Novel Rapid Molecular Detection and Processing Approaches for the Control of *Salmonella enterica* Serovars in the Food Environment

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ABSTRACT

The increase in *Salmonella enterica* outbreaks calls for an urgent need to rapidly detect and control *Salmonella*-associated contamination. Loop-mediated isothermal amplification (LAMP) assay is a novel method that can be completed within 90 min in a simple waterbath. Detection is by simple turbidity, fluorescence, or gel electrophoresis and is more specific than PCR. Reverse-transcriptase LAMP (RT-LAMP) targeting mRNA for the potential detection of live infectious *Salmonella* or recent contamination was used in this study and detection sensitivity to culture-based detection and RT-PCR assays was compared in pure culture, food products, and food processing environments. Our results showed detection limits of 10^1 and 10^2 CFU/ml for *S.* Typhimurium and 10^6 and 10^7 CFU/ml for *S.* Enteritidis by RT-PCR and RT-LAMP assays, respectively. Both assays targeted the specific *Salmonella invA* gene. Enrichment of 10 h was required for equivalent detection to culture-based methods for *S.* Typhimurium in pork products and 16 h for *S.* Enteritidis in liquid whole egg (LWE). For natural LWE and pork samples, 4-h non-selective enrichment followed by 16-h selective enrichment is recommended to ensure sensitive detection.

Effective inactivation/control measures for foodborne pathogens include high intensity ultrasound (HIU, an attractive non-thermal microbial inactivation process). HIU is gaining popularity due to its low cost that also maintains product sensory and functionality attributes. The efficiency of HIU (20 kHz) for *Salmonella* inactivation alone or in combination with nisin (a broad range bacteriocin), in a food model (liquid whole egg, LWE) was studied. Significant *S.* Enteritidis reduction of 3.6 log CFU/ml in pure culture and 1.4 log CFU/25 ml in LWE were obtained after HIU treatment alone for 10 min ($P<0.05$). Scanning electron micrographs revealed microbial structural damage after 5-min HIU. After 10-min HIU, LWE color became visually

and instrumentally lighter along with a lower measured viscosity. However, no additional or synergistic antimicrobial effect was observed with nisin (100 and 1000 IU/ml) in combination with HIU. HIU shows great promise as an alternative non-thermal inactivation process for liquid foods. For use in hurdle approaches, further research on HIU combinations with other natural or generally recognized as safe antimicrobials is needed.

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INTRODUCTION

Salmonella enterica is a leading cause of foodborne bacterial illness in the United States and worldwide. This is most often attributed to the consumption of contaminated foods such as poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce. *S. enterica* serovar Typhimurium and Enteritidis are most frequently associated with pork and egg products, respectively. The consumption of these products contaminated with *S. enterica* poses great risks of outbreaks related to salmonellosis. Therefore, effective *Salmonella* inactivation measures as well as rapid and sensitive detection methods are necessary for the food industry to control the spread and prevent their outbreaks.

As thermal pasteurization may affect the food quality especially in appearance, coagulation, viscosity and flow properties, many non-thermal processes are being researched. High intensity ultrasound (HIU) treatment is an attractive option for microbial inactivation in liquid foods due to its low cost and feasibility for industrial use, while maintaining sensory attributes for consumer acceptability. Nisin is a bacteriocin naturally produced by *Lactococcus lactis* with a positively charged peptide. It holds a GRAS status according to the United States Food and Drug Administration. It is known for its effective antimicrobial properties against Gram-positive bacteria, but not against Gram-negative bacteria under normal condition at neutral pH. This is associated with the outer membrane of Gram-negative bacteria which acts as a permeability barrier. However, by altering the Gram-negative bacterial outer cell structure, nisin may exhibit bacteriostatic or bactericidal effects towards Gram-negative bacteria including *Salmonella*. Nisin and nisin-EDTA were selected for this study to explore the possible

synergistic anti-salmonellae effects when used in combination with HIU, in comparison to the effect by HIU alone.

To determine the absence of *Salmonella* and prevent and control its spread, rapid robust diagnostic assays are crucially needed. The traditional culture-based detection methods for *Salmonella* are labor-intensive and time-consuming, requiring ≥5 days. Therefore, more rapid detection technologies are being extensively researched for testing and field deployment. The real-time polymerase chain reaction (PCR) after optimization can meet the specificity and sensitivity needed for *Salmonella* detection. However, it requires expensive thermal cyclers, which may not be available for routine diagnostics in processing facilities and small industries. A novel nucleic acid amplification assay called loop-mediated isothermal amplification (LAMP) is more rapid, specific and simpler than PCR. It requires only one temperature of 62^{o}C in a simple waterbath for only 90 mins. Amplified products are detected by turbidity, which can be observed visually or by a simple turbidimeter, making it easy and simple for routine diagnostics. The LAMP assay has been successfully applied for the detection of several foodborne bacterial an viral pathogens. Conversion of the described LAMP assay to a Reverse-Transcriptase-LAMP (RT-LAMP) system using mRNA (shorter half-life than DNA) as template can have a higher potential of detecting viable *Salmonella* cells or at the very least recent contamination, compared to LAMP assays that detect DNA. A newly optimized molecular RT-LAMP assay was developed and explored for *Salmonella* detection in food products and processing environments, and further compared for detection sensitivity to traditional culture-based and real-time RT-PCR assays.

CHAPTER I

Literature Review:

Processing and Detection Approaches for the Control of *Salmonella* spp. in the Food Environment

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Introduction

Salmonellosis is a major worldwide foodborne disease that the common manifestations of mild to moderate gastroenteritis, consisting of diarrhea, abdominal cramps, vomiting, and fever (NIAD, 2007). According to the U.S. Centers for Disease Control and Prevention (CDC, 2008), there are approximately 40,000 cases of salmonellosis in the United States annually. Within the U.S., *Salmonella* associated outbreaks cost more than $2.5 billion annually (ERS/USDA, 2008). The illness is most often linked to consumption of contaminated poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce (Foley and Lynne, 2008). Among the >2,500 serovars of *Salmonella* that are capable of causing human disease, *Salmonella enterica* serovar Enteritidis and Typhimurium are most frequently associated with poultry and egg, and swine, respectively (Betancor et al., 2010; Clavijo et al., 2006; Delhalle et al., 2009). Therefore, it is crucial to minimize and eliminate contamination of this foodborne pathogen in at-risk foods.

Salmonella Inactivation

Thermal inactivation has been commonly used for pasteurization and sterilization of food products due to its effectiveness against a wide range of spoilage and pathogenic organisms. However, thermal processing can alter food components and may cause undesirable sensory changes, lowering functional properties and nutritional values. Due to the high demand of consumers for fresh products with high quality and nutritive value, the food industry is interested in non-thermal pasteurization methods which have minimal to no impact on food functionality and sensory quality (Ukuku et al., 2009). Many non-thermal microbial inactivation alternatives, such as high-pressure, pulsed electric field processing, irradiation and ultrasound technologies, have been investigated for their effectiveness against microorganisms, while

maintaining food product quality (Barbosa-Cánovas et al., 1999; Piyasena et al., 2003; Raso and Barbosa-Cánovas, 2003; Ross et al., 2003). Thermal and non-thermal technologies including hurdle approaches for *Salmonella* inactivation that can potentially be applied to liquid whole eggs and egg products are the focus of this review.

High Pressure Processing

High pressure technology has been employed to enhance the safety of food products due to its effectiveness to inactivate foodborne pathogens. Unlike thermal processing, high pressure processing (HPP) has relatively less effect on the product sensory quality, and nutritional attributes (San Martin et al., 2002). Although pressure treatment may cause alterations in the non-covalent bonds of macromolecules such as proteins (which can be reversible, metastable or irreversible depending on the pressure level, treatment time, and other treatment conditions), it is not likely to affect covalently bonded molecules thus maintaining flavor, aroma, vitamins and other pharmacologically active molecules of the food products (Diehl et al., 2008; Masson et al., 2001; Balasubramaniam and Farkas, 2008). However, very high pressure may be required to inactivate food enzymes and bacterial spores, as enzymatic degradation could occur when enzymes are not fully inactivated, and low temperature storage is needed in most pressure-treated products (Yaldagard et al., 2008). Over the years, HPP, including high hydrostatic pressure (HHP) and high pressure homogenization (HPH) technologies have been applied for pathogenic bacterial, fungal, and viral inactivation in foods (Dong-Un, 2002; Préstamo et al., 2000; Kovac et al., 2010; Grove et al., 2006; Wuytack et al., 2002; Diels and Michiels, 2006; Pathanibul et al., 2009; D'Souza et al., 2009). It is also necessary to note the differences between HHP and HPH as described below.

High hydrostatic pressure

High hydrostatic pressure (HHP) has gained interest from the food industry for its ability to enhance food safety by inactivating pathogenic microorganisms, as well as to prolong the product shelf-life due to inactivation of enzymes in food (Kovac et al., 2010; Neetoo et al., 2009; Yaldagard et al., 2008). HHP for food application uses the pressure range of 100 - 1000 MPa, but 400 to 700 MPa are typically used in commercial operations (Yaldagard et al., 2008; San Martin et al., 2002). A typical HHP system consists of a high pressure vessel, a pressure generation system, and a temperature control device, which a pressure treatment process generally involves 3 steps of pressure building up, pressure holding, and depressurizing (Guerrero-Beltran et al., 2005). HHP can be generated either by direct and indirect compression. The pressure medium in the high pressure chamber/vessel is directly pressurized by a piston using a hydraulic pump in the direct-type compression, while a high pressure intensifier is used to pump the pressure medium into the closed and de-aerated high pressure vessel, until the desired pressure is reached (San Martin et al., 2002, Guerrero-Beltran et al., 2005; Yaldagard et al., 2008). Typically, indirect-type pressurization is employed for the industrial cold, warm and hot isostatic pressing systems (Mertens, 1995). Advantages of HHP technology are: (1) It does not depend on size and geometry of the food as it is isostatic; (2) High pressure treatment is uniformly delivered throughout the food so HHP can be use in a wide range of food products (Guerrero-Beltran et al., 2005; Knorr, 1993; Barbosa-Cánova and Rodriguez, 2002); (3) High pressure acts immediately and independently of time/mass, which can reduce the processing time (Yaldagard et al., 2008); and (4) Moreover, it can be applied at room temperature thus reducing the amount of energy comparing to thermal processing.

Microorganisms adapted to normal atmospheric pressure (0.1 MPa) are often able to grow, though at slower rate, under pressure up to around 10 MPa; however, when pressure increases to 100-1000 MPa or more, most of the microorganisms are inactivated (Aertsen et al., 2004). Mechanisms of microbial inactivation of HHP involve cellular membrane damage, which results in leakage of intracellular contents, and dissociation of proteins (Gross and Jaenicke, 1994; Hamada et al., 1992). It is also suggested that protein and nucleic acid complexes in the cell with critical functions, such as ribosomes and septal rings are particularly vulnerable to HPP-induced dissociation (Niven et al., 1999; Kawarai et al., 2004). Bacterial enzymes, such as ATPase, were also reportedly denatured by pressurization, leading to cell death (Simpson and Gilmour, 1997; Wouters et al., 1998).

