immunocompromised individuals (YOPI's) are much more susceptible to infection, with fatality rates up to 34% (5).

A critical component in controlling these pathogens in foods is the implementation of "challenge studies." These studies are used to validate the ability of a food safety process, intervention, or critical control point (CCP) to effectively inactivate high levels of pathogens of concern when present in the food product. Challenge studies are done in specialized research facilities, using stringent experimental protocols, to ensure containment of the pathogen. Traditionally, challenge studies require researchers to enumerate pathogens from the product using culture-based plate and count (PAC) methods. However, the use of traditional, PAC methods for quantification of pathogens in food has several disadvantages: enumeration of pathogens is extremely material-intensive and can generate large amounts of waste (6). This process is also very time-

intensive, both in terms of labor required and total time needed to process and enumerate samples (7). Given the above, challenge studies that rely on PAC methods can be expensive, which may be cost prohibitive to small food processing facilities and therefore, discourage food safety innovation. In addition, many foodborne pathogens can form viable but non-culturable (VBNC) cells that may not be detected using PAC methods (8–11). To overcome these issues, the use of viability qPCR has been proposed. Viability qPCR offers a faster method of quantification that can suppress the DNA of dead cells while allowing for the quantification of living and VBNC cells (12–14). There are several preliminary steps required in the design of a successful viability qPCR quantification assay. First, it is necessary to choose appropriate primers and reagents for the qPCR and pathogens of interest and verify that they can efficiently amplify the amplicons of interest. It is then necessary to test which DNA extraction process allows for the highest quality DNA to be yielded from the matrix and organisms being used. Finally, a DNA intercalating agent must demonstrate high suppression of dead cell DNA and low suppression of living cells. This chapter provides a step-by-step approach to accomplishing these tasks, using ground beef as a model food system.

Materials and Methods

Selection and Validation of qPCR Primers and Probes

Bacterial Cultures

Listeria monocytogenes Scott A and *Salmonella enterica subsp. enterica* serovar Typhimurium (ATCC 14028, isolated from chicken organs) were obtained from the Food Microbiology Culture Collection located in the Department of Food Science, Pennsylvania State University. These organisms were chosen based on pathogenicity

potential as demonstrated by association with previous foodborne disease outbreaks and use in previous challenge studies (6). Freezer stocks of *S.* Typhimurium and *L. monocytogenes* were made by growing cultures in 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson and Company; BD, Sparks, MD) 24 hours at 37°C under aerobic conditions. Colony morphology and presence of pathogen specific target genes were used to confirm identity of the cultures. After incubation, 0.3 mL of a 50% glycerol solution (v/v) was added to 1.5 mL of TSB culture in a cryogenic tube and frozen at -80°C. Cultures were revived from freezer stocks by adding 100 µL of thawed and vortexed culture stock to 10 mL of fresh TSB and incubating for 24 hours at 37°C under aerobic conditions. Bacteria were then streaked for isolation on Tryptic Soy Agar (TSA, Becton Dickinson and Company; BD, Sparks, MD) and incubated for 24 hours at 37°C under aerobic conditions. Stock plates were refrigerated until needed. Regrowth of single colonies in TSB and isolation onto fresh TSA were conducted periodically to maintain culture viability.

Bacterial Growth

A single colony was selected from working cultures of both *L. monocytogenes* and *S.* Typhimurium and individually inoculated into 10 mL of TSB (BD) and then incubated at 37°C under aerobic conditions. After 240 minutes of incubation, the *L. monocytogenes* culture was vortexed and 1 mL of culture was added to a 1.5 mL semi-micro, disposable cuvette (Plastibrand; Wertheim, Germany). The OD_{600} was then measured in a spectrophotometer (BioPhotometer, Eppendorf; Hamburg, Germany) blanked with 1 mL of sterile TSB (BD). If the culture had an OD_{600} of less than 0.9, it was returned to incubation for an additional 30 minutes. This procedure was repeated

until the OD_{600} was between 0.9 and 1.2. *S.* Typhimurium cultures were treated similarly, except measurement commenced 180 minutes after inoculation and was repeated at 20-minute intervals. Upon reaching the desired optical density, cultures were processed for DNA extraction.

DNA extraction

DNA extraction was conducted using the MasterPure DNA and RNA Purification kit (Lucigen Corporation; LC, Middleton, WI) with modifications as follows. All steps were conducted at 21°C ± 3°C unless otherwise noted. Prior to extraction, 1 µL of Proteinase K (LC) was diluted into 300 µL of Tissue and Cell Lysis Solution (LC) for each sample. Five hundred µL of cell culture were pelleted in a 1.5 mL microcentrifuge tube by centrifugation (Galaxy 20R, VWR International; Darmstadt, Germany) at 10,000g for 10 minutes. Immediately after, the supernatant was discarded, leaving approximately 25 µL of liquid. The pellet was then resuspended via vortexing. Three hundred µL of the Proteinase K and Tissue and Cell Lysis Solution was added to the suspension followed by thorough mixing. The sample was then incubated at 65°C for 15 minutes with vortexing every 5 minutes. Samples were cooled to at least 37°C, 1 µL of 5 mg/mL RNase A was added, mixed thoroughly, and incubated at 37°C for 30 minutes. After incubation, samples were placed on ice for 5 minutes. Precipitation of nucleic acids was initiated by adding 150 µL of MPC Protein Precipitation Reagent (LC) and mixing vigorously for 10 seconds. The debris was pelleted by centrifugation at 4°C for 10 minutes at 10,000g. The supernatant was then transferred to a clean 1.5 mL microcentrifuge tube. If the pellet came loose during transfer, centrifugation and transfer were repeated. Five hundred µL of isopropyl alcohol (BDH1133-1LP, VWR BDH

Chemicals; Mississauga, ON, Canada) was added to the recovered supernatant and tubes were inverted 40 times to mix. DNA was pelleted by centrifugation at 4°C for 10 minutes at 10,000g. The supernatant was discarded, and the pellet was rinsed twice with 50 µL of 70% ethanol. The DNA pellet was resuspended in 50 µL of 10 mM Tris-HCl pH 7.5 (Quality Biological, Inc.; Gaithersburg, MD).

DNA Quality Control and Dilution

Nanodrop analysis was used to measure DNA extract quality (NanoDrop One C, ThermoFisher Scientific; Madison, WI). The Nanodrop was set to analyze double stranded DNA and output ng/µL, A260/A280, and A260/A230. The pedestal and cover were cleaned between samples using Kim wipes (Precision Wipes, Kimtech Science; Mississauga, ON, Canada) and sterile, aerosol 10 µL pipette tips (Cat. 89174-520, VWR International, LLC; Radnor, PA) were used to dispense all samples. The Nanodrop was blanked with 1 µL of 10 mM Tris-HCl, pH 7.5. An additional blank was then measured to check for a lack of signal. One µL of the sample DNA was then analyzed; this procedure was repeated in triplicate for each sample. Following analysis, DNA samples were diluted to form a standard curve. Dilution consisted of adding 30 µL of DNA sample to 270 µL of 10 mM Tris-HCl pH 7.5, then mixing. This procedure was repeated 7 times to form a 9-point standard curve of 10x dilutions.

Qubit

Qubit analysis was conducted using a Qubit 3.0 Fluorometer (Sn. 2321609687, Invitrogen, Life Technologies; Malaysia) and broad spectrum double stranded DNA analysis. Qubit double stranded (ds)DNA BR Reagent and Qubit dsDNA BR Buffer from Qubit dsDNA BR Assay Kit (Life Technologies Corporation; Eugene, OR) were mixed

at the recommended concentration and 190 µL of the mixture was added to a clear, 0.5 mL microcentrifuge tube, as well as 10 µL of the sample DNA. Samples were then vortexed and allowed to incubate at 25°C for two minutes before measuring. Qubit standards #1 and #2 from the dsDNA BR Assay Kit were used to calibrate the Qubit Fluorometer and the tubes were then measured using parameters of broad range, ds DNA, 10 µL of sample, and a readout of ng/µL. Measurements were completed in a triplicate for each sample.

qPCR Primer and Probe Selection

Primers were chosen based off of previous research conducted on qPCR primers for *L. monocytogenes* (15) and for *S.* Typhimurium (16). For *L. monocytogenes*, a 113 bp amplicon of *hlyA* (positions 1627 to 1740 under accession no. M24199 in GenBank) was used. Primers were Custom Taqman Primers, forward: 5' TGC AAG TCC TAA GAC GCC A, reverse: 5' CAC TGC ATC TCC GTG GTA TAC TAA, (S.O. 7411095, Life Technologies Corp. (LT), Pleasanton, CA); the probe was a ThermoFisher Custom Taqman MGB Probe, probe: 5' CGA TTT CAT CCG CGT GTT TCT TTT CG containing 5' VIC dye and 3' minor groove binding non-fluorescent quencher (MGBNFQ, LT). For *S.* Typhimurium, a 130 bp amplicon of *invA* (positions 197 to 303 under accession no. M90846 in GenBank) was used. Primers and probes were, forward: 5' CGTTTCCTGCGGTACTGTTAATT, reverse: 5' TCGCCAATAACGAATTGCCCGAAC, probe 5' CCACGCTCTTTCG with ThermoFisher 5' 6FAM dye and 3' MGBNFQ (LT). Primers and probes were mixed with nuclease free sterile water to form a working stock for each organism containing 18 µM of the respective primers and 5 µM of the respective probe per µL. Adding 1 µL of

working stock per reaction resulted in final concentrations of 900 nM for each primer and 250 nM of each probe, as suggested by the manufacturer (LT).

qPCR Plate Construction and Run Parameters

Quantitative Polymerase Chain Reaction (qPCR) was conducted using a QuantStudio 3 Real-Time PCR Instrument (96-well 0.1 mL Block) (SN 272310615, Life Technologies Holdings Ltd; Singapore, 739256) and protocols were based on usage instructions for TaqMan Fast Advanced Master Mix (17). Each reaction used 10 µL of TaqMan Fast Advanced Master Mix (4444557, Thermo Fisher Scientific; Vilnius, Lithuania), 7 µL of UltraPure Distilled Water (Life Technologies; Grand Island, NY), 1 µL of organism specific primer (LT), and 2 µL of template DNA. Two batches of working stock, one for each organism, were made by vortexing the total volume of Master Mix, nuclease free water, and appropriate primer required for each well. Required volume was calculated based on number of samples and controls tested in triplicate plus 10% to account for potential loss. All reagents, primers, and probes were kept on ice. Working stock was dispensed into MicroAmp Optical 96-Well Reaction Plate wells (Life Technologies, China) in 18 µL aliquots under a AirClean 600 PCR Workstation (Model 300, AirClean Systems; Creedmoor, NC). The appropriate sample DNA was then added in 2 µL aliquots, each sample was tested in triplicate; 10 mM Tris-HCl pH 7.5 was used as a control. Plates were sealed with MicroAmp Optical Adhesive Film and vortexed briefly (Applied Biosystems, Life Technologies Corp.; Carlsbad, CA). After vortexing, plates were centrifuged at 300g for 1 minute. Parameters were chosen based on recommendations for TaqMan Fast Advanced Master Mix (Table 1). These were, 2-minute uracil-DNA glycosylases incubation at 50°C, 2-minute polymerase activation at

95°C, and 40 cycles consisting of a 1 second denaturation step at 95°C and a 20 second anneal/extend step at 60°C. Run speed was standard and plate size was 0.1 milliliters.

Pathogen Enumeration

One mL of resuspended 24-hour culture was added to 9 mL of Buffered Peptone Water (BPW; BD) and vortexed. The resulting dilution was then repeated until relevant dilutions were reached. One hundred µL of the dilution was inoculated onto a TSA (BD) plate and spread using a sterile plastic spreader. Each relevant dilution was plated in triplicate; final dilutions of 10^{-6}, 10^{-7}, 10^{-8} resulted in the ideal range of countable colony forming units (CFUs) per plate. Three petri dishes were plated with 100 µL of BPW as a negative control. Plates were left to dry for 10 minutes then inverted. Incubation of plates was for 48 hours at 37°C under aerobic conditions. After 48 hours, plates were counted manually for total colonies. Dilutions resulting in average plate counts under 30 or over 300 were discounted. Abnormal colony morphologies were rare and not included in plate counts. Dilution and enumeration were repeated for both *L. monocytogenes* and *S.* Typhimurium cultures. Presence of pathogen specific qPCR signal and absence of signal in the controls was used as confirmation of organism identity.