In recent years, effectiveness of HHP inactivation has been explored for several target microorganisms in different types of food products (Bertucco and Spilimbergo, 2006). Gram-negative bacteria are shown to be less resistant to HHP than Gram-positive ones (Moerman, 2005). Bozoglu et al. (2004) demonstrated the inactivation of *S.* Enteritidis along with other bacteria, including *Listeria monocytogenes, Staphylococcus aureus,* and *Escherichia coli,* using 350-550 MPa pressure at 30-45°C in UHT 1% milk, with an average of 7 log reduction for these microorganisms. In 0.1% peptone water (pH 7.0), *S.* Typhimurium, *E. coli, Yersinia enterocolitica, Vibrio parahaemolyticus, Bacillus cereus, S. aureus,* and *L. monocytogenes* were decreased by an average of 4 logs after HHP exposure at 20°C with 300 MPa for 5 min, 400 MPa for 1 min, 700-800 MPa for 5 min, and 900 MPa for 1 min, respectively (Yuste et al., 2004). Inactivation of *E. coli* and *L. monocytogenes* on inoculated air-dried alfalfa seeds by HHP was also tested (Ariefdjohan et al., 2004). At 40°C, HHP treatment conditions ranging from 275-575 MPa for 2 min and 475 MPa for 2-8 min resulted in a maximum bacterial reduction of 2

logs. However, this study showed that the HHP-treated seeds required a longer time for germination when compared to the untreated seeds (Ariefdjohan et al., 2004). Recently in 2010, Jofré et al. (2010) reported that populations of overnight grown pure cultures of *S. enterica* serovars Enteritidis, Typhimurium, London, Schwarzergrund and Derby, as well as *L. monocytogenes* were reduced by 8 to 9 log after HHP treatment at 900 MPa for 5 min. *S.* Enteritidis inactivation by HHP was previously investigated in LWE in comparison to pulsed-HHP (Bari et al., 2008). HHP at 300-400 MPa and pulsed-HHP at 350 MPa were evaluated at 25, 40, and 50°C for up to 40 min. HHP treatment at 350 and 400 MPa at 25°C for up to 40 min allowed maximum reduction of *S.* Enteritidis by approximately 4.8 and 6.0 log CFU/ml, respectively. Pulsed-HHP at 350 MPa and 50°C, caused inactivation of *S.* Enteritidis in LWE with no recoverable cells during the storage at 4, 25, and 37°C for 24 h. Other examples of *Salmonella* inactivation using HHP are described in Table 1.1. In addition, HHP processing has also been employed for inactivation of enzymes in foods, such as proteolytic enzymes, peroxidase, polyphenoloxidase, and pectin methylesterase (Bertucco and Spilimbergo, 2006). Currently, HHP is used for pasteurization of commercialized food products in the market. These products include jams, juices, sauces, milk-desserts, fruit jellies, fish, fruit, vegetables, shellfish, meat products (such as ham and beef products), and avocado puree (Ohlsson and Bengtsson, 2002; Cheftel, 1995; Bertucco and Spilimbergo, 2006; Murchie et al., 2005).

High pressure homogenization

Homogenization processing has continuously been employed for sensory quality and shelf-life improvement of food products in the food, especially in the dairy industry. With the creation of uniformly dispersed emulsion of dairy foods (e.g., milk, butter, and cream) by

homogenization, stability, flavor and texture of products can be improved (Diels and Michiels 2006; Dickinson and Stainsby, 1988).

Recently, high pressure homogenization (HPH) was developed by incorporation of high pressure processing into a homogenization system to advance these existing non-thermal processing technologies for industrial use. Valve homogenizer (also called a radius diffuser) is typically used in HPH system and pressure can be controlled by altering the distance between the valve and the valve seat, thus adjusting the force of the valve (Diels and Michiels, 2006; Schultz et al., 2004). One significant advantage of HPH method over HHP processing is that HPH can be operated as a continuous process. This makes the HPH suitable for liquid food processing in a large scale production. Not only HPH is used in chemical, cosmetic, pharmaceutical, and food industry for preparation and stabilization of suspensions/emulsions, as well as for modification of physical properties of products, it is also considered as an alternative microbial inactivation measure due to its ability to cause disruption of microbial cells (Kelemen and Sharp, 1979; Paquin, 1999; Pathanibul et al., 2009; Vachon et al., 2002; Wuytack et al., 2002). Phenomena caused by HPH, which are supposedly responsible for microbial inactivation, include turbulence (Doulah et al., 1975), impingement of a high velocity jet of suspended cells on a stationary surface (Engler and Robinson, 1981), cavitation which is the process of rapid creation and collapse of bubbles in liquid medium (Save et al., 1994), and combination of pressure, turbulence, cavitation, high temperature, and sheer stress (Taylor et al, 2007). These phenomena could result in mechanical destruction of bacterial cell wall, leading to the release of intracellular constituents and cell death (Diels and Michiels, 2006; Kleinig and Moddelberg, 1996).

Several studies have investigated the effect of HPH on microbial inactivation, including on salmonellae in pure culture and food samples (refer to Table 1.1). Taylor et al. (2007) showed

that *E. coli* K-12 could be inactivated by HPH at 100 MPa in combination with heat treatment at 60°C in 0.9% NaCl solution. HPH has also been applied for *E.coli* K-12 inactivation in apple juice (Kumar et al., 2009; Pathanibul et al., 2009) with 200 MPa at 2°C inactivating >4 log CFU/ml of this microorganism. With higher pressure at 250 MPa, Pathanibul et al. (2009) showed the decrease in survival numbers of *E. coli* K-12 in apple juice by 7.5 log CFU/ml after HPH treatment. Wuytack et al. (2002) reported the bacterial inactivation effects by different levels of HPH (100–300 MPa) and HHP (200–400 MPa). Five Gram-positive (*Enterococcus faecalis, S. aureus, Lactobacillus plantarum, L. innocua* and *Leuconostoc dextranicum*) and six Gram-negative (*S. enterica* serovar Typhimurium, *Shigella flexneri, Y. enterocolitica, Pseudomonas fluorescens, E. coli* LMM1010, and *E. coli* MG1655) bacterial strains were used in the study. Among the tested bacteria, diverse resistance to HHP was observed depending on the strain within the group of Gram-positive and Gram-negative bacteria. In HPH treatment, Gram-negative bacteria were found to be more sensitive to HPH treatment than Gram-positive in this study. It has been explained by several researchers that susceptibility of Gram-negative bacteria to HPH is due to their thinner peptidoglycan layer in their cell wall in comparison to Gram-positive bacteria (Kelemen and Sharpe, 1979; Vachon et al., 2002; Wuytack et al., 2002). Approximately 2 logs or more of *E. coli* LMM1010 and MG1655, *S.* Typhimurium, and *Y. enterocolitica* in buffer were inactivated by HPH at 200 MPa at room temperature, while 4.6 logs of *S. flexneri* were decreased after the same treatment (Wuytack et al., 2002). Although HPH technology is still relatively costly, it remains a promising tool for microbial inactivation in a continuous liquid food processing.

Pulsed Electric Fields

Food safety improvement using pulsed electric fields (PEF) is based on utilization of high intensity electric field pulses to inactivate microorganisms in foods (Ravishankar et al., 2008). A typical PEF processing system consists of a pulse modulator using a semiconductor switch that can turn pulses on and off, a set of PEF treatment chambers, and a cooling system for maintaining the temperature of food products (Ravishankar et al., 2008; Amiali, 2005). Food flows through the treatment chamber, whose geometry can be parallel plate, co-field flow or coaxial cylinder, to receive the pulsed field treatment (Amiali, 2005). Factors involving bactericidal efficacy of PEF include the strength of the electric fields, pulse width, pulse number, and delay time (Zhang et al., 2007; Evrendilek and Zhang, 2005). Pulsed field intensity is typically in the range of 15-50 kV/cm, with pulse width between 1-5 μs, and pulse frequency of 200-400 Hz (pulses/s) (Wan et al., 2009). Besides, other factors such as treatment time, ionic strength, pH, medium conductivity, and temperature also have impact on the microbial inactivation efficacy (Palaniappan and Sastry, 1991; Zhang et al., 2007). Although electric pulses are required for inactivating microorganisms, PEF is still considered as a non-thermal process due to the increase in only a few degrees of the temperature of food products (Ravishankar et al., 2008). Therefore, only minimal changes in quality, sensory properties, and nutritional value of foods may occur during PEF processing (Wan et al., 2009). Another advantage of PEF system is that it is a continuous system, which allows the application in the fluid food processing.

Mechanisms of microbial inactivation by PEF have been studied and proposed that PEF mainly causes structural disruption of microbial cell membranes leading to cell inactivation (Amiali, 2005). High trans-membrane potential difference is also known to occur when microorganisms are exposed to PEF, which can cause the osmotic imbalance across cell membrane and the breakdown of lipid membrane (Ravishankar et al., 2008; Wan et al., 2009).

These could result in electroporation-induced effect where the electroconductivity and permeability of cells are increased (Barbosa-Cánovas et al., 1999; Wan et al., 2009). Permanent damage to the membrane may be achieved when cells are exposed to PEF approximately at 5–15 kV/cm (Ravishankar et al., 2008).

As in most microbial inactivation processes, inactivation of vegetative bacterial cells requires smaller doses (less electrical intensity and/or less number of pulses) of PEF when compared to bacterial spores (Barbosa-Cánovas and Rodriguez, 2002; Pothakamury et al., 1996). Gram-negative bacteria are, in general, more sensitive to PEF than Gram-positive ones (Barbosa-Cánovas and Rodriguez, 2002; Vega-Mercado et al., 1996; Mazurek et al., 1995). As Gram-positive bacteria have thicker and more rigid outer cell structures, they can withstand higher osmotic forces that occur during PEF process (Amiali, 2005). Inactivation of *Salmonella* spp. by PEF is shown in Table 1.2.

Pulsed Light

Pulsed light is a non-thermal processing method using intense, short-duration pulses of broad spectrum white light, including wavelengths in the ultraviolet to the near infrared region (Elmnasser et al., 2007; FDA, 2011b). Pulsed light is produced by accumulation of electrical energy in an energy storage capacitor over time and then release the stored energy in a very short time to magnify the power onto materials (Dunn et al., 1995). Typically, a pulse of light used has an energy density in the range between 0.01 to 50 J/cm^2 at the surface of treated materials, with a wavelength distribution at least 70% of the electromagnetic energy between 170 to 2600 nm (FDA, 2011). In pulsed light processing, material is exposed to at least 1 pulse of light for 1 μs to 0.1 s duration (Dunn et al., 1991).

Pulsed light technology has been applied for microbial inactivation on the surface of packaging materials, pharmaceutical products, fresh produce (cabbage, lettuce, alfalfa seeds, and berries), milk, eggs, marine products, and other surfaces (Anderson et al., 2000; Dunn, 1996; Elmnasser et al., 2007; Marquenie et al., 2003). The microbial inactivation efficacy involves the selection of light intensity, wavelength of light, pulse duration, and number of pulses. Moreover, surface texture of the products plays a critical role on the effectiveness of pulsed light treatment. As rough/uneven surface may result in some areas that cannot be reached by light and the microorganisms present on those shadowed areas will not be properly treated. Thus, a smooth or clear material would be more suitable for the pulsed light processing application. Additionally, the level of pulsed light treatment required also depends on the type(s) of target microorganisms. Light pulses are known to induce photochemical or photothermal reactions in food materials, causing microbial inactivation (Rowan et al., 1999). The visual and infrared lights can cause photothermal effects, while the UV-rich light typically results in photochemical reactions (FDA, 2011). The mechanisms of action of pulsed light have been widely studied and proposed. A primary cellular target of the photochemical effect is nucleic acids, where DNA is subjected to chemical modifications and cleavage (Elmnasser et al., 2007). In addition, proteins, membranes, and other cellular materials are potentially affected as a result of cellular DNA destruction, leading to microbial lethality (FDA, 2011). As mentioned earlier, the photothermal changes of microorganisms due to pulsed light treatment can also occur. This causes rapid overheating of microbial cells depending on thermal energy delivered during the process; microbial inactivation in this case is attributed to cell disruption/explosion and loss of cellular contents (Wekhof, 2000).

Reductions of bacteria between 2 to 8 logs and 4.5 log reduction in fungi were obtained using pulsed light technology (MacGregor et al., 1998; Rowan et al., 1999). *S.* Enteritidis

numbers on shelled eggs was shown to be reduced by ~8 logs after treatment by 8 light pulses at 0.5 J/cm^2 (Dunn, 1995). Pulsed light at 5.6 J/cm^2 was used for *E. coli* inactivation in alfalfa seeds, which resulted in bacterial inactivation of ~1 to 2 log CFU/g (Sharma and Demirci, 2003). In 2005, Ozer and Demirci (2006) demonstrated the same treatment conditions of pulsed light for *E. coli* O157:H7 and *L. monocytogenes* inactivation in salmon fillets. Their results showed that bacterial levels were decreased by 0.24-0.91 and 0.72-0.8 log for *E. coli* O157:H7 and *L. monocytogenes* in salmon samples, respectively (Ozer and Demirci, 2006). A pulsed light treatment was also used for inactivation of fungal conidia of *Botrytis cinerea* and *Monilia fructigena* in fresh produce with pulses of 30 µs at 15 Hz frequency and treatment duration ranging from 1 to 250 s (Marquenie et al., 2003). Similar inactivation of conidia of both fungi was observed with the reduction of 3 and 4 log units for *B. cinerea* and *M. fructigena*, respectively (Marquenie et al., 2003). Increased inactivation of conidia was obtained with increasing pulsed light intensity. Thus the duration and intensity of the pulse light treatment are important factors to be considered for inactivation.

Irradiation

Food irradiation is a non-thermal food processing method which exposes food to sufficient radiation energy for shelf-life extension, product quality improvement, and microbial control (FDA, 2001). Electrons within foods are excited by radiation to be above their ionization potential, causing ionization which results in damage of microbial genes and cell death (Barbosa-Cánovas et al., 1998; Farkas, 1988; FDA, 2001). Common sources of radiation include gamma rays (with Cobalt-60 or Cesium-137 radioisotope), electron beams (e-beams; high energy of up to 10 MeV), and X-rays (high energy of up to 5 MeV) (Morehouse and Kamolprasert, 2004;

Hirneisen et al., 2010). As ionizing radiation can cause alterations in chemical properties of foods, the safety of treated product consumption becomes a concern. In 1981, the joint expert committees of the International Atomic Energy Agency (IAEA), the World Health Organization (WHO), and the Food and Agricultural Organization (FAO) of the United Nations reviewed and evaluated the safety of irradiated foods and concluded that the food irradiation process does not present any enhanced toxicological, microbiological, or nutritional hazard beyond the conventional food processing techniques (Diehl, 1995). In the U.S., irradiation was used for food preservation in the early 1920s, and was first approved in 1997 for pathogen control in unprocessed red meat and meat products, which led to numerous studies and interest on other food irradiation applications (Morehouse and Kamolprasert, 2004). Currently, irradiation is considered as a food additive and is regulated for food application by the US FDA (21 CFR 179) (FDA, 2011a). Doses of irradiation are based on target microorganisms of each process (Barbosa-Cánovas and Rodriguez, 2002). Firstly, radurization an irradiation process targeting spoilage microorganisms, uses dosage normally below 10 kGy. Secondly, radicidation is a process that targets non-spore forming bacterial pathogens with the typical dosage between 2.5 to 10 kGy. And lastly, radappertization typically uses dosage between 10 to 50 kGy for inactivation of spore-forming pathogenic bacteria and viral pathogens. Gram-negative bacteria (which have thinner peptidoglycan layer than Gram-positive bacteria) have been shown to be less resistant to irradiation, followed by Gram-positive bacteria, molds, and then viruses (van Gerwen et al., 1999). Radiation has also been proven to be suitable for inactivation of spores in low-moisture foods, such as garlic and onion powders (Schmidt, 1961; Farkas, 1985). The application of radiation is suggested to be an effective means to destroy bacterial spores, and higher inactivation effect can be achieved when used in combination with heat (Nakauma et al., 2004).

Refer to Table 1.3 for salmonellae inactivation efficacy by gamma ray, e-beam, and X-ray irradiation.

Gamma Ray

Gamma radiation used in food processing is produced from radioactive sources, including Cobalt-60 and Cesium-137 radioisotopes. Several advantages of gamma irradiation using Cobalt-60 include high availability (up to 95%) of the emitted energy, high penetration, uniformity of the dose in the food product, and a decay of stable non-radioactive nickel isotope (Environmental Protection Agency, 2011; Satin, 1996). However, some limitations of Cobalt-60 gamma source are that special storage is required with frequent replenishment, radiation emission cannot be turned on and off, and treatment of the food is relatively slow due to its 5.3-year half-life (Environmental Protection Agency, 2011; Shin et al., 2011). Another important gamma source for food irradiation is a Cesium-137 radioisotope. It is known to have a less penetrating gamma beam and has a longer half-life compared to Cobalt-60.

Patterson (1988) reported that *S.* Typhimurium, *E. coli*, and *Moraxella phenylpyruvica* in poultry products were very sensitive to gamma irradiation, especially when subjected to various atmospheric changes. A study conducted by Thayer and Boyd (1991) showed that a gamma irradiation could effectively inactivate *S.* Typhimurium by 2.59 and 5.67 logs at 1.8 and 2.7 kGy, respectively, in mechanically deboned chicken meat. When used in combination with heat, as low as 0.90 kGy of gamma irradiation followed by heating at 60°C for 3 min was shown to reduce 8.9 logs of *S.* Typhimurium in deboned chicken meat, while less effect (6.4 log reduction) was found when samples were heated prior to irradiation (Thayer et al., 1991). In fresh produce, gamma irradiation at 0.35 kGy decreased aerobic microorganisms by 1.5 logs and yeast and mold counts by 1 log in cut romaine lettuce packaged under modified atmosphere, with

reductions remaining the same through the 22 d storage at 4°C (Prakash et al., 2000). D_{10} values for *S. enterica* serovar Enteritidis, Typhimurium, and Infantis were reportedly at 0.29 to 0.43 kGy on minimally processed watercress (*Nasturtium officinalis*) samples in polyethylene bags, and 1.7 kGy was sufficient to reduce 4 logs of *Salmonella* population in the watercress (Martins et al., 2004). Niemira and Solomon (2005) investigated the inactivation of *S. enterica* serovars in planktonic and biofilm-associated forms by gamma irradiation. The D_{10} values of biofilm-associated and planktonic *S.* Anatum were found to be 0.645 and 0.677 kGy, For *S.* Stanley, biofilm-associated cells showed D_{10} value of 0.531 kGy, while D_{10} value for planktonic cells was 0.591 kGy, respectively. D_{10} values of *S.* Enteritidis were shown to be 0.436 and 0.535 kGy for biofilm-associated and planktonic cells, respectively. A feasible dose of irradiation for improving the safety of liquid egg white and liquid egg yolk without causing adverse sensory effects was found to be 3-kGy irradiation after storage at 4±1°C. This dose did not cause alteration in amino acid composition, fatty acid profiles or sensory preference when compared to non-treated samples (Badr, 2006). Levels of total plate count, *Enterobacteriaceae*, *S. aureus* and *Salmonella*, as well as amino acid composition, fatty acid profiles, sensory properties of the products were determined after egg samples were irradiated and then stored at 4±1°C (Badr, 2006).

Although irradiation is approved for various foods and appears to be a promising alternative for food preservation, it may have negative effects of food quality as ionizing radiation disrupts the chemical composition in not only microorganisms, but also food products. Previous research showed that irradiated fruits and vegetables may result in softer texture products, with possible color alteration (Nagai and Moy, 1985; Bourne, 1995; Prakash et al., 2000). In the study by Zhu et al. (2004), color and flavor of ready-to-eat turkey breast rolls were also shown to be affected by irradiation. Hanis et al. (1989) reported that poultry meat treated

with gamma irradiation at 1.0 kGy resulted in an increased level of oxidation, which is noticeable by consumers as the product received lower scores for flavor and taste attributes comparing to non-irradiated meat. Moreover, the high in cost and low in consumer acceptance make this technology still limited for commercial use.

Electron Beam

Electron beam (e-beam) irradiation uses accelerators to generate up to 10-MeV e-beams, which are directed for product treatment by a magnet (Nieto-Sandoval et al., 2000). As accelerators are used for e-beam generation, unlike radioisotopes in gamma irradiation, the process can be turned on and off with no nuclear waste generation. However, e-beams have less penetration within only 2-4 inches, when compared to gamma radiation (Lewis et al., 2002). This may not be a total drawback as some food products are subjected to only surface contamination. This level of beam penetration could be sufficient for microbial inactivation on food surfaces, while minimizing adverse effect on product quality. E-beam irradiation can also be applied in a bi-directional manner, from top and bottom of food products; therefore, uniform irradiation of a product irradiation can be achieved (Lewis et al., 2002).

E-beam irradiation has been explored for its efficacy on *Salmonella* spp. inactivation. Heath et al. (1990) reported that low dose e-beam irradiation at 1.0 kGy was sufficient to reduce numbers of *Salmonella* and other aerobic bacteria in broiler thighs and breasts. Pork chops and ham inoculated with *S.* Typhimurium have also been irradiated with e-beam (Fu et al., 1995; Song et al., 2011). *Salmonella* levels were reduced by 1 log on pork chops and 3 logs on ham after e-beam irradiation at 0.75 or 0.90 kGy, respectively (Fu et al., 1995). At 2 kGy, e-beam treatment showed 3.78 log reduction of *S.* Typhimurium in sliced ham (Song et al., 2011), and 2.04 log reduction in powdered weaning foods (Hong et al., 2008). In 2005, Sarjeant et al. (2005)

tested the *Salmonella* inactivation effect in inoculated frozen raw chicken breast strips (8 logs of *S.* Typhimurium per strip) by e-beam irradiation at 0, 1, 2 or 3.0 kGy, and with ≥2 kGy e-beam doses. *Salmonella* could not be detected by direct plating; however, injured *Salmonella* cells were recovered at all irradiation levels after enrichment. In peanut butter, 3-kGy e-beam could reportedly reduce 6.75 and 4.85 logs of *S.* Tennessee and *S.* Typhimurium, respectively (Hvizdzak et al., 2010).