Statistical Analyses

For PAC, the dilution resulting in an average of between 30 and 300 colonies per plate was used for statistical analysis. The average plate count of this dilution was calculated and used to estimate DNA copy levels for reactions. In addition, the mean and standard deviation for plate counts and undiluted DNA C_t values across all three runs were calculated. Standard curves were plotted for each run and trimmed to remove outlier dilutions and form five-point standard curves as recommended by QuantStudio Design

and Analysis Software (v1.5.1, ThermoFisher Scientific). The trimmed curves were then evaluated by QuantStudio Design and Analysis Software for slope, R^2 value, and percent efficiency of the reaction. Ideal slope and efficiency are -3.32 and 100%, respectively. A slope of -3.1 to -3.6, corresponding to 90% to 110% efficiency were considered acceptable (18), as well as a R^2 value over 0.99 (19). In addition, curves were created for the maximum range of dilutions over which there was minimal variation in variance between replicates or failure of amplification. This approach was done to derive a tentative range over which the standard curve would be accurate. The maximum range dilution curves were then evaluated by QuantStudio Design and Analysis Software (v1.5.1, ThermoFisher Scientific) for slope, R^2 value, and percent efficiency of the reaction.

Selection and Comparison of DNA Extraction Kits Using Ground Beef Model Bacterial Growth

A single colony was selected from the refrigerated stock plate and inoculated into 10 mL of TSB (BD) then incubated at 37°C for 24 h under aerobic conditions, for both *L. monocytogenes* and *S.* Typhimurium. One milliliter of the appropriate broth culture was added to 9 g of 80% lean:20% fat irradiated ground beef (Wegmans' Food Markets, Inc.; Rochester, NY) for each sample in a 400 mL sterile lateral filtered stomacher bag (BagSystem, Interscience; Saint Nom, France) and incorporated thoroughly. Ten milliliters of BPW (BD) were added and the sample was homogenized (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK) for 1 min at 260 rpm. The appropriate volume of homogenate was removed for each extraction from the filtered side

of the stomacher bag. The various DNA extraction and plate count methodologies were then carried out as later described.

Plate Count Enumeration

One mL of resulting homogenate also was removed from the filtered stomacher bag and added to 9 mL of BPW to form a 10-fold dilution. This process was repeated for a total of 7 dilutions. Starting with the 10^{-5} dilution, 0.1 mL of diluent was removed from the dilution tube and deposited onto a TSA plate. The plate was then spread with a sterile hockey stick and manual plate spinner. Spreading for each dilution was completed in a triplicate for the 10^{-5}, 10^{-6}, and 10^{-7} dilutions, yielding final dilutions of 10^{-6}, 10^{-7}, and 10^{-8}. The ground beef control was not diluted, and 0.1 mL of homogenized sample was added directly onto the TSA plate, then spread-plated in triplicate. Plates were then incubated at 37°C for 48 h. After incubation, plates containing approximately 30 to 300 colonies were counted and the average of the counts was multiplied by the inverse of the dilution factor to determine colony forming units per mL (CFU/mL).

DNA Extraction Kit Selection and Comparison

To decide which DNA extraction kits were to be used in the study, a search was conducted for research comparing various methods for the extraction of bacterial DNA, preferably from food and for use with qPCR. Based on previous research, the Applied Biosystems PrepMan Ultra Sample Preparation Reagent (P), Biotecon foodproof StarPrep Two Kit (S), Qiagen DNeasy PowerFood Microbial Kit (Q), and Indicating FTA Elute Micro Card (F) were chosen for evaluation (20–23). Extractions were carried out following the manufacturers' instructions with modifications as necessary; protocols are

provided below. Preliminary testing of DNA yield and quality was done using Nanodrop and Qubit. Kits failing to consistently yield quantifiable levels of DNA as measured using a broad range Qubit (>2 ng/µL) were eliminated from further experiments. Each DNA extraction from kits yielding higher than 2 ng/µL were measured in duplicate using qPCR to determine the cycle threshold (C_t) value. The trial was repeated in four, temporally distinct replicates to increase statistical strength. Below are the instructions for the individual kits that were evaluated.

Indicating FTA Elute Micro Card (F, Cat. WB120412, E Healthcare UK Limited; Little Chalfont, Buckinghamshire, UK):

1. Apply 40 µl of liquid sample onto FTA Elute card. Air dry for 3 h at room temperature or 15 to 20 min at 80°C.

2. Use a Harris punch tool to remove the section of the disc that was soaked with liquid culture. Place punch into a 2 ml microcentrifuge tube.

3. Add 500 µl of sterile water and vortex 5 times.

4. Centrifuge at 13,000 x g for 2 minutes (VWR High Speed Microcentrifuge; Radnor, PA) and remove rinse water. Use a pipette tip to transfer the washed disc to a clean 0.5 ml microcentrifuge tube.

5. Add 30 µl of sterile distilled water and incubate in a calibrated VWR Advanced Dry Block Heater (Cat. 75838-270; Radnor, PA) at 95°C for 30 min. After incubating the disc, vortex for 1 min by pulsing the tube 60 times to dislodge the DNA from the matrix.

6. Centrifuge the tube to recover the condensation from the top of the tube and to pellet the disc. Withdraw the disc from the solution and store eluted DNA at -20°C.

Applied Biosystems PrepMan Ultra Sample Preparation Reagent (P, Ref. 4318930, Life Technologies LTD; Woolston, Warrington, UK):

1. Using 100 µL per reaction and a sterile pipette, transfer the appropriate quantity of PrepMan Ultra Sample Preparation Reagent into a 50-mL sterile conical tube or other sterile container.

2. Pipet 1 mL of culture broth containing bacteria or fungi into a new 2-mL or other appropriate microcentrifuge screw-cap tube that can be tightly closed.

3. Centrifuge the tubes in the microcentrifuge at 13,000 x g for 2 minutes.

4. Aspirate and discard the supernatant.

5. Using a 1-mL pipette, aseptically add 100 µL of the PrepMan Ultra Sample Preparation Reagent into each tube.

6. Tightly cap the tubes, then vigorously vortex the sample for 10–30 seconds.

7. Heat the tubes for 10 minutes at 100°C in a heat block.

8. Cool the tubes to room temperature for 2 minutes.

9. Centrifuge the tubes 13,000g for 2 minutes.

10. Transfer all the supernatant into new labeled microcentrifuge screw-cap tubes and discard the remaining supernatant. Store at -20°C.

Biotecon foodproof StarPrep Two Kit (S, Ord. S40008.1, Biotecon Diagnostics; Potsdam, Germany):

1. Shake the sample gently and let settle for 5-10 min at 25°C.

2. Transfer 800 µl of sample (supernatant) to a 1.5 ml reaction tube.

3. Centrifuge at 13,000 x g for 1 minute.

4. Remove the supernatant with a pipette immediately after centrifugation.

5. Add 800 µl sterile double-distilled water to wash the pellet.

6. Resuspend the pellet by vortexing or by pipetting gently up and down.

7. Centrifuge for 5 min at 8,000 × g.

8. Remove the supernatant with a pipette immediately after centrifugation.

9. Add 300 µl of Lysis Buffer. Place the container of Lysis Buffer on the magnetic stirrer (Ord. S40008.1, Biotecon Diagnostics; Potsdam, Germany). Stir using the provided stir bar at 400 rpm on a magnet stir plate to mix the Lysis Buffer gently and yield a homogeneous solution. Use a 1,000 µl filter tip to transfer 300 µl Lysis Buffer to the sample.

10. Resuspend the pellet by vortexing or by pipetting gently up and down.

11. Place the tube in the horizontal vortexer setup at 13,000 x g for 10 min.

12. Incubate the suspension in a heating unit for5 min at 95 – 100°C

13. Remove the reaction tube from the heating unit, and allow the tube to sit 1 min at 15 – 25°C.

14. Mix by vortexing for 2 s.

15. Centrifuge for 5 min at 13,000 × g. Transfer supernatant to a clean 1.5 mL microcentrifuge tube and store at -20°C.

Qiagen DNeasy PowerFood Microbial Kit 2 mL tubes (Q, Ref. 21000-100-MON, Qiagen GmbH, Hilden, Germany):

1. Stomach the sample for 1 minute at 260 rpm (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK) and remove homogenate for use in DNA extraction.

2. Add 1.8 ml of microbial food culture to a 2 ml Collection Tube (provided) and centrifuge at 13,000 x g for 1 min at room temperature. Decant the supernatant and spin the tubes at 13,000 x g for 1 min. Remove remaining supernatant completely with a pipette tip.

3. Resuspend the cell pellet in 450 µl of Solution MBL (write out).

4. Transfer the resuspended cells to a PowerBead Tube.

5. Secure PowerBead Tubes horizontally to a Vortex Adapter.

6. Vortex at maximum speed for 10 min.

7. Centrifuge the tubes at a maximum of 13,000 x g for 1 min at room temperature.

8. Transfer the supernatant to a clean 2 ml Collection Tube.

9. Add 100 µl of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min.

10. Centrifuge the tubes at 13,000 x g for 1 min at room temperature.

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube.

12. Add 900 µl of Solution MR and vortex to mix.

13. Load 650 µl of supernatant onto an MB Spin Column and centrifuge at 13,000 x g for 1 min. Discard the flow-through and repeat until all the supernatant has been loaded onto the MB Spin Column.

14. Place the MB Spin Column into a clean 2 ml Collection Tube.

15. Add 650 µl of Solution PW. Centrifuge at 13,000 x g for 1 min at room temperature.

16. Discard the flow-through and add 650 µl of ethanol and centrifuge at 13,000 x g for 1 min at 25°C.

17. Discard the flow-through and centrifuge at 13,000 x g for 2 min.

18. Place the MB Spin Column into a clean 2 ml Collection Tube.

19. Add 100 µl of Solution EB to the center of the white filter membrane and centrifuge at 13,000 x g for 1 min.

20. Discard the MB Spin Column. Store the extract at -20°C.

qPCR Primers and Run Parameters

The qPCR components and parameters were selected as described previously (see page 72 "qPRC Primer and Probe Selection", see page 82 "qPCR Plate Construction and Run Parameters").

Plate Count Enumeration

One mL of resuspended 24-hour culture was added to 9 mL of Buffered Peptone Water (BPW; BD) aseptically and vortexed. The resulting dilution was then repeated until relevant dilutions were reached. One hundred µL of the dilution was placed on a TSA (BD) plate and spread plated using a sterile plastic spreader. Each relevant dilution was plated in triplicate. Typically, final dilutions of 10^{-6}, 10^{-7}, 10^{-8} resulted in the ideal range of colony forming units (CFU) per plate. Three petri dishes were plated with 100 µL of BPW as a negative control. Plates were left to dry for 10 minutes then inverted. Incubation of plates was for 48 hours at 37°C under aerobic conditions. After 48 hours, plates were counted manually for total colonies. Dilutions resulting in average plate counts under 30 or over 300 were discounted. Abnormal colony morphologies were rare

and not included in plate counts. Dilution and enumeration were repeated for both *L. monocytogenes* and *S.* Typhimurium cultures.

Statistical Analyses

For plate counting, the dilution resulting in an average of between 30 and 300 colonies per plate was used for statistical analysis. The average plate count of this dilution was calculated and used to estimate DNA copy levels for reactions. In addition, the mean and standard deviation for plate counts and undiluted DNA C_t values across all three runs were calculated.

The C_t values for each sample were determined using QuantStudio^T Design and Analysis Software (v1.5.1, ThermoFisher Scientific) and automatic baseline settings. The ANOVA and Tukey pairwise tests were used to compare C_t values (values ($P \leq 0.05$, C.I.=95%). The C_t values for each kit were measured at both 10^0 and 10^{-4} dilutions of DNA. A Bonferroni correction was applied to the one set of data in which qPCR, Qubit, and Nanodrop results were measured. No statistics were done on the C_t values obtained for the extracts from the 10^{-3} and 10^{-5} dilutions of microbial culture due to a there only being two, time-independent trials, but results were checked for any large deviations from the expected numbers.

Selection and Comparison of DNA Intercalation Agents

Bacterial Growth and Inoculation

A single colony was selected from the refrigerated stock plate and inoculated into 10 mL of TSB (BD) then incubated at 37°C for 24 h under aerobic conditions, for both *L. monocytogenes* and *S.* Typhimurium.

Plate Count Enumeration

Plate Count enumeration was conducted as outlined previously (see page 74, "Pathogen Enumeration").

DNA Intercalation

DNA intercalating agents were chosen based on a review of literature for both proven viability qPCR agents and agents shown to stain nucleic acids, but not yet tested for viability qPCR (*24, 25*). Reagents included in the study were Reagent D (Biotecon Diagnostics; Potsdam, Germany), Ethidium homodimer-2 (Life Technologies Corporation; Eugene, OR), SYTOX Blue (Life Technologies Corporation; Eugene, OR), PMAxx (Biotium Inc.; Fremont, CA), Live or Die NucFix Red (Biotium Inc.; Fremont, CA), and PMA Enhancer for Gram Negative Bacteria (In conjunction with PMAxx, Biotium Inc.; Fremont, CA). Each dye was added to 1 mL of resulting homogenate (ground beef with pathogen). If the original dye protocol was meant for a smaller or larger sample volume, the dye volume was adjusted to maintain the same concentration for 1 mL. If a range of dye concentrations were given, the highest dye concentration suggested was used. Dye addition volumes for 1 mL of sample were: 428 µL Reagent D (D), 1 µL Ethidium homodimer-2 (2), 5 µL SYTOX Blue (B), 1.25 µL PMAxx (P), 1 µL Live or Dye NucFix Red (R), and 250 µL PMA Enhancer for Gram Negative Bacteria preceding addition of 1.25 µL of PMAxx (E).