In natural samples, e-beam irradiation was shown to be effective in eliminating low levels of bacterial contamination. Lewis et al. (2002) demonstrated that approximately 40% of boneless, skinless chicken breasts could be naturally contaminated with *Salmonella*. E-beam treatment at 1.0 kGy was found to completely eliminate *Salmonella* contamination in chicken breast samples. Although e-beam irradiation can be used for decontamination of natural food products, it is also important that irradiated products are maintained at appropriate cold temperature storage (e.g., refrigeration) after irradiation as bacterial survivors (including injured cells) can still grow in the products if storage temperature is abused. Fu et al. (1995) showed that *Salmonella* counts in irradiated pork products remained the same when samples were stored at 7°C; however, *Salmonella* growth was observed when stored at 25°C for 7 days.

X-Ray

Irradiation by X-ray is the newest in ionizing irradiation technologies which has been applied commercially in food products (Shin et al., 2011). The high-energy photons are produced by the interaction of charged particles with matters using a high-energy beam generated by a machine (Farkas, 2006). It is generally lower in energy and therefore less penetrating than gamma rays (Environmental Protection Agency, 2007). X-irradiation has advantages over other currently approved ionizing irradiation methods for food industrial use (such as gamma rays) as

it is generated by machine, can be turned on and off, and does not have a radioactive source, while gamma rays are obtained from radioisotopes (Janatpour et al., 2005). Therefore, X-irradiation does not require a special processing facility and exposes less risk to handling personnel and the environment. The ability to control dosage and exposure with an on and off mode is very beneficial to the industry as the process can be easily controlled, unlike the gamma ray which is constantly emitted from radioisotopes. Also, consumers show a better acceptability to X-irradiation compared to gamma irradiation (Robertson et al., 2006). This is due to consumers' familiarity with X-ray use in the medical area. Thus, X-rays could be a great candidate as gamma irradiation alternatives for commercial foodborne pathogen inactivation in foods.

Previously, studies have demonstrated that X-ray sanitation technology can result in high microbial reduction (>6 log reduction) for pathogens on various food products including *V. parahaemolyticus, V. vulnificus* in pure culture, half shell and whole shell oysters (Mahmoud and Burrage, 2009; Mahmoud, 2009a), *E. coli* O157:H7, *S. enterica, S. flexneri* and *V. parahaemolyticus* in ready-to-eat shrimp (Mahmoud, 2009b), Cronobacter species (*Enterobacter sakazakii*) in dairy products (skim milk, low-fat milk and whole-fat milk) (Mahmoud, 2009c), and *E. coli* O157:H7, *L. monocytogenes, S. enterica* and *S. flexneri* in fresh produce (spinach leaves and shredded iceberg lettuce) (Mahmoud et al., 2010; Mahmoud, 2010). Although X-irradiation shows promise for foodborne pathogen inactivation in foods, it may also cause biochemical changes in food products. Shin et al. (2011) reported that the phenylalanine ammonia-lyase activity of asparagus in vacuum skin-package was increased after X-ray treatment after storage up to 8 days. Therefore, further investigation on the effect of X-irradiation

on changes in sensory quality, nutritional value, and other functional properties is needed for evaluation of its commercial feasibility.

Ultraviolet Light

Ultraviolet (UV) light is commonly used as a non-thermal disinfection method for air, water, packaging and other food contact surfaces (Dinçer and Baysal, 2004; Wells et al., 2010; Koutchma, 2008). UV radiation between 220 and 300 nm is known for its germicidal effect (typically a 254-nm UV is used for decontamination). This range of wavelength can cause photochemical reactions within the nucleic acid of target bacteria causing cross-linking between the neighboring pyrimidine nucleoside bases (thymine and cytosine) in the same DNA strand, which can result in bacterial DNA denaturation and cellular inactivation (Bachman 1975; Sizer and Balasubramaniam 1999; Guerrero-Beltrán and Barbosa-Cánovas, 2004; Wells et al., 2010). Typical UV units consist of UV lamps, UV exposure detection sensors and concentric tubes where product flows as a thin film and are exposed to UV light in case of liquid foods (Donahue et al., 2004). The main advantage of UV processing is its low cost, thus the system can be a great alternative for non-thermal food pasteurization, especially for small processing facilities.

Many studies have reported bactericidal effect of UV treatment at different doses in several food products (Guerrero-Beltrán and Barbosa-Cánovas, 2004). Chavez et al. (2002) found that aerobic bacteria on eggshell were reduced by 2 to 3 log CFU/egg after 60-s UV light exposure at 75 mW/cm^2 intensity. In 2004, Yuan et al. demonstrated the application of UV for bactericidal effects on the surface of fruits and vegetables inoculated with *Salmonella* spp. and *E. coli* O157:H7. UV treatment on apples inoculated with *E. coli* O157:H7 resulted in 3.3 log reduction at 24 mW/cm^2. On tomatoes inoculated with *Salmonella* spp., 2.19 log reduction was

achieved when treated with UV treatment at the same dose. UV treatment on green leaf lettuce inoculated with *Salmonella* spp. and *E. coli* O157:H7 resulted in 2.65 and 2.79 log reduction, respectively. Inactivation of *E. coli* O157:H7 in apple cider using UV treatment was shown by Wright et al. (2000) and Donahue et al. (2004). Apple cider containing a mixture of acid-resistant *E. coli* O157:H7 strains was treated using a thin-film UV disinfection unit at 254 nm ranging from 9,402 to 61,005 mW-s/cm^2 (Wright et al., 2000). A reduction of *E. coli* O157:H7 by 3.81 log CFU/ml was reported after cider was treated with UV light (Wright et al., 2000). Later in 2004, Donahue et al. demonstrated inactivation of *E. coli* O157:H7 in unpasteurized apple cider by 8777 μW-s/cm^2 UV (at 254.7 nm) per pass through the system. The treatment was shown to be effective in reducing bacteria in inoculated apple cider by 2.20 logs per pass, and multiple passes could result in higher log reduction (Donahue et al., 2004). On poultry skin, *S.* Typhimurium was eliminated by UV treatment (Sumner et al., 1996). *S.* Typhimurium at ~7 X 10^5 CFU on the surface of poultry skin was reduced by 80.5% when treated with UV light at 2,000 μW-s/cm^2. On pork muscle and skin, UV treatment could effectively reduce *S.* Senftenberg and *E. coli* (Wong et al., 1998). For fresh pork muscle, after 1920 s exposure, a 1.5 log reduction at ≥100 mW/cm^2 for *E. coli* and 2.0 log reduction at ≥80 mW/cm^2 for *S.* Senftenberg were observed. For pork skin, 1.6 log reduction for *S.* Senftenberg was observed after treated with UV at 100 mW/cm^2. *E. coli* on pork skin was reduced by 4.6 logs when UV intensity was increased to 1000 mW/cm^2. Kim et al. (2002) showed that UV intensity of 500 mW/cm^2 was able to completely destroy *E. coli* O157:H7 on stainless steel after 3 min. Under the same treatment conditions, *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium on chicken meat with or without skin were reduced by 0.36 to 1.28 logs (Kim et al., 2000). *Campylobacter jejuni* was inactivated by UV irradiation at 32.9 mW/s per cm^2 on broiler meat, skin, and carcasses with

bacterial reductions of 0.7, 0.8, and 0.4 log, respectively, without any significant changes in sensory quality of products (visual appearance, odor, and fatty acid composition) (Isohanni and Lyhs, 2009). Wells et al. (2010) revealed that UV exposure of eggshells for 8 min yielded significant bacterial reduction of 2 log CFU/egg without excessive egg heating. They also showed that the combination of 1.5% H_2O_2 and UV for 8 min could reduce bacterial counts by up to 3 log CFU/egg (Wells et al., 2010). Due to its germicidal efficacy, ease of installation, and cost effectiveness, UV irradiation seems to be an appealing non-thermal, chemical-free alternative for pathogen inactivation in foods and food processing environment, but only for surfaces and has low penetration ability.

Pulsed-UV light is a novel UV technology for non-thermal pathogen inactivation on the food surfaces within a short time period. A pulsed-UV system produces a continual UV range below 400 nm, which is germicidal, with microsecond pulse duration by a xenon gas lamp (Dunn, 1996). In 1999, pulsed-UV light treatment was approved for food application by the US FDA (Keklik et al., 2010b). Several studies have demonstrated the application of pulsed-UV light as a foodborne pathogen inactivation tool. Ozer and Demirci (2006) showed that 1 log reduction of *E. coli* O157:H7 and *L. monocytogenes* Scott A in raw salmon was obtained after 60-s (3 pulses/s) pulsed-UV light treatment at an 8-cm distance from the quartz window, in the pulsed-UV light chamber (5.6 J/cm^2 per pulse on the strobe surface) with no change in product quality. Similar studies using the same pulsed-UV treatment conditions in blueberries for bacterial inactivation was conducted by Bialka and Demirci (2007), where *S. enterica* and *E. coli* O157:H7 levels were reduced by up to 4.3 and 2.9 log CFU/g blueberries, respectively, after a 60-s treatment (Bialka and Demirci, 2007). Complete inactivation of *S. aureus* was obtained in milk after pulsed-UV light treatment with an 8-cm distance from the quartz window in a single

pass at a 20-mL/min flow rate or with an 11-cm distance in 2 passes at the same flow rate (Krishnamurthy et al., 2007). Recently, *S.* Typhimurium was reportedly reduced by 1.2 log CFU/cm^2 after a 5-s/13-cm and 2.4 log CFU/cm^2 after a 60-s/5-cm distance from the quartz window of pulsed-UV light treatment in unpackaged boneless chicken breast samples (Keklik et al., 2010b). Comparable or less effect was obtained when the same treatments were used in vacuum-packaged samples. The researchers suggested that the optimum treatment conditions were 15-s/5-cm for unpackaged boneless chicken breast and 30-s/5-cm for vacuum-packaged samples, with ~2 log reduction of *S.* Typhimurium (Keklik et al., 2010b). Examples of inactivation of *Salmonella* spp. in culture media and food products are presented in Table 1.4.