The DNA intercalation protocol was adapted from procedures used in a similar trial evaluating the use of DNA intercalation agents to suppress DNA from dead cells in qPCR quantification (26). Briefly, 1 mL of filtered homogenate from the sample was

placed in a clear, low binding, 2 mL tube (Biotix Inc., San Diego, CA). This procedure was repeated for a total of 7 tubes per species for the sample containing a mix of live and dead cells; one tube for each DNA intercalating agent as well as one live and dead cell control tube. One tube for each species was prepared using the homogenate containing only live cells as a live cell control, and two tubes were prepared using the homogenate from the uninoculated ground beef as a negative control. Each of the 6 tubes for each species receiving DNA intercalating agents was given the appropriate volume of DNA intercalating agent and thoroughly mixed via 20 inversions. Both sample and control tubes were then covered to prevent light exposure and placed in a 37°C incubator for 15 minutes to allow for dispersion and permeation of the DNA intercalating agents. The tubes were shaken every 5 minutes to encourage even distribution. After the 15 minutes of incubation, tubes were transferred to a holder suspended in water 20 cm away from the 500-watt T-3 halogen bulb and 14F7 work light used for photoactivation (Mod. PQS45, Cooper Lighting; Peachtree City, GA; Figure 1). The bulb was then switched on for 15 minutes to photoactivate the DNA intercalating agents and cause crosslinking. Water temperature was monitored by immersion thermometer to ensure it never exceeded 30°C and the holder was rotated manually at 7.5 minutes to allow for even exposure of the samples to light. After the 15 minutes of photoactivation, the samples were removed and placed on ice for 2 minutes before proceeding to DNA extraction. Multiple sets of DNA intercalation experiments were conducted to test how the intercalating agents responded under a variety of conditions.

Set one tested the efficacy of the DNA intercalating agents on raw DNA. To do this, DNA was extracted from 1 mL of a 24 h *L. monocytogenes* or *S.* Typhimurium culture (in TSB) using the Qiagen DNeasy PowerFood Microbial Kit (Ref. 21000-100-MON, Qiagen GmbH, Hilden, Germany). The approximately 100 µL of resulting DNA extract was then added to 7.9 mL of sterile, UltraPure Distilled Water and vortexed to mix. The DNA mixture was divided into 2 mL tubes, seven tubes per species, each containing 1 mL of the mixed DNA extract. Treatments were: Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), PMA Enhancer for Gram Negative Bacteria (E), and non-intercalated DNA (DNA). The DNA extract was treated with the appropriate DNA intercalating agent as outlined above and frozen for qPCR analysis. Preliminary attempts to precipitate and resuspend the intercalated DNA were unsuccessful, so the intercalated DNA was added directly to the qPCR reaction.

Set two tested the efficacy of the DNA intercalating agents on a mixture of dead and live cells. A 10 mL, 24 h culture of *L. monocytogenes* or *S.* Typhimurium was vortexed and divided into an 8 mL and 2 mL portion. The 8 mL portion was heat-killed by immersing the 15 mL centrifuge tube in a 90°C water bath for 10 minutes. The live and dead cell mixtures were then used to form eight treatments. Seven of the treatments contained a ratio of 900 dead cells to every 1 live cell (approximately 10^8 dead cells to 10^5 live cells per mL). Six of these mixtures were treated with one of the six DNA intercalating treatments each. The seventh mixture contained the 900:1 ratio of dead to live cells and no DNA intercalating treatment; it was used as a live/dead cell control to

test if the intercalation treatment reduced qPCR signal. The eighth mixture consisted of only the quantity of live cells used to prepare the live/dead cell mixture (approximately 10^5 live cells) and was used as a live cell control. Ideally, complete suppression of the dead cells and no suppression of the dye treated live cells in the live/dead cell mixture would result in a qPCR C_t value matching that of the live cell control and higher than the C_t value of the untreated live/dead cell mixture. After being intercalated as needed, the samples underwent DNA extraction using the Qiagen DNeasy PowerFood Microbial Kit and the extract was frozen at -20°C until qPCR analysis could be conducted.

Set three tested the efficacy of the DNA intercalating agents on a mixture of dead and live cells in 80% lean:20% fat irradiated ground beef (Wegmans' Food Markets, Inc.; Rochester, NY). A 10 mL, 24 h culture of *L. monocytogenes* or *S.* Typhimurium was vortexed and divided into an 8 mL and 2 mL portion. The 8 mL portion was heat killed by immersing the 15 mL centrifuge tube in a 90°C water bath for 10 minutes. The live and dead cell broth cultures were mixed at a ratio of 900 dead cells to 1 live cell then 1 mL of the mixed cell culture was added to 9 g of irradiated ground beef in a 400 mL sterile lateral filtered stomacher bag (BagSystem, Interscience; Saint Nom, France) and incorporated thoroughly via 30 seconds of stomaching at 230 rpm (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK). The ground beef inoculation was repeated using only the quantity of live cells used to make the live/dead cell mixture (approximately 10^5 live cells) as a live cell control. Ten mL of PBS were then added to both inoculated ground beef samples and the sample was homogenized for 1 min at 260 rpm. The appropriate volume of homogenate was then removed for each extraction from

the filtered side of the stomacher bag. The live and mixed cell mixtures were then used to form eight treatments. Seven of the treatments contained a ratio of 900 dead cells to every 1 live cell (approximately 10^8 dead cells to 10^5 live cells per mL); six of these were treated with one of the six DNA intercalating treatments each, and the seventh was used as a live/dead cell control (not treated with a dye) to test if the intercalation treatment reduced qPCR signal. The eighth treatment consisted of only the quantity of live cells used to make the live/dead cell mixture (approximately 10^5 live cells) with no dye treatment and was used as a live cell control. Ideally, complete suppression of the dead cells and no suppression of the live cells in the live/dead cell mixture would result in a qPCR C_t value matching that of the live cell control. After being intercalated as needed, the samples underwent DNA extraction using the Qiagen DNeasy PowerFood Microbial Kit and the extract was frozen at -20°C until qPCR analysis could be conducted.

Set four also tested the efficacy of the DNA intercalating agents on a mixture of dead and live cells in irradiated ground beef. However, in this set, the live to dead cell inoculation ratio was approximately 100,000 dead cells to 1 live cell, mimicking the results of a 5-log reduction in bacteria such as might be seen in a challenge study. In addition, only the top two performing DNA intercalating agents from previous experiments were used in this set of experiments, with several additional controls added. A 10 mL, 24 h culture of *L. monocytogenes* or *S.* Typhimurium was vortexed and divided into an 8 mL and 2 mL portion. The 8 mL portion was heat killed by immersing the 15 mL centrifuge tube in a 90°C water bath for 10 minutes. The live and dead cell broth cultures were mixed at a ratio of 100,000 dead cells to 1 live cell then 1 mL of mixed cell

culture was added to 9 g of irradiated ground beef in a 400 mL sterile lateral filtered stomacher bag (BagSystem, Interscience; Saint Nom, France) and incorporated thoroughly via 30 seconds of stomaching at 230 rpm (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK). The ground beef inoculation was repeated using only the quantity of live cells used to prepare the live/dead cell mixture (approximately 10^3 live cells). A treatment containing 1 mL of BPW and 9 g of uninoculated irradiated ground beef was used as a control. Ten mL of PBS were then added to both inoculated ground beef samples and the samples were homogenized for 1 min at 260 rpm. The appropriate volume of homogenate was then removed for each extraction from the filtered side of the stomacher bag. Samples were then used to form eight treatments.

Treatments were Reagent D plus the live cells from the 100,000:1 mixture (DL), PMAxx plus the live cells from the 100,000:1 mixture (PL), the non-intercalated live cells from the 100,000:1 mixture (L), Reagent D plus the 100,000:1 mixed cells (DM), PMAxx plus the 100,000:1 mixed cells (PM), the non-intercalated dead cells from the 100,000:1 mixture (D), the non-intercalated 100,000:1 mixed cells (M), and an uninoculated ground beef control. After intercalation, the samples underwent DNA extraction using the Qiagen DNeasy PowerFood Microbial Kit and the extract was frozen at -20°C until qPCR analysis could be conducted.

DNA Extraction

DNA extraction of the intercalated samples was carried out using the Qiagen DNeasy PowerFood Microbial Kit as described previously (see page 80; “DNA Extraction Kit Selection and Comparison”).

qPCR Primers and Run Parameters

The qPCR components and parameters were selected as described previously (see page 72 "qPRC Primer and Probe Selection", see page 82 "qPCR Plate Construction and Run Parameters

Statistical Analyses

For plate counting, the dilution resulting in an average of between 30 and 300 colonies per plate was used for statistical analysis. The average plate count of this dilution was calculated and used to estimate DNA copy levels for reactions. In addition, the mean and standard deviation for plate counts and undiluted DNA C_t values across all three runs were calculated.

The C_t values for each sample were determined using QuantStudio Design and Analysis Software (v1.5.1, ThermoFisher Scientific) and automatic baseline settings. The ANOVA and Tukey pairwise tests were used to compare C_t values. An alpha of 0.05 was used for the p-value and the confidence interval was 95% ($P \leq 0.05$, C.I.=95%).

Results

Selection and Validation of qPCR Primers and Probes

L. monocytogenes plate counts were 9.3 ± 0.06 $\log_{10}$ CFU/mL (mean, σ). *S.* Typhimurium plate counts were 8.8 ± 0.1 $\log_{10}$ CFU/mL (mean, σ). *L. monocytogenes* undiluted DNA C_t values were 19.3 ± 1.4 (mean, σ). *S.* Typhimurium undiluted DNA C_t values were 13.7 ± 1.1 (mean, σ). Standard curves measuring the qPCR reaction efficiency for *L. monocytogenes* yielded efficiencies of 96.9±2.1% (mean, σ, n=9). *S.* Typhimurium yielded standard curves with qPCR reaction efficiencies of 96.1±1.5% (mean, σ, n=9). R^2 values for all curves exceeded 0.99. The maximum dilution range

while maintaining acceptable qPCR and R^2 values for *L. monocytogenes* was 6, 6, and 7 ten-fold dilutions, corresponding with percent efficiencies of 95.9%, 95.0%, and 96.1%, respectively. All R^2 values were greater than 0.99. This result suggests a maximum range of 6 $\log_{10}$ of quantification using this organism, extraction kit, and medium. *S.* Typhimurium yielded maximum range curves of 8, 8, and 7 points corresponding with percent efficiencies of 100.4%, 94.8%, and 94.7%, respectively. All R^2 values were greater than 0.99. This result suggests a maximum range of 7 $\log_{10}$ of quantification using this organism, extraction kit, and medium.

Selection and Comparison of DNA Extraction Kits

All sets of inoculated irradiated ground beef contained 8 ± 0.5 $\log_{10}$ CFU/mL for both *L. monocytogenes* and *S.* Typhimurium. DNA extraction kits P, Q, and S yielded detectable levels of DNA according to Qubit analysis of *L. monocytogenes* DNA extract (N=12; means: kit Q=22.6, kit S=2.56, P=1.76). Kit F did not consistently yield detectable levels of DNA and was eliminated from further analysis. The ANOVA and Tukey pairwise tests were used to compare C_t values ($P \leq 0.05$, C.I.=95%). For *Listeria*, kits Q and S resulted in the lowest C_t values at 10^0 dilution (means: kit P=24.5, kit Q=17.7, kit S=17.6) and at 10^{-4} dilution (means: kit P=37.7, kit Q=31.3, kit S=31.2). For *Salmonella*, kit Q exhibited significantly lower C_t values at both 10^0 (means: kit P=20.7, kit S=18.3, Kit Q=17.5) and 10^{-4} dilutions (means: kit P=34.3, kit S=32.6, kit Q=31.2). Kit Q also demonstrated favorable C_t values at 10^{-3} and 10^{-5} dilutions of the starting microbial culture. Nanodrop and Qubit values did not correlate with C_t values, likely due to the ground beef contributing large amounts of beef DNA to the extract, and were therefore, not analyzed.