High Intensity Ultrasound

Ultrasound is one of the novel techniques that have caught the attention of the food industry. While low intensity ultrasound has been employed for biomedical purposes as a therapeutic, operative, and diagnostic tool (Rubin et al., 2001), higher intensity ultrasound has become more common for use in equipment cleaning (especially in laboratory and medical area), compound (such as essential oils) extraction, emulsification, liquid degassing, homogenization, crystallization, dewatering, low temperature pasteurization, defoaming, activation and inactivation of enzymes, particle size reduction and viscosity alteration (Patist and Bates, 2008). High intensity ultrasound (HIU) treatment (10-1000 W·cm^{-2} and 20-100 kHz in frequency range) seems to be an attractive option for microbial inactivation in liquid foods due to its low cost and feasibility for industrial use, while maintaining the sensory and nutritional attributes of food for consumer acceptability (McClements, 1995; Mason, 1998; Villamiel et al., 1999). HIU effect on microbial cell destruction depends on the transmission of sound waves at varying frequencies

causing vibration throughout the medium (Sala et al., 1995; Su et al., 2010). The displacement of particles in the medium then occurs, creating extremely rapid formation and collapse of bubbles due to expansion and compression of medium, called cavitation (Earnshaw 1998). The HIU frequency, medium viscosity, temperature and pressure play important roles in the cavitation phenomena (Betts et al., 1999; Piyasena et al., 2003; Suslick, 1988). The mechanisms of microbial killing are mainly due to localized changes in pressure and temperature caused by cavitation, resulting in cell membrane disruption and thinning, shear-induced breakdown of cell walls, enzyme inactivation, biocomponent separation, and DNA damage via production of free radicals in bacterial cells (Butz and Tauscher, 2002; Fellows, 2000; Seymour et al., 2002; Earnshaw et al., 1995; Lillard, 1994; Sala et al., 1995; Su et al., 2010).

HIU has been continually researched for its bactericidal effect in food applications. Liquid foods, including milk and fruit juices, are primarily selected to use as food models due to the ease to implement HIU technology into the process (Yuan et al., 2009; Ferrante et al., 2007; Bermúdez-Aguirre et al., 2008; Cheng et al., 2007). D'Amico et al. (2006) reported that HIU treatment at 150 W power, 118 W/cm^2 intensity and 20 kHz frequency for 18 min could reduce aerobic microbial levels in raw milk by 5 logs, *L. monocytogenes* levels inoculated in UHT pasteurized milk by 5 logs, and *E. coli* O157:H7 in apple cider by 6 logs when mild heat (57°C) was used in combination. Although shorter exposure of HIU alone in foods without incorporation of heat treatment could result in bacterial reduction, less inactivation effect was obtained. Effect of HIU along with heat on *L. innocua* inactivation was also determined in milk with 4 butterfat contents (Bermúdez-Aguirre et al., 2008). HIU at 400 W and 24 kHz was found to be effective in killing *L. innocua* in milk when system was run at 63 °C for 30 min without causing degradation of protein content or color variation of the product. These researchers

reported that butterfat in milk can be sonoprotective to microorganisms as the rate of inactivation decreases with increasing fat content. With these HIU tested conditions, 2.5 log reduction of *L. innocua* could be achieved when used in whole milk, while ~5.0 log reduction was reached in skim milk (Bermúdez-Aguirre et al., 2008). Later in 2009, Bermúdez-Aguirre et al. (2009) continued to investigate the bactericidal effect using similar HIU settings, except increasing the power from 400 W to 600 W, and more than 5 log reduction of *L. innocua* was obtained in full-fat whole milk when the HIU power was raised (Bermúdez-Aguirre et al., 2009). Lee et al. (2003) reported a1 to 2 log reduction of *E. coli* after HIU treatment in spiked liquid whole egg (LWE) at 5°C with 20-kHz HIU for 5 min. Inactivation of *S. enterica* serovars by HIU has also been studied. Wrigley and Llorca (1992) demonstrated that indirect HIU treatment at 20, 40 and 50°C for 15 and 30 min could inactivate *S.* Typhimurium. LWE inoculated with *S.* Typhimurium was treated with HIU for 30 min at 50°C and found to decrease by 1 to 3 logs after treatment. *S.* Enteritidis in LWE was shown to be reduced by 0.65 log when samples were treated with 40-W ultrasound for 5 min at 55°C (Huang et al., 2006). Summary of salmonellae inactivation using HIU processing is presented in Table 1.5. Many studies have shown that bacterial spores are more resistant to HIU treatment than vegetative bacterial cells, and Gram-positive bacteria are more resistant than Gram-negative bacteria (Barbosa- Cánovas and Rodriguez, 2002; Raso et al., 1998; Earnshaw, 1998).

Although HIU is shown to be promising for microbial control in the food industry, it currently poses some limitations. High intensities and/or long exposure time may be required to completely inactivate microorganisms; however, higher doses and longer exposure of HIU treatments could result in alterations of functional and nutritional properties of foods which could be undesirable. Therefore, appropriate levels of HIU treatment are needed to balance the

advantages and disadvantages of this processing technology. Hurdle approaches using HIU in combination with other antimicrobial compounds (such as bacteriocins and organic acids) and/or processing methods (such as heat treatment and pressure processing) could enable better usage of HIU technology.

Natural Antimicrobials

With the growth of consumers' demand for natural and minimally processed foods, biopreservatives derived from nature have extensively been researched for their antimicrobial properties and application in food systems. Natural antimicrobials can be widely found in the environment, which their origins include animals, plants, and microbes (Stopforth et al., 2005). Examples of natural antimicrobials and their sources are demonstrated in Table 1.6.

Animal Origin Antimicrobials

Animal origin antimicrobial agents generally evolved as host defense mechanisms, and typically are in the form of polypeptides (Stopforth et al., 2005; Tiwari et al., 2009). Many animal-derived compounds are immune factors and antimicrobials produced and transferred from the mother to the offspring (unborn or newborn) (Floris et al., 2003). These antimicrobial agents can be isolated from animal products, such as lactoperoxidase, lactoferrin, lactoferricin B and lactoglobulins from milk, and lysozyme, ovotransferrin, ovoglobulin and avidin from eggs (Vigil et al., 2005).

Antimicrobial peptides (AMPs) can be categorized into 4 major groups, cationic peptides, anionic peptides, aromatic dipeptides, and peptides derived from oxygen-binding proteins (Vizioli and Salzet, 2002). Cationic peptides are the most common type of AMPs isolated from animals, especially insects, with stronger antimicrobial properties when compared to other

structural groups of AMPs (Bulet et al., 1999; Vizioli and Salzet, 2002). Inhibitory effects of cationic AMPs are commonly caused by the interaction of AMPs with microbial plasma membrane resulting in destabilization and increase permeabilization of the membrane. Due to the positive charge of AMPs, they electrostatically interact with the negatively charged elements of microbial membrane such as phospholipid composition, sterol content, or other polyanions (Andreu and Rivas, 1999; Floris et al., 2003; Zasloff, 2002). Studies have shown that the mechanisms of membrane damage caused by AMPs include the generation of oxidation products, blocking of receptor-ligand interactions, iron deprivation, and antibody-mediated mechanisms (e.g., complement activation, agglutination, opsonization, adherence-blocking, or neutralization) (Stopforth et al., 2005). Other mechanisms proposed include inhibition of specific membrane protein synthesis, synthesis of stress proteins, interaction with DNA or interference of DNA synthesis, production of hydrogen peroxide, triggering self-destructive mechanisms (e.g., autolysis in bacteria), alteration of cytoplasmic membrane septum formation, inhibition of cell-wall synthesis, or interference of microbial enzyme activity (Andreu and Rivas, 1999; Brogden, 2005). While several studies have investigated and proposed the modes of action of cationic AMPs, research in this area of other AMPs is not well established.

Lactoperoxidase was tested to effectively inactivate *L. monocytogenes* in dairy products (Boussouel et al., 2000; Kangumba et al., 1997; Rodriguez et al., 1997). Marks et al. (2001) reported that lactoperoxidase could still actively act against *P. aeruginosa*, *S. aureus* and *Streptococcus thermophilus* in milk even after pasteurization at 72°C for 15 s. *P. fluorescens* levels were shown to reduce by 1.69 and 1.85 logs at 4 and 8°C, respectively, in lactoperoxidase-activated goat milk within 24 h (Zapico et al., 1995). And in the same study, a 2 d lag phase of *E. coli* in lactoperoxidase-activated goat milk at 8°C was observed, resulting in lower counts than

the control milk. Similarly, only a bacteriostatic effect against *E. coli* was obtained in lactoperoxidase-activated goat milk when stored at 30°C (Seifu et al., 2004). *L. monocytogenes* and *S. aureus* inactivation effects by lactoperoxidase and its combinations with other preservatives in cuajada (curdled milk) were also reported (Arqués et al., 2008). UHT pasteurized cuajada samples with lactoperoxidase, nisin, reuterin, or their combinations were inoculated with 4 log CFU/ml of each pathogen, and stored at 10°C. After 3 day storage, *L.monocytogenes* number in lactoperoxidase system was lower than in control by 4 logs, and a lower number by 8 logs in cuajada with lactoperoxidase, nisin and reuterin combination. For *S. aureus*, only 1 log lower counts were obtained after 3 day storage when compared to the control. Lactoperoxidase, nisin and reuterin combination showed improved anti-bacterial activity against *S. aureus* of at least >3 log lower counts comparing to non-preservative added sample after storing for 3 days and up to 12 days. Lactoferricin B is bactericidal and lactoferricin H is bacteriostatic against a wide variety of Gram-negative, including *E. coli, Klebsiella pneumoniae, Proteus vulgaris, P. aeruginosa, P. fluorescens, S.* Enteritidis, *S.* Montevideo, *S.* Salford, *S.* Typhimurium, and *Y. enterocolitica*, and Gram-positive bacteria, including *Bacillus cereus, B. circulans, B. natto, B. subtilis, Clostridium paraputrificum, C. perfringens, Corynebacterium ammoniagenes, C. diphtheria, C. renal, E. faecalis, Lactobacillus casei, L. monocytogenes, S. aureus, S. epidermidis, S. haemolyticus, S. hominus, Streptococcus bovis S. cremoris, S. lactis, S. mutans*, and *S. thermophilus* (Gifford et al., 2005; Yamauchi et al., 1993). In 2001, Masschalck et al. reported that lactoferrin at 500 µg/ml could reduce the populations of *S. sonnei*, *P. fluorescens* and *S.* Typhimurium, while lactoferricin and lactoferrin hydrolysate treatments resulted in 1 to >2 log reduction of *E. coli*, *S.* Enteritidis, *S.* Typhimurium, *S. sonnei*, *S. flexneri*, and *P. fluorescens*. Recently, López-Expósito et al. (2008) determined the concentration of

lactoferrin and lactoferricin B against *E. coli*, and *S.* Choleraesuis. They reported that 0.075 and 1.25 µM of lactoferrin was required to give a log (N_0/N_f) value at least 0.25. For lactoferricin B, 0.0125 µM was shown to be sufficient to exhibit the similar reduction in *E. coli*.

Another animal origin antimicrobial compound which has been researched continuously is lysozyme. Poultry eggs and milk are typical sources for lysozyme isolation (Hugkey and Johnson, 1987). Lysozyme has been shown to have antimicrobial activity against several foodborne bacteria, such as *L. monocytogenes*, *C. botulinum*, *C. tyrobutyricum*, *C. thermosaccharolyticum*, *B. stearothermophilus*, *B. cereus*, *C. jejuni*, *Y. enterocolitica*, *E. coli*, *E. coli* O157:H7, *P. vulgaris*, *P. aeruginosa*, and *S.* Enteritidis (Hugkey and Johnson, 1987; Chander et al., 1984; Branen and Davidson, 2004; Naknukool et al., 2009; Cegielska-Radziejewska et al., 2008). Naknukool et al. (2009) reported that duck lysozyme at 0.1 mg/ml is effective in reducing *S.* Enteritidis population (initial population at 10^5 CFU/ml) after 1 h incubation at 30°C, which >1 log (N_0/N_f) value was achieved. Their results also indicated that antibacterial activity against *S.* Enteritidis could be enhanced with reduced lysozyme from both chicken and duck eggs when compared to their native forms. At least 1 log (N_0/N_f) higher values were obtained when *S.* Enteritidis was treated with reduced lysozyme from chicken and duck eggs (Naknukool et al., 2009). Hughey and Johnson (1987) reported that *C. tyrobutyricum*, *C. thermosaccharolyticum*, and *B. stearothermophilus* were completely inhibited by lysozyme hydrochloride treatment at 20 or 200 mg/l in complex media. Lysozyme was also investigated for its application in food samples. Lysozyme at 12 to 24 x 10^3 U/ml was able to extend shelf-life of cut-up poultry at 4°C for 48 to 72 h (Kijowski et al., 2002). However, at the microbial inhibitory activity of lysozyme at this concentration and treatment condition, lysozyme did not reduce the growth of *Salmonella* in this study. Similarly, *S.* Typhimurium was shown to be

insensitive to lysozyme treatment in the study by Nakimbugwe et al. (2006). Generally, Gram-positive bacteria were found to be more sensitive to lysozyme treatment than Gram-negative bacteria due to additional protective barrier of Gram-negative bacterial inner membrane compositions (proteins, phospholipids and lipopolysaccharides) (Cegielska-Radziejewska et al., 2008). However, some staphylococcal bacteria can completely resist antibacterial effect of lysozyme. It has been suggested that the peptidoglycan-specific O-acetyltransferase and OatA protein (intergral membrane protein) are responsible for the resistance of these bacteria to lysozyme (Bera et al., 2005).

Although many of antimicrobial substances derived from animal sources are GRAS, caution is needed for their consumption in people with food allergy as they may cause adverse health issues. Other challenges of animal origin antimicrobial application in food systems include high concentration potentially required in foods to achieve the desired microbial inhibitory effects, and cost of antimicrobial isolation and purification.

Plant Origin Antimicrobials

Plant essential oils (PEOs) are volatile aromatic compounds formed as secondary metabolites by plants which can be obtained from various parts of plant materials, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, via a variety of processes. Steam/hydro-distillation is the most commonly used for essential oil production; super critical carbon dioxide, microwaves, pressure distillation, expression, fermentation, enfleurage or extraction can also be employed to obtain essential oils (Van de Braak and Leijten, 1999; Bakkali et al., 2008). PEOs are produced by plants as a natural defensive mechanism against plant bacteria, viruses, fungi, insects, herbivores and undesirable others (Bakkali et al., 2008). PEOs comprise various individual components, which mainly are alcohols, aldehydes, esters,

ethers, ketones, phenols, and terpenes (Ouattara, 1997; Bowles, 2003; Pichersky et al., 2006). Due to their aromatic properties, PEOs have been used in cosmetics for their fragrances and as flavoring agents in the food and beverage industry. Additionally, PEOs are also important in the pharmaceutical and medicinal fields. Some PEOs or their components are well recognized for their functional properties and use as natural remedies and therapeutics (Bauer et al., 2001; Hussain et al., 2011; Iranshahy and Iranshahi, 2011; Momtaz and Abdollahi, 2010). Besides, it has also been known that some PEOs have properties in controlling microorganisms and pests (Daferera et al., 2003; Elgayyar et al., 2001; Tassou et al., 2000; Grande et al., 2007; Sinigaglia et al., 2008; Viuda-Martos et al., 2011). PEOs have been explored for their antimicrobial function along with their potential application in the food, agricultural and marine production system. Various PEOs derived from plants used as herbs, spices or infusions in foods have been studied for their inhibitory properties against important foodborne pathogens and food spoilage microorganisms. Oregano, thyme, clove, basil, cinnamon, geranium, lemon, lime, orange rosemary and coriander oils have shown inhibitory activity against *E. coli, E. coli* O157:H7, *Salmonella* spp., *S. aureus, L. monocytogenes, Y. enterocolitica, L. plantarum, P. aeruginosa, K. pneumoniae, B, subtilis, E. feacalis, P. vulgaris, S. cerevisiae, Aspergillus niger, Candida albicans, Geotrichum candidum* and *Rhodothorula* (Prabuseenivasan et al., 2006; Elgayyar et al., 2001; Prudent et al., 1995; Hammer et al., 1999; Burt and Reinders, 2003; Cosentino et al., 1999). Some minor/trace components of PEOs such as phenolics and terpenoids appear to be the major active compounds playing a significant role in antimicrobial activity as well as possible combined effect (Marino et al., 2001; Davidson and Naidu, 2000; Burt, 2004). PEOs containing a high percentage of components, such as eugenol, thymol, carvacrol, cinnamaldehyde, and linalool, were shown to effectively limit growth of a variety of microorganisms, including

Shigellae sp., *E. coli*, *L. monocytogenes*, *B.acillus cereus*, and *S. aureus* (Bagamboula et al., 2004; Delgado et al., 2004; Ettayebi et al., 2000; Ultee et al., 2000; Karatzas et al., 2001; Vrinda Menon and Garg, 2001; Gill and Holley, 2004 and 2006; Lis-Balchin and Deans, 1997; Lis-Balchin et al., 1998).

PEOs are complex mixtures which can comprise more than 60 individual components with different concentrations (Bakkali et al., 2008; Russo et al., 1998). Phenolic compounds, quinones, alkaloids, flavanols/flavonoids and lectins are known to mainly contribute to the antimicrobial efficacy of PEOs (Gupta and Abu-Ghannam, 2012). The chemical composition of PEOs normally defines their biological and functional properties. As mentioned above, several methods can be employed for PEO extraction. Method selected for PEO production can affect the chemical composition of extracts thus consequently leading to different sensory and functional properties (including solubility and antimicrobial activity) when different extraction methods are used (Corbo et al., 2009). Therefore, the PEO extraction techniques need to be appropriately selected for specific use to control particular microorganism(s) in particular food. As the sufficient level of PEOs/PEO components is required to have adequate interaction with target microorganisms for the inactivation, the concentration and solubility of compounds in the food systems are crucial. Too high concentration of PEOs could have an adverse effect on the sensory properties of foods, which could limit their application in foods when being used alone.

Antimicrobial activity and modes of action of PEOs and PEO components against bacterial organisms along with the potential application in food system have widely been explored. Several mechanisms of their inhibitory effects against bacteria have been proposed, including interference with intracellular pH gradient (ΔpH), intracellular ATP and proton motive force (PMF). These actions can cause leakage of specific ions, alteration in nucleic acids and

amino acids, structural and functional damage of cell membrane, disruption of metabolic system, and inhibition of synthesis of essential elements (Kreydiyyeh et al., 2000; Gill and Holley, 2004; Evans and Martin 2000). Phenolic compounds in PEOs are suggested to be responsible for the antimicrobial activity against various types of organisms by inhibiting DNA, RNA, protein, lipid and polysaccharide synthesis, and inhibiting the respiratory chain, electron transfer, substrate oxidation and active transport system (Nes and Eklund, 1983; Denyer, 1990; Nychas, 1995). Moreover, the interaction of phenolic compositions with enzymes located on bacterial cell wall is also found to be another mechanism of microbial inhibition by PEOs (Farag et al., 1989; Wendakoon and Sakaguchi, 1995; Kreydiyyeh et al., 2000).

Various studies have evaluated the antimicrobial activity of PEOs and their components against salmonellae. In 2010, Gündüz et al. (2010) applied oregano oil onto tomatoes to investigate the antimicrobial properties against nalidixic acid resistant *S.* Typhimurium. Oregano oil at 100 ppm successfully reduced the tested *Salmonella* population by 2.78 logs in tomato (Gündüz et al., 2010). Anti-salmonellae effect of carvacrol was tested against *S.* Enteritidis (5×10^3 CFU) on $10\times10\times5$ mm^3 raw chicken (Burt et al., 2007). A minimum concentration of carvacrol at 20% v/v in ethanol was required to show significantly reductions of viable *S.* Enteritidis at 4, 20 and 37°C. And carvacrol vapor at 40% v/v resulted in a complete elimination of all viable cells after at least 3 h treatment at 37°C. PEOs have also been experimented in combination with other antimicrobial agents for possible enhanced antimicrobial effect. Govaris et al. (2010) investigated the efficacy of oregano oil for *S.* Enteritidis inhibition in minced sheep meat. Also, nisin and oregano oil with nisin combination were tested for their anti-salmonellae efficiency. Application of oregano oil at 0.6 and 0.9% resulted in constant numbers of <3.0 and <1.0 log CFU/g of *S.* Enteritidis survivors, respectively, during 12 d refrigerated storage. When

0.9% oregano oil was applied in combination with nisin at 500 and 1000 IU/g, *S.* Enteritidis population in sheep meat was completely inhibited after 2 d storage at refrigeration temperature. The inhibitory effect found in this experiment was higher at 10°C storage when compared to at 4°C.

Microbial Origin Antimicrobials

Microorganisms also produce wide range of compounds which exhibit antimicrobial properties against foodborne spoilages and pathogens. These substances are usually produced by microorganisms to promote their survival and proliferation by limiting growth of other microbial strains. Compounds such as bacteriocins, metabolites from fermentation processes, as well as other antagonistic substances can be isolated from microorganisms and have been reported for their application as biopreservatives (Tiwari et al., 2009).

One of the most important groups of microorganisms yielding biopreservatives for food application is lactic acid bacteria (LAB). LAB have been employed for shelf-life extension of foods, which are more shelf stable, such as cheese, sausage, and sauerkraut (Smid and Gorris, 1999). LAB produce acids, which can lower the pH of foods and act as natural antimicrobials, from their fermentation process. Although high acidity is effective in inhibiting growth of several microorganisms, in often cases, high amount of acids may not be desirable in food products as the sensory quality of food could be altered and may be unacceptable. Bacteriocins are AMPs produced by bacteria, including LAB, to inhibit the closely related bacterial strains (Cleveland et al., 2001). Various Gram-positive bacteria, including bacterial spores, were shown to be sensitive to bacteriocin treatment. As bacteriocins have no/minimal effect on sensory properties of foods, LAB which can produce minimum amount of acids while yielding sufficient amount of bacteriocins to enhance food safety are very much useful for the food application (Smid and

Gorris, 1999). Bacteriocins can be classified into 3 groups, Class I: lantibiotics, a small (<5 kDa) peptides containing lanthionine and β-methyl lanthionine (such as nisin and mersacidin), Class II: small, heat-stable, non-modified peptides (such as pediocin PA-1, leucocin A, carnobacteriocins, lactacin F, plantaricin EF and JK, and lactococcins G and F), and Class III: large, heat-sensitive molecules (such as helveticins J and V-1829, acidophilucin A, and lactacins A and B) (Klaenhammer, 1993; Hoover and Chen, 2005). Another group of bacteriocins, which are complex molecules with lipid and carbohydrate moieties, is sometimes included in the bacteriocin classifications and known as Class IV (Papagianni and Anastasiadou, 2009).