Selection and Comparison of DNA Intercalation Agents

In these experiments, the ability of Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), and PMA Enhancer for Gram Negative Bacteria (E) to suppress DNA from heat-killed dead cells without suppressing amplification signal from the live cells was tested. Experiments tested the ability of the agents to suppress the signal of dead DNA under a variety of conditions. Results for the experiments are shown in Tables 2a through 5b. In the raw DNA intercalation experiments, treatment B demonstrated no significant reduction of DNA signal for either organism (Tables 2a and 2b). Treatment E demonstrated the highest suppression of DNA signal for both organisms. The suppression by other treatments was higher than suppression by treatment B, but lower than suppression by treatment E. In the broth 900:1 experiment, treatments D and P demonstrated optimal suppression for both organisms (signal was not significantly different from live cell signal, Tables 3a and 3b). Treatments B, R, and 2 did not exhibit significant suppression of dead cell DNA for either organism. Treatment E demonstrated optimal suppression of dead cell DNA signal for *S.* Typhimurium but *L. monocytogenes* signal for treatment E was significantly higher than the live cell control. The ground beef 900:1 experiments found almost the exact same results as the broth 900:1 experiments, except for *S.* Typhimurium treatment E, which demonstrated significantly higher suppression than treatment D, although neither treatment was significantly different from the live cell control (Tables 4a and 4b). In the ground beef 100:000:1 experiments, neither treatments D nor P demonstrated significant suppression of live cell DNA (Table 5a and 5b). For *L. monocytogenes;* treatments D and P had significantly higher C_t values than the unintercalated controls, but C_t values were

significantly lower than the live cell control. The two treatments were not significantly different from each other. For *S.* Typhimurium, neither treatments D nor P exhibited significant suppression of live cell DNA. For *L. monocytogenes*, treatments D and P had significantly higher C_t values than the unintercalated controls, but C_t values were significantly lower than the live cell control. Treatment P signal was significantly closer to the live cell control signal than treatment D signal was. The dead and mixed cell controls for both organisms were not significantly different from each other.

Discussion

Selection and Validation of qPCR Primers and Probes

The results of these trials suggest that one can expect over 9 $\log_{10}$ CFU/mL of *L. monocytogenes* and over 8 $\log_{10}$ CFU/mL of *S.* Typhimurium per milliliter of TSB broth after 24 h growth. In addition, it is important to note that the CFU/mL of *L. monocytogenes* was higher than that of *S.* Typhimurium even though the starting volume for DNA extraction was identical for both organisms. Given this information, it would be expected that *L. monocytogenes* should yield a lower undiluted C_t value than *S.* Typhimurium, due to a higher number of DNA copies being present before amplification, when in fact the opposite occurred. The mean C_t value for *S.* Typhimurium was 5.6 cycles lower than that of *L. monocytogenes*. If DNA extraction and qPCR amplification efficiencies were the same for both organisms, this experiment would correlate with a 48.5-fold greater starting concentration of *S.* Typhimurium. Since plate counts demonstrate a discrepancy and that *S.* Typhimurium had a 3.2-fold lower starting concentration, it could be concluded that the DNA extraction and amplification efficiency for *L. monocytogenes* was over 100-fold lower. The most likely reason for this finding is

a lower DNA extraction efficiency associated with for Gram-positive organisms that are often more difficult to extract DNA from due to a thick layer of peptidoglycan (27). In addition, DNA extraction kits are optimized for a certain range of cells. The high cell concentrations used in these experiments also may cause reduced extraction efficiency. The primers and probes chosen yielded acceptable curves for both organisms with qPCR efficiencies well within the acceptable range of 90% to 110% and R^2 values above the 0.99 cutoff. The maximum range of quantification possible with the conditions and protocols used was 6 $\log_{10}$ CFU/mL for *L. monocytogenes* and 7 $\log_{10}$ CFU/mL for *S.* Typhimurium, although the maximum ranges that could be consistently covered were 5 $\log_{10}$ and 6 $\log_{10}$ CFU/mL, respectively. Although these ranges would be able to demonstrate a 5 $\log_{10}$ reduction in pathogens under optimal conditions, a wider range is desired to ensure that the protocol can be used when dealing with higher or lower starting and ending pathogen populations. It is also important that the curve be effective in subpar conditions, such as complex food matrices. A more effective DNA extraction protocol could be very useful in both increasing the range of quantification and allowing for quantification of pathogens from complex food matrices.

Selection and Comparison of DNA Extraction Kits

Kit F was eliminated in these experiments due to extremely low DNA yields. Kit P was tested further, but had consistently higher C_t values than kit S and kit Q. Kit Q, the Qiagen DNeasy PowerFood Microbial Kit, was the most effective of the kits evaluated for extracting target DNA from a complex food matrix for use in qPCR quantification, confirming other results where pathogen DNA was extracted from food systems (22). Kit S, the Biotecon foodproof StarPrep Two Kit, was as effective at extracting DNA for *L.*

monocytogenes, but resulted in C_t values approximately 1 C_t value lower than kit Q for *S.* Typhimurium. Since accurate and sensitive quantification of bacteria is of importance in challenge studies, kit Q was selected for use in future protocol development. It should be noted that kit S did exhibit several advantages over kit Q, such as being quicker (30 min vs 1.5 h), cheaper ($2.82 vs $3.88), and having a lower number of steps and reagents. In addition, kit S may exhibit more specificity towards bacterial DNA in complex matrices. Qubit analysis demonstrated close to a 10-fold difference between DNA extraction levels by kit Q and kit S (means 22.6 ng/µL vs 2.56 ng/µL, respectively). This finding would suggest that kit S should exhibit an approximately 3 C_t higher qPCR value than kit Q if both kits extracted beef and bacterial DNA at similar proportions. In actuality, the difference in C_t value between the two kits ranged from approximately 0 to 1. The ≥ 2 C_t difference between expected and actual values suggests that the ratio of bacterial DNA to beef DNA extracted by kit S was roughly four times greater than the ratio of bacterial DNA to beef DNA extracted by kit Q; however, further experimentation would be needed to verify this finding. These results also helped validate the decision to not utilize Nanodrop and Qubit values in further selection of a DNA extraction kit as their values can be misleading when large amounts of background DNA or contaminants from the matrix may be present. Other studies have also found that absorbance ratios have little value in predicting PCR success (28, 29).

Selection and Comparison of DNA Intercalation Agents

Of the six DNA intercalating agents tested (Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), and PMAxx plus PMA Enhancer for Gram Negative Bacteria (E)), the three that were sold for use in

viability qPCR (Reagent D (D), PMAxx (P), and PMAxx plus PMA Enhancer for Gram Negative Bacteria (E)) were far more effective at intercalating dead cell DNA than the three agents that were only marketed as having DNA intercalating properties. Although this trend generally held throughout the various sets of DNA intercalation experiments, there were some interesting and unexpected results of interest. Due to the novelty of viability qPCR, it was often difficult or impossible to find similar comparisons in other studies to strengthen or propose mechanisms for these results.

The maximum mean intercalated DNA C_t values were 33.6 for *L. monocytogenes* and 34.2 for *S.* Typhimurium, meaning that even without the interference of cell components, none of the treatments were able to fully suppress amplification of all the pathogen DNA present (Table 2a and 2b). The most likely explanation is the intercalating agents were overwhelmed by high levels of DNA used. This observation suggests that systems with extremely high levels of target DNA may require modifications, such as an increased dye concentration. Another unexpected result was that treatment 2 yielded 8.6 C_t cycles more of suppression in *L. monocytogenes* than in *S.* Typhimurium, despite there only being 0.3 C_t cycle difference between the raw, non-intercalated DNA values for the species. The other sets of experiments did not support this trend with treatment 2 showing poor intercalation ability for both organisms. This finding would suggest that the raw *L. monocytogenes* DNA was much more susceptible to intercalation. The mechanism for this susceptibility is unknown, it is possible that differences such as amplicon GC content or binding of the intercalating agent to primers may have hindered amplification. It is also unknown why treatment D exhibited a higher C_t value than treatment P for both species, 6.0 C_t for *L. monocytogenes* and 6.6 C_t for *S.* Typhimurium, when the greatest

difference between the two in any of the other experiments was a 1.6 C_t greater reduction (higher value) in signal by treatment P for *Salmonella* in the ground beef with live:dead cells of 100,000:1. It is possible that the ability of treatment P to reduce dead cell DNA signal relies on the agent crosslinking between the DNA and cell components during activation, allowing for removal of the DNA/cell component complex during DNA extraction. If this finding is correct, and treatment D acts through a solely DNA-dependent mechanism, the lack of cell components in this experiment could have resulted in the reduced efficacy of treatment P.

The results from the broth 900:1 intercalation experiments indicated that treatments P and D were very effective at reducing the dead cell in a mix of live and dead cells without causing suppression of the live cell signal (Table 3a and 3b). Treatment E was very effective for *S.* Typhimurium, but also seemed to significantly suppress both dead and live cell signal for *L. monocytogenes*. Treatments B, R, and 2 did not demonstrate significant suppression of the dead cells in the live/dead cell mixture. The only unexplained result was the large standard deviation in *Salmonella* live cell treatment values. The most likely explanation is that since the reduced number of cells resulted in an invisibly small pellet for DNA extraction, the pellet may have undergone incomplete suspension or recovery in one of the replicates. Even if this was the case, the differences between treatments were still large enough to yield significantly different results and the results agreed with those for both *L. monocytogenes* and follow up experiments.

The results from the ground beef 900:1 intercalation experiments yielded similar results (Table 4a and 4b). The only significant difference was that while treatment E still demonstrated artificial suppression of live cell signal for *L. monocytogenes*, for *S.*

Typhimurium, it was significantly more effective at reducing signal than treatment D. However, neither treatment E nor D was significantly different from the live cell control.

Although treatment E often resulted in the greatest suppression of dead cell DNA, it often resulted in a significantly higher C_t value than the live cell control for *L. monocytogenes*. This finding suggests that while it may be a very effective intercalation treatment for dead cells, in the case of Gram-positive bacteria, it may suppress not just the dead cell signal, but also a significant portion of the live cell signal. This treatment would result in underestimation of these pathogens, an unacceptable result in food safety applications. Although it would be possible to utilize treatment E for only Gram-negative bacteria, this treatment would result in the need for two different protocols for qPCR quantification. In addition to increased complexity of protocols, this approach would eliminate the ability to use multiplex qPCR quantification in future development of methods. For these reasons, only treatments P and D were considered for final testing of DNA intercalating agents.

The much greater dead cell ratio (100,000:1) and additional controls used in experiments with ground beef were valuable in deciding on a final intercalation treatment (Table 5a and 5b). The addition of the DNA intercalating agents to samples containing only live cells did not result in significant reductions in the live cell signal. This finding suggests that the agents will not cause artificial reduction in live cell signal, although the treated samples did trend towards higher C_t values. This trend may become significant if even lower live cell numbers were used; however, it could also be explained by an artificially high “live” cell signal due to low numbers of dead cells being present in the “live” cell samples. The DNA intercalating agents may have been removing these dead

"live" cells resulting in a slight, non-significant difference in signal. The dead cell controls did not exhibit a significantly different signal from the mix of dead and live cells despite containing less cells. This observation was most likely due to the live cell concentration being 5 $\log_{10}$ less than the dead cell concentration and thus forming an insignificant proportion of total cell makeup. The C_t difference between the dead cell control and the live cell control was 15.5 C_t for *L. monocytogenes* and 14.8 C_t for *S.* Typhimurium. Given that there was a 5 $\log_{10}$ difference between the quantity of cells in these treatments, and a difference of 3.32 C_t is expected for every 1 $\log_{10}$ difference in starting DNA template, a difference of 16.6 C_t was expected. There are several possible explanations for this discrepancy. It is possible that there is a significant proportion of dead cells in the live cell culture; these cells would be picked up by qPCR but not by culturing, thus making the expected gap artificially large. Although standard curves were not conducted for the experiment, it is possible that differences in qPCR efficiency could also result in deviation from the expected value. The generally accepted range of 90% to 110% qPCR reaction efficiency expands the expected C_t difference between 5 $\log_{10}$ of starting material from 16.6 C_t to a range of 14.9 C_t to 18.3 C_t, fully encompassing the *L. monocytogenes* value and accounting for the majority of difference in *S.* Typhimurium value. Finally, it is possible that reduced qPCR or DNA extraction efficiency at higher starting concentrations could result in a reduced gap in C_t values. This finding was observed in the first set of cell-based experiments in which the C_t values for treatments P and D were significantly different from the live cell control, and in the case of *S.* Typhimurium, each other. The differences between the intercalated mixed cell treatments and the live cell control were 1.8 C_t (D) and 2.4 C_t (P) for *L. monocytogenes* and 1.5 C_t

(P) and 3.1 C_t (D) for *S.* Typhimurium. The presence of a significant difference between intercalated mixed cells and the live cell control at 100,000:1 dead:live cells in irradiated ground beef but not 900:1 dead:live cells in ground beef, despite similar total cell concentrations, suggests that the DNA intercalating agent concentrations used become overwhelmed with dead cells at a certain point. One possible solution to this problem would be to increase the intercalating dye concentration. However, PMA may decrease live cell signal in certain cases, and increasing dye concentration could potentially result in a significant reduction in live cell signal. The struggle to use PMA to achieve complete suppression of high dead cell signal and not cause a reduction in live cell signal has been documented by others (16, 30–33). It is possible that the addition of sodium deoxycholate, cold-shock treatment, higher incubation temperatures, multiple intercalations, and/or sarkosyl may increase DNA intercalating dye efficiency (34–39). However, this increased efficacy may come at the cost of reduced signal from VBNC and injured cells. Increased amplification length has also been showed to increase suppression by DNA intercalation but must be balanced with qPCR efficiency (16, 25, 40, 41).