Nisin, which belongs to bacteriocin Class I, was first discovered in 1928 (Hurst, 1967). It is naturally produced by *Lactococcus lactis*, a bacterial dairy starter culture, as a primary metabolite with a positively charged peptide of 34 amino acids by ribosomal transcription and translation processes (Thomas and Delves-Broughton, 2005). It is recognized as GRAS substance by the US FDA as stated under the Code of Federal Regulations section 184.1538 (Millette et al., 2007). Due to its antimicrobial activity against a broad spectrum of bacteria, nisin is widely used in various food products such as processed and hard cheeses, desserts, milk, yoghurt, cottage cheese, fermented beverages, meat products, fish and canned vegetables (Holzapfel et al., 1995). It has been shown that nisin has no significant taste and cannot be detected even at 200 mg/l in mineral water (Thomas and Delves-Broughton, 2005). This makes nisin favorable for use in foods. Nisin has been known for its antimicrobial against Gram-positive bacteria, but not Gram-negative bacteria under normal conditions. As the site of action is the cytoplasmic membrane, the resistance of Gram-negative bacteria is due to the outer membrane containing lipopolysaccharide that acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander et al., 1997). However, by altering

and/or degrading the Gram-negative bacterial cell envelope, such as combining with chelating agents, nisin exhibits bactericidal effects towards Gram-negative bacteria including *Salmonella* spp., *S. flexneri,* and *E. coli* (Stevens et al., 1991). Nisin can bind to the fatty acyl proteoglycan anchor in the bacterial membrane with high affinity, and diffuse into the surrounding membrane (Brötz et al., 1998). It subsequently causes alteration of the cell membrane of organisms resulting in leakage of low molecular weight cytoplasmic components and the destruction of PMF (Bruno et al., 1992; Driessen et al., 1995).

Nisin has been used for controlling growth of vegetative cells as well as spores of Gram-positive bacteria. Although nisin has a strong bactericidal effect against vegetative cells, a bacteriostatic effect is typically obtained or higher concentration of nisin is required when used against spores (Thomas and Delves-Broughton, 2005). *L. monocytogenes* can be inhibited by nisin in cottage cheese and ricotta cheese (Ferreira and Lund, 1996; Davies et al., 1997). After 7 day storage at 20°C, a 1000-fold reduction of *L. monocytogenes* populations was achieved in cottage cheese spiked with 10^4 CFU/g when 2000 IU/g of nisin was added, comparing to a 10-fold decrease obtained in control sample (Ferreira and Lund, 1996). In ricotta cheese, 10^2-10^3 CFU/ml of *L. monocytogenes* was inhibited up to 55 day storage at 6-8°C in nisin added samples at a concentration of 2.5 mg/l (Davies et al., 1997). Branen and Davidson (2004) showed that nisin at 7.8 µg/ml provides a bactericidal effect against *L. monocytogenes* Scott A and 19115 strains in pure culture. *B. sporothermodurans,* which is a heat-resistant sporeforming bacteria, at 10^4 CFU/ml was reportedly controlled for at least 7 days when treated with nisin at 0.125 µg/ml at 37°C (Thomas and Delves-Broughton, 2001). Choi and Park (2000) reported that 100 IU/ml of nisin was sufficient to inactivate lactobacilli in kimchi. While for bacterial spores, 4000 IU/ml of nisin was needed to be effective against *B. cereus* spores in skim milk (Wandling et al., 1999).

As nisin is effective at pH 3.5-8.0, it can be used in liquid eggs whose pH range typically lies between 7.3 to 7.8 (Thomas and Delves-Broughton, 2005). Application of nisin in pasteurized liquid eggs (whole, yolk, and white) is intended to control bacterial spores and heat-resistant Gram-positive bacteria, which can survive the pasteurization. The nisin concentrations recommended for use in liquid eggs and their products, such as omelettes, scrambled eggs, and pancake mixes, are between 2.5-5 mg/l (Delves-Broughton, 2005). Levels of nisin typically used in foods are presented in Table 1.7.

Ethylenediaminetetraacetic acid (EDTA) is a chelator used in foods to prevent food deterioration cause by reactions catalyzed by metal ions, including oxidation reaction (Jacobsen et al., 2001; Let et al., 2003; Nielsen et al., 2004). EDTA also exhibits antimicrobial activity and can enhance microbial inhibitory effects of antimicrobials, especially against Gram-negative bacteria (Branen and Davidson, 2004). Studies have shown that improved antimicrobial effect of nisin could be achieved by incorporation of EDTA (Stevens et al., 1991; Branen and Davidson, 2004). Branen and Davidson (2004) reported that nisin could effectively inhibit the tested Gram-negative bacteria, including *E. coli* O157:H7, *E. coli* O104:H21, and *P. fluorescens* ATCC 13525, with minimum inhibition concentrations of nisin + EDTA at 31.3 + 313 or 7.8 + 625, 31.3 + 313 or 7.8 + 1250, and 46.9 + 2500 µg/ml, respectively, while >46.9 µg/ml of nisin or 1250-5000 µg/ml of EDTA was needed to achieve similar inhibition when used alone. For *S.* Enteritidis, 46.9 µg/ml of nisin in combination with 1250 µg/ml of EDTA, and 46.9 µg/ml of nisin in combination with 2500 µg/ml of EDTA were shown to be adequate to obtain bactericidal effect in *S.* Enteritidis 13076 and *S.* Enteritidis Φ01, respectively (Branen and Davidson, 2004).

Pediocin is a bacteriocin produced by LAB, such as *Pediococcus* spp. (*P. acidilactici, P. pentosaceus, P. parvulus* and *P. damnosus*), *L. plantarum* and *B. coagulans* (Devi and Halami,

2011; Papagianni and Anastasiadou, 2009). It belongs to Class II bacteriocins (subgroup IIa) which exhibit a very strong antilisterial activity, as well as activity against other Gram-positive pathogenic bacteria, such as *Clostridium* spp., and *Enterococcus* spp. (Rodríguez et al., 2002; Papagianni and Anastasiadou, 2009). It has already been commercialized as a biopreservative used in the food industry. Pediocin PA-1 is one of the most common pediocins, which has been researched for its antimicrobial activity and for food applications. Similar to nisin and other bacteriocins, pediocin PA-1 primarily targets Gram-positive bacteria. It reportedly attacks inner membrane of bacteria causing rapid collapse of bacterial membrane potential and PMF, loss of protons, and inhibition of glucose transport (Ray and Miller, 2000). The antilisterial effect of pediocin PA-1 against *L. monocytogenes* in cottage cheese, half-and-half cream and cheese sauce has also been reported (Pucci et al., 1988). Samples with 100 AU/ml of pediocin showed 3 and 4-5 log CFU/ml of *L. monocytogenes* lower in half-and-half cream and cheese sauce, respectively, than controls after samples were stored at 4°C for 7 to 14 days. In cottage cheese, addition of 50-100 AU/g of pediocin resulted in at least 1 log reduction of *L. monocytogenes* after 1 day at refrigeration temperature. Altuntas et al. (2010) showed that pediocin isolated from *P. acidilactici* 13 has strong antimicrobial activity against *L. monocytogenes* at 37°C with optimal pH at 6.0. Other than antilisterial activity, pediocin was also found to have activity against other Gram-positive bacteria such as *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Staphylococcus, Enterococcus,* and *Clostridium* (Piva and Headon, 1994; Klaenhammer et al., 1988). Pediocin-producing *Pediococcus* species were effectively used as starter cultures in fermented sausage for *L. monocytogenes* control (Berry et al., 1990; Foegeding et al., 1992). *L. monocytogenes* populations (initial count of 10^6 CFU/g) were decreased by 2 logs in sample with *Pediococcus* added, compared to 1 log reduction in control (Berry et al.,

1990). Although pediocin does not show antimicrobial activity against Gram-negative bacteria, it has been shown that stressed/injured Gram-negative strains, such as *Salmonella* sp., *E. coli*, *Serratia* sp., and *Pseudomanas* sp. could become susceptible to this bacteriocin (Ray and Miller, 2000).

Hurdle Technologies

Although non-thermal inactivation processing or use of natural preservatives can overcome adverse effects caused by heat treatment, other drawbacks may be present. To reach a sufficient level of microbial inactivation by any single inactivation approach, high treatment doses may be required which can result in undesirable product attributes such as changes in physical and functional properties of food due to treatment with mechanical forces or pressures, flavor alteration from addition of PEOs, and limitation of automation or continuous processing. As each method has its own advantages and limitations as presented in Table 1.8, using hurdle approach by combining two or more processing technologies can simultaneously enhance microbial control efficiency while overcoming the drawbacks of one particular method when used alone. The antimicrobial effect obtained using hurdle approach can be from a combination of effects from each method, with also possible synergistic effects with intelligent use. However, a careful method selection is needed as antagonistic effect might be obtained with inappropriate choice of hurdles.

Non-thermal processing may be combined with other non-thermal processes, mild heat treatment (which does not unfavorably affect the products), additives, and/or processing factors (e.g., water activity, pH, and acidity). The criteria for hurdle selection depend on type of foods, target microorganism(s), as well as modes of action of each method choice (Gupta and Abu-

Ghannam, 2012). Synergistic effects can be accomplished by multi-target hurdles; thus, understanding the mechanisms of microbial inhibition/inactivation by each technique is crucial (Ross et al., 2003). Applications of many microbial inactivation processes have been explored when combined with other processing factors, such as thermosonication (heat + ultrasound), manosonication (pressure + ultrasound), manothermosonication (pressure + heat + ultrasound), UV + high intensity pulsed light, manothermosonication + PEF, heat +PEF, HPP + heat, heat + irradiation, UV + antimicrobials, HPP + antimicrobials, and PEF + antimicrobials (Knorr et al., 2002; Palgan et al., 2011; Alvarez et al., 2006; Aronsson and Rönner, 2001; Ohshima et al., 2002; Bazhal et al., 2006; Sommers et al., 2010; Hermawan et al., 2004; Lee and Kaletunç, 2010; Viedma et al., 2008).