This set of experiments also allowed for selection of a final DNA intercalating treatment, 1.25 µL of PMAxx per 1 mL of homogenate. There was no significant difference between the two treatments in the ground beef 100,000:1 experiments for *L. monocytogenes*, but the reduction in dead cell signal was significantly higher for *S.* Typhimurium using treatment P than for treatment D (means= 33.3, 31.7 respectively). Although neither treatment was able to match the signal of the live cell control, the ability of treatment P to be significantly closer to matching the live cell for *S.* Typhimurium resulted in its selection for further protocol development. An additional advantage of

treatment P was that it only required 1.25 µL per mL of homogenate versus 428 µL per mL of homogenate for treatment D. This finding is important as it allows for the reaction to be carried out in a 1.5 or 2 mL microcentrifuge tube and gives greater flexibility in increasing the amount of homogenate or DNA intercalating agent used with the 2 mL setting when further optimizing the process. Further optimization of the DNA intercalating protocol may allow for greater suppression of dead cell signal, less suppression of live cells, and greater applicability.

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Tables and Figures

Table 1. qPCR Run Parameters.

Real-time PCR System	**UNG Incubation**	**Polymerase Activation**	**PCR (40 cycles)**
QuantStudio 3 Real-Time PCR Instrument	50°C Hold, 2 minutes	95°C Hold, 2 minutes	Denature: 95°C, 1 second Anneal/extend: 60°C, 20 seconds

Table 2a and 2b. Results of experiments in which raw DNA extracts from *L. monocytogenes* or *S.* Typhimurium were treated with intercalating agents. Treatments were: Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), PMA Enhancer for Gram Negative Bacteria (E), and non-intercalated DNA (DNA). Mean, StDev, and 95% CI are measures of C_t value. Means that do not share a letter are significantly different by Tukey testing.

Table 2a. *L. monocytogenes* Raw DNA Intercalation Results

Treatment	N	Mean	StDev	95% CI
E	9	33.6[a]	0.597	(33.0, 34.2)
D	9	30.6[b]	1.023	(30.1, 31.2)
2	9	27.4[c]	1.684	(26.9, 28.0)
P	9	24.6[d]	0.252	(24.0, 25.1)
R	9	21.0[e]	0.656	(20.4, 21.5)
B	9	18.9[f]	0.558	(18.3, 19.5)
DNA	9	18.1[f]	0.405	(17.5, 18.7)

Table 2b. *S.* Typhimurium Raw DNA Intercalation Results

Treatment	N	Mean	StDev	95% CI
E	9	34.2[a]	1.284	(33.6, 34.9)
D	9	32.5[b]	1.001	(31.9, 33.2)
P	9	25.9[c]	0.746	(25.2, 26.6)
R	9	22.0[d]	1.610	(21.3, 22.6)
2	9	18.8[e]	0.613	(18.1, 19.5)
B	9	18.7[e]	0.762	(18.0, 19.4)
DNA	9	17.8[e]	0.531	(17.1, 18.5)

Table 3a and 3b. Results of experiments in which TSB broth culture containing a ratio of 900 dead cells to 1 live cell from *L. monocytogenes* or *S.* Typhimurium were treated with intercalating agents. Treatments were Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), PMA Enhancer for Gram Negative Bacteria (E), non-intercalated 900:1 mixed cells (M), and the equivalent number of live cells to the live cells in the 900:1 mixture (L). Mean, StDev, and 95% CI are measures of C_t value. Means that do not share a letter are significantly different by Tukey testing.

Table 3a. *L. monocytogenes* Broth 900:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
E	9	30.6[a]	1.075	(30.0, 31.2)
D	9	25.0[b]	0.457	(24.4, 25.6)
L	9	24.6[b]	1.406	(24.0, 25.2)
P	9	24.5[b]	0.267	(23.9, 25.2)
B	9	21.2[c]	1.492	(20.6, 21.8)
R	9	20.7[c]	0.855	(20.1, 21.3)
2	9	20.4[c]	0.426	(19.8, 21.0)
M	9	20.2[c]	0.354	(19.6, 20.8)

Table 3b. *S.* Typhimurium Broth 900:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
L	9	27.1[a]	4.70	(25.8, 28.4)
P	9	26.0[a]	0.284	(24.7, 27.3)
D	9	25.7[a]	0.527	(24.5, 27.0)
E	9	24.8[a]	0.451	(23.5, 26.1)
B	8	20.3[b]	2.086	(19.0, 21.7)
R	9	19.8[b]	1.506	(18.6, 21.1)
2	9	19.0[b]	0.221	(17.7, 20.3)
M	9	18.9[b]	0.295	(17.6, 20.2)

Table 4a and 4b. Results of experiments in which ground beef containing a ratio of 900 dead cells to 1 live cell from *L. monocytogenes* or *S.* Typhimurium were treated with intercalating agents. Treatments were Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), PMA Enhancer for Gram Negative Bacteria (E), non-intercalated 900:1 mixed cells (M), and the equivalent number of live cells to the live cells in the 900:1 mixture (L). Mean, StDev, and 95% CI are measures of C_t value. Means that do not share a letter are significantly different by Tukey testing.

Table 4a. *L. monocytogenes* Ground Beef 900:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
E	9	35.8[a]	2.391	(35.1, 36.5)
D	9	29.1[b]	1.232	(28.3, 29.8)
P	9	28.7[b]	1.194	(27.9, 29.4)
L	9	28.0[b]	0.745	(27.2, 28.7)
2	9	24.3[c]	0.376	(23.6, 25.0)
R	9	24.2[c]	0.591	(23.5, 25.0)
B	9	24.1[c]	0.387	(23.3, 24.8)
M	9	24.0[c]	0.142	(23.3, 24.8)

Table 4b. *S.* Typhimurium Ground Beef 900:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
E	9	28.3[a]	0.243	(28.1, 28.5)
L	9	28.2[ab]	0.218	(28.0, 28.4)
P	9	28.1[ab]	0.397	(28.0, 28.3)
D	9	27.8[b]	0.153	(27.6, 28.0)
M	9	20.9[c]	0.302	(20.7, 21.0)
B	9	20.8[c]	0.246	(20.6, 20.9)
R	9	20.8[c]	0.282	(20.6, 21.0)
2	9	20.7[c]	0.201	(20.5, 20.8)

Table 5a and 5b**.** Results of experiments in which ground beef containing a ratio of 100,000 dead cells to 1 live cell from *L. monocytogenes* or *S.* Typhimurium were treated with intercalating agents. Treatments were Reagent D plus the live cells from the 100,000:1 mixture (DL), PMAxx plus the live cells from the 100,000:1 mixture (PL), the non-intercalated live cells from the 100,000:1 mixture (L), Reagent D plus the 100,000:1 mixed cells (DM), PMAxx plus the 100,000:1 mixed cells (PM), the non-intercalated dead cells from the 100,000:1 mixture (D), and the non-intercalated 100,000:1 mixed cells (M). Mean, StDev, and 95% CI are measures of C_t value. Means that do not share a letter are significantly different by Tukey testing.

Table 5a. *L. monocytogenes* Ground Beef 100,000:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
DL	9	35.9[a]	0.938	(35.3, 36.5)
PL	8	35.7[a]	1.640	(35.1, 36.3)
L	9	34.8[a]	0.275	(34.2, 35.4)
DM	9	33.0[b]	0.877	(32.4, 33.6)
PM	9	32.4[b]	0.471	(31.8, 33.0)
D	9	23.4[c]	0.990	(22.8, 24.0)
M	9	23.2[c]	0.469	(22.6, 23.8)

Table 5b. *S.* Typhimurium Ground Beef 100,000:1 Intercalation Results

Treatment	N	Mean	StDev	95% CI
PL	9	35.2[a]	0.798	(34.7, 35.7)
DL	9	35.1[a]	0.599	(34.6, 35.6)
L	9	34.8[a]	0.724	(34.3, 35.4)
PM	9	33.3[b]	1.398	(32.8, 33.9)
DM	9	31.7[c]	0.722	(31.2, 32.3)
M	9	20.1[d]	0.236	(19.5, 20.5)
D	9	20.0[d]	0.113	(19.4, 20.5)

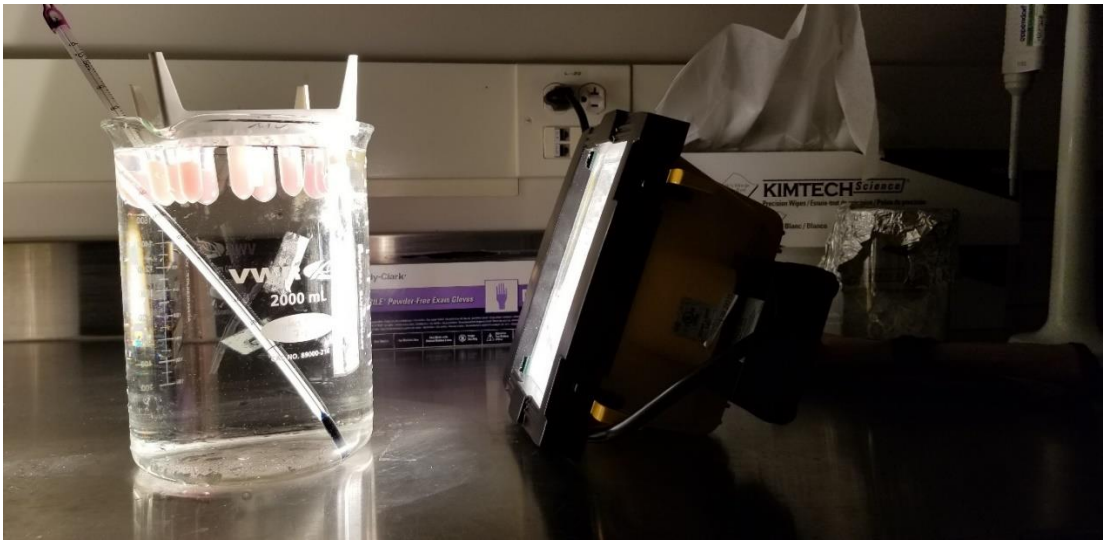

Figure 1. The photoactivation setup utilized for activation of the DNA intercalating agents. Clear 2 mL microcentrifuge tubes containing the samples and dyes were immersed in a 25°C water-filled beaker; water temperature was monitored and did not exceed 30°C during photoactivation. The halogen lamp was placed 20 cm away from the beaker and turned on for 15 minutes; the samples were rotated 180° at 7.5 minutes to allow for even distribution of light.

Chapter 3

Application of Viability qPCR in Challenge Studies

Abstract

Food safety challenge studies play a crucial role in creating a safe food supply chain by ensuring that interventions or critical control points (CCPs) can reduce high levels of pathogens that may be present in the product. Traditionally, monitoring of pathogen levels is done by culture-based plate and count (PAC) methodologies. The rise of methods that use quantitative polymerase chain reaction (qPCR) may offer a faster and more versatile method to enumerate pathogens in food safety challenge studies. The goals of these experiments were to compare a viability qPCR protocol to PAC methods for quantification of pathogens during challenge studies. The first set of experiments used a 60°C water bath as an intervention to reduce the pathogens *Listeria monocytogenes* and *Salmonella* Typhimurium in ground beef. The second set of experiments evaluated lauric arginate to reduce the pathogens in rose meat (IMPS No. 194). The results indicate that viability qPCR can yield similar results to PAC under certain conditions. However, further modifications of these methods may be needed to overcome challenges when quantifying the number of living cells present in a complex food matrix.

Introduction

Salmonella ssp. and *Listeria monocytogenes* are two of the most ubiquitous and harmful food pathogens in the United States. *Salmonella* spp. can survive across a broad range of conditions and possess a wide range of virulence factors allowing for the invasion of multiple cells types and production of a cytolethal distending toxin (1–3). These factors contribute to its ability to cause serious foodborne infections. *Salmonella* ssp. were responsible for 66% of total hospitalizations and 70% of deaths from foodborne disease outbreaks in 2017 (4). The ubiquitous nature of *L. monocytogenes* in the environment, combined with its ability to grow despite high salt and low temperatures, makes it especially problematic in ready-to-eat foods and subsequent outbreaks associated with the pathogen (5). Although *L. monocytogenes* rarely causes disease in healthy individuals, the young, old, pregnant, and immunocompromised (YOPI's) are much more susceptible to the pathogen, with fatality rates up to 34% (5).

A critical component in controlling these pathogens in foods is the implementation of food safety "challenge studies." These studies are used to validate the ability of a food safety process, intervention, or critical control point (CCP) to effectively inactivate high levels of pathogens of concern when present in the food product. Challenge studies are done in specialized research facilities using stringent experimental protocols to ensure containment of the pathogen. Traditionally, challenge studies require researchers to enumerate pathogens from the product using culture-based plate and count (PAC) methods. However, the use of PAC methods for quantification of pathogens in food has several disadvantages; enumeration of pathogens is extremely material intensive and can generate large amounts of waste (6). This process is also very time intensive,

both in terms of labor required and total time needed to process and enumerate samples (7). Given the above, challenge studies that rely on PAC methods can be expensive, which may be cost prohibitive to small food processing facilities and therefore, discourage food safety innovation. In addition, many foodborne pathogens can form viable but non-culturable (VBNC) cells that may not be detected using standard culturing methods (8–11). To overcome these issues, the use of viability qPCR has been proposed. Viability qPCR offers a faster method of quantification that can suppress the DNA of dead cells, while allowing for the quantification of living and VBNC cells (12–14).

Numerous studies have been conducted using viability qPCR to quantify the number of pathogens present in food samples. Limited success in quantifying pathogens using viability qPCR was achieved, but potential limitations have been found. Complete suppression of high dead cell counts can be difficult to achieve with the level of DNA intercalating agent used. Additionally, high levels of DNA intercalating agents may cause live cell signal suppression (15–19). Intercalation of dead cell DNA is also much more successful after inactivation treatments causing high levels of membrane disruption (ex. ultrasound and high heat treatment), than after low membrane disruption methods (ex. mild heat, UV light, and certain disinfectants: (15, 20–23). Little research has been conducted using viability qPCR to enumerate pathogens during challenge studies. The following experiments were designed to compare quantification of pathogens in challenge studies by viability qPCR and PAC methods using two different intervention steps. The first intervention was heat, arguably the most common intervention step used

in meat processing. The second intervention was LAE, an antimicrobial derivative of N-alpha-lauroyl-L-arginine ethyl ester that has a wide range of proposed activities.

Materials and Methods

Bacterial Cultures

Listeria monocytogenes Scott A and *Salmonella enterica subsp. enterica* serovar Typhimurium (ATCC 14028, isolated from chicken organs) were obtained from the Food Microbiology Culture Collection located in the Department of Food Science, Pennsylvania State University. These organisms were chosen based on pathogenicity potential as demonstrated by association with previous foodborne disease outbreaks. Freezer stocks of *S.* Typhimurium and *L. monocytogenes* were made by growing cultures in 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson and Company; BD, Sparks, MD) 24 hours at 37°C under aerobic conditions. After incubation, 0.3 mL of a 50% glycerol solution (v/v) was added to 1.5 mL of TSB culture in a cryogenic tube and frozen at -80°C. Cultures were revived from freezer stocks by adding 100 µL of thawed and vortexed culture stock to 10 mL of fresh TSB and incubating for 24 hours at 37°C under aerobic conditions. Bacteria were then streaked for isolation on Tryptic Soy Agar (TSA, Becton Dickinson and Company; BD, Sparks, MD) and incubated for 24 hours at 37°C under aerobic conditions. Stock plates were refrigerated until needed. Regrowth of single colonies in TSB and isolation onto fresh TSA were conducted periodically to maintain culture viability.

Bacterial Growth and Intervention Treatment

A single colony was selected from the refrigerated stock plate and inoculated into 10 mL of tryptic soy broth (BD) then incubated at 37º C for 24 h under aerobic conditions, for both *L. monocytogenes* and *S.* Typhimurium.

For the heat treatment experiments, one loop of 24 h TSB culture was inoculated into 10 mL of TSB (BD) and incubated at 37º C for 24 h under aerobic conditions, for both *L. monocytogenes* and *S.* Typhimurium to obtain approximately 9 $\log_{10}$ CFU/mL for each organism. One milliliter of the appropriate broth culture was added to 9 g of 80% lean:20% fat irradiated ground beef (Wegmans Food Markets, Inc. (WFM); Rochester, NY) for each sample in a 400 mL sterile lateral filtered stomacher bag (BagSystem, Interscience; Saint Nom, France) and homogenized at 230 rpm for 30 seconds. After stomaching, experimentally-inoculated samples with *L. monocytogenes* and S. Typhimurium were subjected to heat treatments by submerging the bags into a 60°C circulating water bath (Mod. TSCIR35, Thermo Fisher Scientific; Newington, NH) for 0, 18, or 19 minutes for *L. monocytogenes*, and 0, 3.5, or 3.75 minutes for *S*. Typhimurium, followed by immersion in cooling water at <25°C. Times were chosen based on preliminary experiments needed to achieve a 5-log reduction of pathogens in samples that were prepared as outlined. Samples were weighted to ensure complete immersion. Controls were made with uninoculated ground beef (WFM) and buffered peptone water (BD). After cooling, 10 mL of BPW (BD) were added and the samples were homogenized again for 1 min at 260 rpm. Ten mL of homogenate was removed from the filtered side of the stomacher bag, transferred to a sterile tube, and stored at 4°C until subjected to DNA intercalation and extraction procedures described below.

For the lauric arginate (LAE) treatment experiments, one loop of 24-hr TSB culture was inoculated into 10 mL of TSB (BD) and incubated at 37º C for 24 hr under aerobic conditions, for both *L. monocytogenes* and *S.* Typhimurium to obtain 9 $\log_{10}$ CFU/mL for each organism. Vacuum packaged rose meat (IMPS No. 194, flank portion) was obtained from the Penn State Meats Lab, stored at ≤0°F, and thawed at 2-4°C 24 hours prior to use. On the day of the experiment, rose meat was prepared by aseptically cutting into several 10 cm^2 pieces, and immersing these pieces in 70% ethanol for 30 minutes at 25ºC to kill any background microflora. This process did not appear to visibly denature the meat surface. Rose meat pieces were then removed from the ethanol solution and vigorously washed via submersion in sterile BPW (BD) for 15 seconds to remove and dilute any residual ethanol. Rose meat pieces were immersed into 20 mL of 24 h TSB culture for 30 minutes at 25ºC to allow for attachment of the pathogens; one piece was immersed in 10 mL BPW (BD) as a control. A 6% v/v solution of CytoGuard LA 2X (A&B Ingredients (LAE), Fairfield, NJ) was prepared by mixing 0.6 mL of LAE and 9.4 mL of deionized water in sterile 50 mL tubes. The experimentally inoculated samples were dipped into the LAE solution (approx. 15 seconds), excess solution was allowed to drip off, then the pieces were transferred to sterile, 50 mL capped tubes and stored at 4º C for 24 hours. Additional pieces (one control and one for each organism) were added to 10 mL of BPW (BD) and the samples were homogenized for 1 min at 260 rpm (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK). For each sample, 8 mL of homogenate was removed from the filtered side of the stomacher bag, transferred to a sterile capped tube, and used for DNA intercalation and extractions. Control samples consisted of uninoculated meat and the individual pathogens.

Plate Count Enumeration

One mL of resulting homogenate also was removed from the filtered stomacher bag and added to 9 mL of BPW to form a 10-fold dilution. This process was repeated for a total of 7 dilutions. Starting with the 10^{-5} tube, 0.1 mL of diluent was removed from the dilution tube and deposited onto a TSA plate. The plate was then spread with a sterile plastic spreader and manual plate spinner. Spreading for each dilution was completed in triplicate for the 10^{-5}, 10^{-6}, and 10^{-7} dilutions, yielding final dilutions of 10^{-6}, 10^{-7}, and 10^{-8}. The controls were not diluted and 0.1 mL of homogenized sample was added directly onto the TSA plate, then spread-plated in triplicate. Following the interventions, spreading for each dilution was completed in triplicate for the 10^{0}, 10^{-1}, and 10^{-2} dilutions, yielding final dilutions of 10^{-1}, 10^{-2}, and 10^{-3}. Plates were then incubated at 37°C for 48 h. After incubation, plates containing approximately 30 to 300 colonies were counted and the average of the counts was multiplied by the inverse of the dilution factor to determine colony forming units per mL (CFU/mL). Using the above methodology, the limit of detection was 10 CFU/mL.

DNA Intercalation

Based on the results of previous experiments, PMAxx (Biotium Inc.; Fremont, CA) was chosen for the DNA intercalation treatment used in these experiments. One mL of the inoculated, intervention treated homogenate was placed in a clear, low binding, 2 mL tube (Biotix Inc., San Diego, CA), mixed with 1.25 µl of PMAxx by inverting 20 times, covered with aluminum foil to prevent light exposure, and placed in a 37°C incubator for 15 minutes to allow for dispersion and permeation of the DNA intercalating

agents. The tubes were shaken manually every 5 minutes to encourage even distribution of the agent with the sample, and suspended in water 20 cm away from the 500 watt T-3 halogen bulb and 14F7 work light used for photoactivation (Mod. PQS45, Cooper Lighting; Peachtree City, GA, Figure 1). The bulb was then switched on for 15 minutes to photoactivate the DNA intercalating agents and cause crosslinking. Water temperature was monitored by an immersion thermometer to ensure it never exceeded 30°C and the holder was rotated manually at 7.5 minutes to allow for even exposure of the samples to light. After photoactivation, the samples were removed and placed on ice for 2 minutes before proceeding to DNA extraction.

DNA Extraction

DNA extraction of the intercalated samples was carried out using the Qiagen DNeasy PowerFood Microbial Kit 2 mL tubes as follows (Q, Ref. 21000-100-MON, Qiagen GmbH, Hilden, Germany):

1. Homogenize the sample for 1 minute at 260 rpm (Seward Stomacher Model 400, Seward; Worthing, West Sussex, UK) and remove homogenate for use in DNA extraction.

2. Add 1.8 ml of microbial food culture to a 2 ml Collection Tube (provided) and centrifuge at 13,000 x g for 1 min at room temperature. Decant the supernatant and spin the tubes at 13,000 x g for 1 min. Remove remaining supernatant completely with a pipette tip.

3. Resuspend the cell pellet in 450 µl of Solution MBL (write out).

4. Transfer the resuspended cells to a PowerBead Tube.

5. Secure PowerBead Tubes horizontally to a Vortex Adapter.

6. Vortex at maximum speed for 10 min.

7. Centrifuge the tubes at a maximum of 13,000 x g for 1 min at room temperature.

8. Transfer the supernatant to a clean 2 ml Collection Tube.

9. Add 100 µl of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min.

10. Centrifuge the tubes at 13,000 x g for 1 min at room temperature.

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube.

12. Add 900 µl of Solution MR and vortex to mix.

13. Load 650 µl of supernatant onto an MB Spin Column and centrifuge at 13,000 x g for 1 min. Discard the flow-through and repeat until all the supernatant has been loaded onto the MB Spin Column.

14. Place the MB Spin Column into a clean 2 ml Collection Tube.

15. Add 650 µl of Solution PW. Centrifuge at 13,000 x g for 1 min at room temperature.

16. Discard the flow-through and add 650 µl of ethanol and centrifuge at 13,000 x g for 1 min at 25°C.

17. Discard the flow-through and centrifuge at 13,000 x g for 2 min.

18. Place the MB Spin Column into a clean 2 ml Collection Tube.

19. Add 100 µl of Solution EB to the center of the white filter membrane and centrifuge at 13,000 x g for 1 min.

20. Discard the MB Spin Column. Store the extract at -20°C.

qPCR Primer and Probe Selection

A search was conducted for papers containing qPCR primers that had been validated for effective detection of the target organisms and validation for a lack of amplification of closely related species and sequences. Based on these criteria, primers and probes were selected for *L. monocytogenes* (24) and for *S.* Typhimurium (16). For *L. monocytogenes*, a 113 bp amplicon of *hlyA* (positions 1627 to 1740 under accession no. M24199 in GenBank) was used. Primers were Custom Taqman Primers, forward: 5' TGC AAG TCC TAA GAC GCC A, reverse: 5' CAC TGC ATC TCC GTG GTA TAC TAA, (S.O. 7411095, Life Technologies Corp. (LT), Pleasanton, CA); the probe was a ThermoFisher Custom Taqman MGB Probe, probe: 5' CGA TTT CAT CCG CGT GTT TCT TTT CG containing 5' VIC dye and 3' minor groove binding non-fluorescent quencher (MGBNFQ, LT). For *S.* Typhimurium, a 130 bp amplicon of *invA* (positions 197 to 303 under accession no. M90846 in GenBank) was used. Primers and probes were, forward: 5' CGTTTCCTGCGGTACTGTTAATT, reverse: 5' TCGCCAATAACGAATTGCCCGAAC, probe 5' CCACGCTCTTTCG with ThermoFisher 5' 6FAM dye and 3' MGBNFQ (LT). Primers and probes were mixed with nuclease free sterile water to form a working stock for each organism containing 18 µM of the respective primers and 5 µM of the respective probe per µL. Adding 1 µL of

working stock per reaction resulted in final concentrations of 900 nM for each primer and 250 nM of each probe, as suggested by the manufacturer (ThermoFisher).

qPCR Plate Construction and Run Parameters

Quantitative Polymerase Chain Reaction (qPCR) was conducted using a QuantStudio 3 Real-Time PCR Instrument (96-well 0.1 mL Block) (SN 272310615, Life Technologies Holdings Ltd; Singapore, 739256) and protocols were based on usage instructions for TaqMan Fast Advanced Master Mix (25). Each reaction used 10 µL of TaqMan Fast Advanced Master Mix (4444557, Thermo Fisher Scientific; Vilnius, Lithuania), 7 µL of UltraPure Distilled Water (Life Technologies; Grand Island, NY), 1 µL of organism specific primer (LT), and 2 µL of template DNA. Two batches of working stock, one for each organism, were made by vortexing the total volume of Master Mix, nuclease free water, and appropriate primer required for each well. Required volume was calculating based on number of samples and controls tested in triplicate plus 10% loss. All reagents, primers, and probes were kept on ice. Working stock was dispensed into MicroAmp Optical 96-Well Reaction Plate wells (Life Technologies, China) in 18 µL aliquots under a AirClean 600 PCR Workstation (Model 300, AirClean Systems; Creedmoor, NC). The appropriate sample DNA was then added in 2 µL aliquots, each sample was tested in triplicate; 10 mM Tris-HCl pH 7.5 was used as a control. Plates were sealed with MicroAmp Optical Adhesive Film and vortexed briefly (Applied Biosystems, Life Technologies Corp.; Carlsbad, CA). After vortexing, plates were centrifuged at 300g for 1 minute. Parameters were chosen based on recommendations for TaqMan Fast Advanced Master Mix (Table 1). These were, 2-

minute uracil-DNA glycosylases incubation at 50°C, 2-minute polymerase activation at 95°C, and 40 cycles consisting of a 1 second denaturation step at 95°C and a 20 second anneal/extend step at 60°C. Run speed was standard and plate size was 0.1 milliliters.

qPCR Enumeration

Standard curves were designed using 1 mL of broth culture and an 8-point dilution series. One mL of culture underwent DNA intercalation and extraction as described previously in this chapter (see page 119, "DNA Intercalation"; see page 120, "DNA Extraction"). The resulting extract was measured in triplicate and three-time independent trials were used in curve construction (n=9).

The threshold value for qPCR was manually set to 0.3 and the baseline was manually set to start at cycle 3 and end at cycle 8 for all qPCR runs to ensure uniformity. These parameters were based on the baselines and threshold values automatically selected by the program for qPCR reactions containing undiluted DNA. The 8-point standard curves were made by adding 30 µL of DNA extract to 270 µL of 10 mM Tris-HCl pH 7.5, thus forming 1:10 dilutions. Standard curve points were assigned CFU/mL values by assigning the mean CFU/mL enumerated via plate and count enumeration to the undiluted points; dilution points were auto-populated based on the 1:10 dilution series. Standard curves were also checked for any points deviating more than 10-fold (3.32 C_t) from the mean C_t value for that dilution; only one such point was found and removed.

Enumeration of qPCR samples in the challenge study experiments was done by importing the standard curves into the experiments and setting parameters to a threshold value of 0.3 and baseline of cycles 3 to 8.

Statistical Analyses

For plate counting, the dilution resulting in approximately 30 to 300 colonies per plate was used to calculate $\log_{10}$ CFU/mL for statistical analysis. The qPCR estimations for $\log_{10}$ CFU/mL were calculated using the standard curves designed for each organism and QuantStudio™ Design and Analysis Software (v1.5.1, ThermoFisher Scientific). The ANOVA and Tukey pairwise tests were used to compare qPCR and PAC enumeration values (values ($P \leq 0.05$, C.I.=95%).

Results

The purpose of these experiments was to determine if viability qPCR would result in quantification similar to results found through PAC. Standard curves for both organisms exhibited high efficiency and R^2 values and had a much broader quantification range than the 5 $\log_{10}$ minimum typically required in food safety challenge studies (Table 2). The first set of experiments tested the resulting protocol in a challenge study with heat treatments (Table 3a and 3b). In the original, untreated inoculations, qPCR and PAC yielded very highly similar estimates for the starting $\log_{10}$ CFU/mL. *L. monocytogenes* samples were estimated to contain 8.45 $\log_{10}$ CFU/mL by qPCR and 8.38 $\log_{10}$ CFU/mL by PAC ($P \leq 0.05$, C.I.=95%). *S.* Typhimurium samples were estimated to contain 8.28 $\log_{10}$ CFU/mL by qPCR and 8.27 $\log_{10}$ CFU/mL by PAC. Unfortunately, agreement between qPCR and PAC quantification was not found after heat treatment of the

inoculated samples. Heat treated *L. monocytogenes* samples were estimated to contain 6.40 $\log_{10}$ CFU/mL by qPCR and 3.09 $\log_{10}$ CFU/mL via PAC. Heat treated *S.* Typhimurium samples were estimated to contain 7.88 $\log_{10}$ CFU/mL via qPCR and 2.94 $\log_{10}$ CFU/mL via PAC. The difference between PAC and qPCR quantification estimates for heat treated cells were significantly different for both organisms ($P \leq 0.05$, C.I.=95%).

The viability qPCR protocol also was compared to PAC using LAE as the intervention step (Table 4a and 4b). *S.* Typhimurium-inoculated samples without treatment were estimated to contain 8.10 $\log_{10}$ CFU/mL via PAC and 7.77 $\log_{10}$ CFU/mL via qPCR; these results were not significantly different. After LAE treatment, PAC yielded a live cell estimate of 5.97 $\log_{10}$ CFU/mL and qPCR yielded a live cell estimate of 3.38 $\log_{10}$ CFU/mL, which were significantly different ($P \leq 0.05$, C.I.=95%). Untreated *L. monocytogenes* samples were estimated to contain 8.31 $\log_{10}$ CFU/mL via PAC and 7.49_{10} $\log_{10}$ CFU/mL via qPCR; these values were significantly different ($P \leq 0.05$, C.I.=95%). The *L. monocytogenes* data for LAE-treated samples was not analyzed due to: extreme variance in level of kill between the time independent trials; a lack of reproducibility and statistical strength; and the level of kill was so great that bacterial cells and qPCR amplification was so low that counts were below the limit of detection for 3 of the 4 replications.

Discussion

The results of these experiments demonstrate that more research is needed using viability qPCR as a replacement for PAC in food safety challenge studies. The method appears to work well for quantifying high levels of live cells not subject to intervention.

In the heat treatment experiments using a ground beef matrix, qPCR and PAC gave estimates that were not statistically different from the population of live cells present for both pathogens examined. In the LAE experiments using a rose meat matrix, qPCR and PAC yielded similar results for *S.* Typhimurium, although the difference between pre-intervention means was greater than that seen in the heat challenge experiments. For *L. monocytogenes,* there was a significant difference between untreated rose meat sample estimates by qPCR and PAC. It is unclear why untreated cell estimates were so much closer in the ground beef system than in rose meat. One possibility is that the smaller, mixed particles in ground beef form a more homogenous system than the whole muscle rose meat, and thus yields more similar results. This observation seems less likely given that qPCR yielded a lower mean value for both organisms. For the heat-treated samples, qPCR yielded a 3.31 $\log_{10}$ higher estimate than PAC for *L. monocytogenes*, and a 4.94 $\log_{10}$ higher estimate for *S.* Typhimurium. The most likely reason for the very large overestimation of live cells is that the 60°C heat treatment used may not disrupt the cell wall sufficiently for DNA intercalating agent penetration. This theory is supported by another study which demonstrated that PMA inactivation of dead cell DNA was highly effective using heat treatments over 80°C, but not in the range of 52°C to 72°C (23). The researchers also found that the addition of 0.5% to 1% sodium deoxycholate had limited success in helping overcome the lack of DNA inactivation at lower heat treatments. In the current study, experiments comparing qPCR and PAC for the estimation of cells in LAE-treated samples also found a lack of agreement. However, qPCR had a 2.59 $\log_{10}$ CFU/mL lower estimated value than PAC. This observation is the opposite of what was seen in the heat treatment experiments and suggests that qPCR was artificially

underestimating the number of live cells. One hypothesis is that the LAE may reduce qPCR efficiency, thus artificially lowering the estimated number of cells present in a sample. Further studies would need to be conducted to confirm this hypothesis. It should also be noted that the reduction in bacteria caused by LAE varied by several $\log_{10}$ between trials. This variability could have been caused by a number of factors such as the proportion of fat present in each sample, which has been shown to influence LAE efficacy (26, 27).

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Tables and Figures

Table 1: qPCR Run Parameters.

Real-time PCR System	**UNG Incubation**	**Polymerase Activation**	**PCR (40 cycles)**
QuantStudio 3 Real-Time PCR Instrument	50°C Hold, 2 minutes	95°C Hold, 2 minutes	Denature: 95°C, 1 second Anneal/extend: 60°C, 20 seconds

Table 2. Properties of the standard curves made for qPCR quantification of *L. monocytogenes* and *S.* Typhimurium.

Organism	**Slope**	**y-intercept**	**R^2**	**Efficiency (%)**	**Error**	**Range (log_{10} CFU/mL)**
L. monocytogenes	-3.441	45.86	0.998	95.26	0.018	min: 2.43, max: 9.42
S. Typhimurium	-3.437	44.033	0.996	95.401	0.024	min: 2.01, max: 9.00

Table 3a and 3b. Results of the experiments testing viability qPCR protocol in a heat treatment challenge study application. Treatments were quantified by PAC of the starting inoculation (PAC Live), quantified by viability qPCR of the starting inoculation (qPCR Live), quantified by PAC of the heat-treated inoculation (PAC Heat), and quantified by qPCR of the heat-treated inoculation (qPCR Heat). Mean, StDev, and 95% CI are measures of $\log_{10}$ CFU/mL. Means that do not share a letter are significantly different by the Tukey test.

Table 3a. *L. monocytogenes* Heat Treatment Challenge Results

Treatment	N	Mean	StDev	95% CI
qPCR Live	9	8.28[a]	0.203	(8.13, 8.43)
PAC Live	9	8.27[a]	0.062	(8.13, 8.42)
qPCR Heat	9	7.88[b]	0.027	(7.74, 8.03)
PAC Heat	9	2.94[c]	0.380	(2.79, 3.09)

Table 3b. *S.* Typhimurium Heat Treatment Challenge Results

Treatment	N	Mean	StDev	95% CI
qPCR Live	9	8.45[a]	0.129	(7.95, 8.94)
PAC Live	9	8.38[a]	0.161	(7.88, 8.88)
qPCR Heat	9	6.40[b]	1.284	(5.90, 6.89)
PAC Heat	9	3.09[c]	0.673	(2.60, 3.59)

Table 4a and 4b. Results of the experiments testing the viability qPCR protocol in challenge studies using LAE. Treatments were quantified by PAC of the starting inoculation (PAC Live), quantified by viability qPCR of the starting inoculation (qPCR Live), quantified by PAC of the LAE-treated inoculation (PAC LAE), and quantification by qPCR of the heat-treated inoculation (qPCR LAE). Mean, StDev, and 95% CI are measures of $\log_{10}$ CFU/mL. Means that do not share a letter are significantly different by a Tukey test.

Table 4a. *S.* Typhimurium Lauric Arginate Challenge Results

Treatment	**N**	**Mean**	**StDev**	**95% CI**
PAC Live	12	8.10[a]	0.077	(7.77, 8.43)
qPCR Live	12	7.77[a]	0.288	(7.44, 8.10)
PAC LAE	12	5.97[b]	0.583	(5.64, 6.30)
qPCR LAE	12	3.38[c]	0.924	(3.06, 3.71)

Table 4b. *L. monocytogenes* Lauric Arginate Challenge Results

Treatment	**N**	**Mean**	**StDev**	**95% CI**
PAC Live	12	8.31[a]	0.162	(8.11, 8.52)
qPCR Live	12	7.49[b]	0.454	(7.29, 7.70)

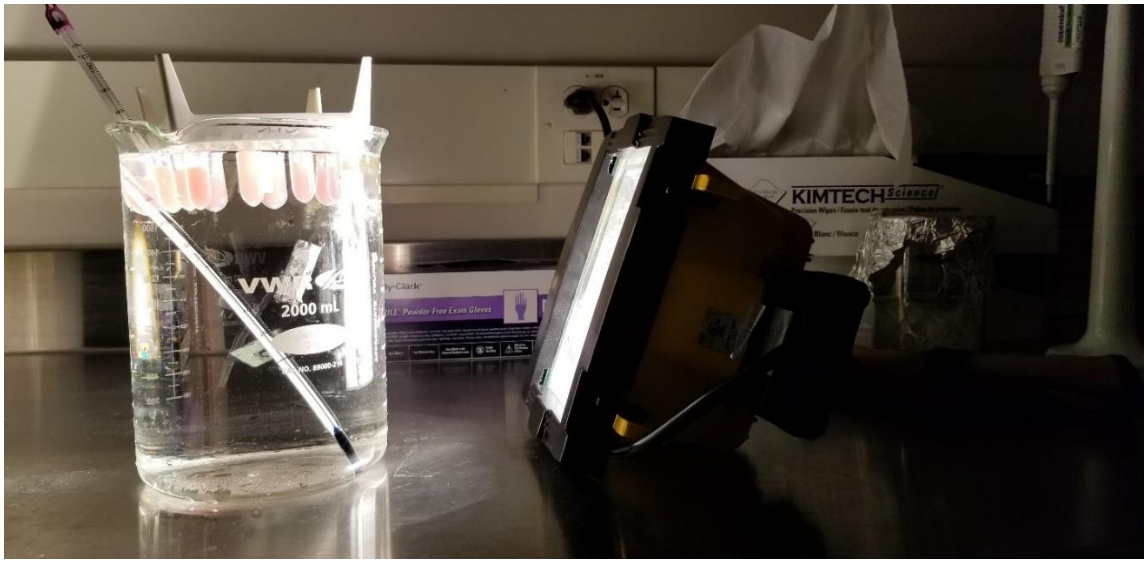

Figure 1: The photoactivation setup utilized for activation of the DNA intercalating agents. Clear 2 mL microcentrifuge tubes containing the samples and dyes were immersed in a 25°C water-filled beaker; water temperature was monitored and did not exceed 30°C during photoactivation. The halogen lamp was placed 20 cm away from the beaker and turned on for 15 minutes; the samples were rotated 180° at 7.5 minutes to allow for even distribution of light.

Chapter 4

Conclusions and Future Directions

Conclusions

There is a distinct lack of published research that addresses the use of quantitative Polymerase Chain Reaction (qPCR) in challenge studies for quantification of pathogens in food matrices. The replacement of culture-based plate and count (PAC) methods with qPCR would allow for faster,and potentially, more accurate detection and quantification of pathogens in challenge studies. However, this application faces challenges, such as the inability of qPCR to distinguish between living and dead cells. The most promising approach to distinguish DNA from live and dead cells in qPCR is the use of DNA intercalating agents, which can be used to remove or inactivate DNA from dead cells before extraction and amplification of live cell DNA, in what is known as viability qPCR. The focus of this thesis was to design a highly effective viability qPCR protocol and then apply it in several challenge study applications.

The first steps necessary in this process were selection of effective qPCR reaction components, selection of a suitable DNA extraction kit, and selection of a DNA intercalating agent. These selections were then applied to viability qPCR for quantification of pathogens in two, small-scale challenge studies. Quantification estimates were then compared between qPCR and PAC to determine efficacy.

Listeria monocytogenes and *Salmonella* Typhimurium were chosen as pathogens of interest for the experiments. A 113 bp amplicon of *hlyA* was selected for *L. monocytogenes* and a 130 bp amplicon of *invA* was selected for *S.* Typhimurium in qPCR experiments. The primers and probes chosen yielded acceptable curves for both organisms, with qPCR efficiencies well within the acceptable range of 90% to 110% and

R^2 values above 0.99. This approach validated the use of these primers and probes in further experimentation.

Based on previous research and additional recommendations, Applied Biosystems PrepMan Ultra Sample Preparation Reagent (P), Biotecon foodproof StarPrep Two Kit (S), Qiagen DNeasy PowerFood Microbial Kit (Q), and Sigma Aldrich Whatman FTA Elute (F) were evaluated for DNA extraction (1–4). Kits were evaluated by inoculating *L. monocytogenes* or *S.* Typhimurium into irradiated ground beef, extracting the DNA from resulting samples, then using qPCR to determine cycle threshold (C_t) values of undiluted and 10^{-4} diluted DNA extracts. Kit Q, the Qiagen DNeasy PowerFood Microbial Kit, was the most effective for extracting target DNA from a complex food matrix and chosen for use in qPCR quantification, confirming results from similar research (3).

Subsequently, six DNA intercalating agents were evaluated for suppressing dead cell DNA: Reagent D (D), Ethidium homodimer-2 (2), SYTOX Blue (B), PMAxx (P), Live or Die NucFix Red (R), and PMAxx plus PMA Enhancer for Gram Negative Bacteria (E). Intercalating agents were evaluated under a variety of conditions: in raw pathogen DNA; in a mix of 900 dead pathogen cells to 1 live cell in tryptic soy broth; in a mix of 900 pathogen dead cells to 1 live cell in irradiated ground beef; and in a mix of 100,000 dead pathogen cells to 1 live cell in irradiated ground beef. Three intercalating agents: Reagent D (D), PMAxx (P), and PMAxx plus PMA Enhancer for Gram Negative Bacteria (E) were far more effective at intercalating dead cell DNA than the other agents. Unfortunately, none of the treatments were able to fully suppress amplification of all the pathogen DNA present in the raw DNA experiments. The most likely explanation for this

finding is the intercalating agents were overwhelmed by high levels of DNA used in these experiments. This observation suggests that systems with extremely high levels of target DNA may require modifications, such as an increased dye concentration. The results from the broth 900:1 intercalation experiments indicated that treatments P and D were effective at reducing the dead cell signal in a mix of live and dead cells, without causing suppression of the live cell signal. Treatment E was very effective at suppressing dead cell DNA from *S.* Typhimurium but resulted in a significantly higher C_t value than the live cell control for *L. monocytogenes*. Treatments B, R, and 2 did not demonstrate significant suppression of the dead cells. The results from the ground beef 900:1 intercalation experiments were similar. Although treatment E resulted in the greatest suppression of dead cell DNA, it also resulted in significantly higher C_t values than the live cell control for *L. monocytogenes*. This finding suggests that while treatment E may be an effective intercalation treatment for dead cells, it suppresses a significant portion of the live cell signal of Gram-positive bacteria, resulting in underestimation of these pathogens, which is unacceptable in food safety applications. Given these results, treatments P and D were evaluated with greater dead cell ratio (100,000:1) in experiments with irradiated ground beef. The addition of the DNA intercalating agents to samples containing only live cells did not result in significant reductions in the live cell signal. This finding suggests that the agents will not cause artificial reduction in live cell signal, although the treated samples did trend towards higher C_t values. The C_t difference between the dead cell control and the live cell control was 15.5 C_t for *L. monocytogenes* and 14.8 C_t for *S.* Typhimurium. Given that there was a 5 $\log_{10}$ difference between the quantity of cells in these treatments, and a difference of 3.32 C_t is expected for every 1

$\log_{10}$ difference in starting DNA template, a difference of 16.6 C_t was expected. The most likely explanations for this discrepancy are: inefficiencies in PAC quantification; PAC does not detect viable but non-culturable cells; or possible differences in qPCR efficiency. The presence of a significant difference between intercalated mixed cells and the live cell control at 100,000:1 dead:live cells, but not at a 900:1 dead:live cells, despite similar total cell quantities, suggests that the DNA intercalating agent concentrations used were overwhelmed with dead cells at a certain point. One possible solution to this problem would be to increase the intercalating dye concentration. However, PMA may decrease live cell signal in certain cases, and increasing dye concentration could potentially result in a significant reduction in live cell signal. The 100,000:1 experiments also allowed for selection of a final DNA intercalating treatment, 1.25 µL of PMAxx per 1 mL of sample.

There was no significant difference between treatments P and D in the ground beef 100,000:1 experiments for *L. monocytogenes*, but the reduction in dead cell signal was significantly higher for *S.* Typhimurium using treatment P than for treatment D (means= 33.3, 31.7 respectively). Although neither treatment was able to match the signal of the live cell control, the ability of treatment P to be closer to matching the live cell values for *S.* Typhimurium resulted in its selection for further protocol development. Further optimization of the DNA intercalating protocol may allow for greater suppression of dead cell signal, less suppression of live cells, and greater applicability.

The results of the challenge study trials demonstrated that more research is needed before viability qPCR can be used as a replacement for PAC methods in food safety challenge studies. The viability qPCR method appears to work well for quantifying high

levels of live cells under certain conditions. In the heat treatment challenge study experiments using a ground beef matrix, qPCR and PAC gave similar estimates for the number of live cells present for both organisms used. In the LAE challenge study experiments using beef carcass surfaces (ex. rose meat), qPCR and PAC yielded similar results for *S.* Typhimurium; although the difference between means was greater than that seen in the heat challenge study experiments. For *L. monocytogenes,* there was a significant difference between untreated rose meat sample estimates by qPCR and PAC when treated with LAE. It is unclear why untreated cell estimates were so much closer in the heat-treated ground beef challenge study than in LAE-treated rose meat challenge study. It is speculated that differences in fat covering on the rose meat may have resulted in significant differences in attachment of pathogens, despite the use of multiple replicates. For the heat-treated samples, qPCR yielded a 3.31 $\log_{10}$ CFU/g higher estimate than PAC for *L. monocytogenes*, and a 4.94 $\log_{10}$ CFU/g higher estimate for *S.* Typhimurium.

The experiments comparing viability qPCR and PAC for the estimation of cells in LAE-treated samples also found a lack of agreement. However, viability qPCR had a 2.59 $\log_{10}$ CFU/cm^2 lower estimated value than PAC. This finding is the opposite of what was seen in the heat treatment challenge experiments and suggests that viability qPCR was artificially underestimating the number of live cells. It should also be noted that the reduction in bacteria caused by LAE varied by several $\log_{10}$ between trials. This variability could have been caused by the proportion of fat present in each sample, which has been shown to influence LAE efficacy (5, 6).

Future Directions

There are many additional experiments that would help in understanding and reducing limitations for the use of viability qPCR in challenge studies. Perhaps the biggest issue, as demonstrated in the challenge study experiments conducted, is that certain interventions result in poor dead cell DNA intercalation and viability qPCR quantification. The mild (60°C) heat treatment used in these experiments resulted in overestimation of the number of live cells by qPCR which was consistent with findings by other researchers (7). This overestimation has been observed with other intervention treatments resulting in low cell membrane disruption, such as UV light and certain disinfectants (8–11). Intercalation of cells also has been shown to be improved by using multiple intercalations, sodium deoxychloate, higher incubation temperatures, cold-shock, and sarkosyl (7, 12–16). Future challenge studies could implement one or more of these treatments to increase dead cell DNA intercalation. The use of controlled studies for these treatments would help determine which of the treatments are most effective, and if they can completely overcome the challenges posed by intact membranes in dead cells.

The LAE experiments resulted in underestimation of live cells by viability qPCR. A likely hypothesis for this observation is that LAE is interfering with qPCR amplification, although additional research is needed to confirm. The use of the viability qPCR protocol over a wide variety of intervention steps is also recommended to help determine its limitations. Another important variable that could be evaluated is the use of additional pathogenic organisms in challenge studies, such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, or *Campylobacter jejuni*.

Another important aspect to consider is to test if viability qPCR is efficient and reliable using multiplex qPCR. Having to run only one set of samples and reactions to quantify all pathogens of relevance would result in major reductions in the amount of time and resources needed, compared to both single qPCR and current, PAC methods utilizing a wide variety of selective media.

To increase the versatility of viability qPCR, it would also be helpful to increase the limit of quantification beyond the 10^2 or 10^3 CFU/mL limit found in similar studies (10, 14, 15, 17–21). In the case of applications where detection is more important than quantification, a 4 to 12 hour enrichment of the sample has been shown to greatly increase sensitivity and could be combined with most probable number (MPN) methods to allow for quantification at lower pathogen concentrations (8, 18, 22–24). It is also possible that a hybrid protocol that incorporates qPCR and PAC methods could allow for faster and better enumeration of pathogens in challenge studies.

Finally, more research is needed to address the presence and enumeration of injured and VBNC cells during challenge studies to determine if PAC methods are underestimating pathogen presence. If underestimation by PAC methods is found, viability qPCR may play an important role in addressing this issue.

Although several limitations were found, the results of this research project demonstrate the potential of viability qPCR to enumerate pathogens in challenge studies. With further development, viability qPCR holds promise for improving food safety interventions by replacing costly, resource-intensive, PAC quantification.

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