Alvarez et al. (2006) conducted a study on combined process inactivation of *Salmonella* serovars using heat treatment (55 and 57°C) and gamma irradiation (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kGy). Radiation at as low as 0.1 kGy prior to heat treatments resulted in synergistical reduction of the $D_{55°C}$ and $D_{57°C}$ values of *S.* Senftenberg, *S.* Typhimurium, and *S.* Enteritidis by 3.6- and 2.5-fold, 2- and 1.4-fold, and 2- and 1.6-fold, respectively. Heating time required was decreased by 86 and 30% at 55 and 57°C, respectively, with samples previously irradiated. Combined effect of UV treatment (0.5 J/cm^2) and potassium lactate, lauric arginate ester and sodium diacetate was studied by Sommers et al. (2010). UV in combination with 3 tested antimicrobials resulted in 2.32 to 2.80 log reductions of *S.* Senftenberg, *S.* Typhimurium, *S.* Enteritidis, *L. monocytogenes* and *S. aureus* on frankfurter surface, which was more effective than when used individually. After 12-week storage at 10°C, 3.6 to 4.1 log reductions of tested pathogens were achieved when compared to the control (Sommers et al., 2010). When PEF (25kV/cm, 250 μs in pulses of 2.12 μs) was used in liquid whole egg (LWE) followed by heat

treatment at 55°C for 3.5 min, *S.* Enteritidis population was decreased by 4.3 log CFU/ml (Hermawan et al., 2004). This result shows significant promise for *S.* Enteritidis control as PEF treatment alone resulted in only 1 log of bacterial reduction. No changes in color, pH, viscosity or degree brix were observed when PEF and heat were used together, and the shelf-life of LWE was significantly increased. Lee and Kaletunç (2010) reported that HHP at 200 MPa or nisin at 200 IU/ml did not individually show any inhibitory effect against *S.* Enteritidis strains. Yet, HHP treatment at 500 MPa or a combined treatment of 200 IU/ml of nisin and HHP at 350-400 MPa were effective against *S.* Enteritidis, resulting in an 8 log reduction. This study demonstrated that although certain antimicrobials, such as nisin, do not affect Gram-negative bacteria due to bacterial outer membrane barrier, additional processing treatment can result in sublethally injured cells and assist in penetration of antimicrobial substance into cells causing inactivation.

Salmonella Inactivation: Conclusions and Perspectives

Processing technologies for microbial control in the food industry have been continuously advancing to enhance the safety of foods while maintaining the excellent quality of the products. Several non-thermal processes were successfully tested and implemented for microbial inactivation; however, many challenges still exist. Extensive studies on the mechanisms of inactivation by each non-thermal method are necessary for further process development and for hurdle selection in a multi-target approach. An intelligent combined process seems to be very promising for food preservation as milder treatments used in conjunctioncan result in effective microbial inactivation with minimal effect on sensory and functional quality of foods. Moreover, cost effective and convenient intervention strategies should be targeted for practical and sustainable food preservation.

Salmonella Detection

Culture-based detection has been used in the food industry for almost a century as a standard microbial diagnostic tool. Although high in sensitivity, it requires several days for completion, and is therefore time-consuming, labor-intensive, and cumbersome. Culture based detection also represents numerous difficulties, such as variability in interpretation of some biochemical or morphological tests, as well as the high cost associated not only with supplies and reagents, but labor as well (Tomás et al., 2009). To curb the release of food commodities contaminated with bacterial and viral pathogens in the market, improved rapid detection assays with high speed, specificity, and sensitivity are essential. Tremendous efforts have been devoted to develop novel detection technologies with these attributes that are also low in cost and labor requirements to enhance and ensure food safety.

In recent years, rapid detection assays, such as molecular techniques, immunoassays, and biosensors, have gained popularity and are being developed for use as routine monitoring and screening tools in the food industry. As these current detection methods have their own limitations, many researchers try to overcome their drawbacks by merging the advantages of several techniques to maximize the robustness of the newly developed detection assay. Moreover, automated systems have been implemented to improve the practical applications of detection assays for industrial use. Currently, most of the detection methods still require sample preparation and enrichment for the detection of the low number of microorganisms in foods, e.g., cell concentration or sample enrichment, and removal or minimizing the interference associated with the presence of inhibitors of assay detection, resulting in the extension of assay time. Faced with these challenges, emphasis is placed on improvement of detection sensitivity using simple,

economical, and user-friendly procedures along with appropriate sample concentration and sampling strategies. However, detection is dependent on the sample size and the sample being tested. Hence, adequate sample representation and concentration schemes are keys to improve the detection sensitivity of an assay to help facilitate sensitivity and speed of downstream detection.

Molecular assays which are based on the specific detection of nucleic acid of foodborne pathogens have gained popularity due to their speed and sensitivity of detection. These methods have been developed and optimized for improved robustness and reliability, as well as for routine detection in foods and the food processing environment. This chapter will focus on RNA-based detection methods for live pathogen detection with discussion on their advantages, disadvantages, and their current application status in foods.

Reverse-transcriptase PCR

The polymerase chain reaction (PCR) was first invented in 1983 by Kary Mullis (Mullis, 1990), and was described as a practical application for diagnosis in 1985 by Saiki et al. (Saiki et al., 1985). PCR is the most widely used oligonucleotide directed DNA amplification technique that targets and synthesizes specific DNA sequences, resulting in several fold increase in DNA copies (Kang et al., 2005). This technique is similar or analogous to a photocopy machine. It is one of the most popular and powerful detection tools studied and currently used in the food industry. The PCR reaction uses a thermostable DNA polymerase isolated from *Thermus aquaticus*, a thermophilic bacterium found in hot springs, so it can withstand the high temperatures associated with thermal PCR cycling. In addition, deoxyribonucleoside triphosphates (dNTPs), selected and specific forward and reverse primers (oligonucleotides) that selectively amplify only the target gene/nucleic acid, buffer, and magnesium chloride are

required for amplification of template DNA. Amplification relies on 3 temperature-dependent steps, denaturation of double stranded DNA (at around 90°C), annealing of primers to target sequence (dependent on primers and target sequence, can be between 50 to 65°C), and extension of DNA complementary strand by polymerase enzyme (at around 72°C), in a thermal cycler. These three steps are repeated about 30 to 40 times, depending on the length of the amplicon to achieve the desired copy number suitable for detection. Amplified products (amplicons) can be detected by using agarose gel electrophoresis after staining with dyes such as ethidium bromide and observing under ultraviolet light. Specific target amplicons are identified by size (sequence length) based on their mobility in the gel in comparison to a standard DNA marker.

To improve the robustness or reliability of nucleic acid amplification assays, including PCR, an internal amplification control (IAC) is recommended in every nucleic acid amplification reaction. In the amplification reaction without IAC, negative results obtained may represent either no target sequence or false negatives. The false negative results can be caused by the presence of inhibitors from the food matrix, machine malfunction, incorrect reaction mixture, degradation of reagents, or low enzyme activity (Hoorfar et al., 2003). False negative results may lead to severe consequences such as contaminated food products being released into the market. Therefore, an IAC is included in the reaction to ensure the presence of an IAC signal when the target is not present to eliminate the possibility of false negatives.

While traditional DNA-based PCR assays are able to sensitively and rapidly detect DNA targets, they cannot distinguish between viable and dead cells. In the food industry when pathogen inactivation measures are typically implemented during food manufacturing, DNA from pathogens can still be present and detected in foods although the cells are killed. Thus, the detection of DNA may lead to misinterpretation of results, when there is a need for the detection

of mainly infectious viable cells. In contrast, RNA has a shorter half-life than DNA, therefore, having greater potential of detecting the presence of viable cells or recent contamination (Maurer, 2006). In addition, the RNA-based amplification assays allow detection of foodborne RNA viruses, which cannot be detected by DNA-based PCR methods. Several researchers have reported on the use of reverse-transcriptase PCR (RT-PCR) targeting mRNA for the detection of viable foodborne organisms. Prior to regular PCR process, target RNA is reverse-transcribed into cDNA, by using the reverse transcriptase enzyme; where typically AMV-Reverse transcriptase is used. Then, the PCR steps of DNA amplification are carried out. It is crucial that only RNA be isolated from samples and that DNA carry-over be removed to avoid false positive results arising from DNA amplification. Burtscher and Wuertz (2003) demonstrated the RT-PCR assay for *Salmonella* spp., *L. monocytogenes*, *Y. enterocolitica*, and *S. aureus* detection in inoculated organic waste samples, with detection limits of <10 CFU/g in all tested strains after 20 to 24-h enrichment. Detection limits of 1 CFU/g Shiga-toxin-producing *E. coli* in ground meat after 12-h enrichment (McIngvale et al., 2002) and as few as 1 CFU of *E. coli* O157:H7 in pure culture (Liu et al., 2008) were reported using RT-PCR. Traditional RT-PCR has been typically used for the detection of foodborne RNA viruses such as hepatitis A virus in pure culture (Bhattacharya et al., 2004; Gúevremont et al., 2006), green onions (Gúevremont et al., 2006), spring water (Brassard et al., 2005), shellfish (Kingsley and Richards, 2001), and human noroviruses in pure culture (Gúevremont et al., 2006), green onions (Gúevremont et al., 2006), produce, and shellfish (Kingsley and Richards, 2001).

Considering the constraints associated with traditional RT-PCR, such as additional time to run the gel followed by confirmation, as well as possibility of cross-contamination, technology has advanced towards using RT-PCR in a real-time format. This allows the simultaneous

monitoring and detection of amplification by using fluorescence and detection directly as amplification progresses and target amplicons are formed. In these real-time reactions, either non-specific fluorescence dyes (such as SYBR Green I), or specific fluorescence probes (hydrolysis, hybridization, or scorpion probes) are incorporated in the reaction to provide the fluorescence signal associated with amplification. The earlier the fluorescence is detected along with increased signal indicates a larger amount of initial target DNA in the sample. This approach enables the quantification of target using threshold cycle (C_T), which is the number of PCR cycles that fluorescence is generated greater than the background signal, to estimate the initial number of template copies (Klein, 2002). Additional advantages of real-time RT-PCR over traditional RT-PCR assays are that the process does not involve the opening of reaction tubes, agarose gel electrophoresis, therefore, avoiding cross-contamination, with shortened total assay time as further confirmation by DNA hybridization, sequencing, or restriction digestion is not needed. Real-time RT-PCR using fluorescence dyes or TaqMan probes have also been developed for the detection of pathogenic bacteria, including *Salmonella* in pure culture and food matrices such as spinach, tomatoes, jalapeno and serrano peppers, lettuce, pork chop, pork sausage, pork carcass rinse, shell egg, liquid whole egg, water, and environmental samples (D'Souza et al., 2009; Gonsalez-Escalona et al., 2009; Miller et al., 2010a and in press; Techathuvanan et al., 2010a, in press a and b; Fey et al., 2004; Jacobsen and Holben, 2007; Day et al., 2009; Balaji et al., 2005). Real-time RT-PCR has also been reported for the detection of *E. coli*, including *E. coli* O157:H7 (Sheridan et al., 1998; Fitzmaurice et al., 2004; Matsuda et al., 2007; Liu et al., 2008) in pure culture, water samples and clinical samples, *Helicobacter pylori* (Rokbi et al., 2001), *Enterococcus faecalis* (Matsuda et al., 2007), *C. perfringens* (Matsuda et al.,

2007), *S. aureus* (Matsuda et al., 2007). RT-PCR assays for *Salmonella* spp. detection are shown in Table 1.9.

As indicated earlier, advantages of real-time RT-PCR include speed, sensitivity, and most importantly potential to detect viable cells or recent contamination. However, the initial cost of equipment, as well as skilled labor may be the main drawbacks that make it unsuitable for routine use in small scale industries or for small scale farmers or field deployment.

RNA-Based Isothermal Amplification

For application of assays in routine testing and rapid and sensitive detection of pathogens, especially by small scale industries and processors, hand-held devices or portable devices that do not require skill, labor, or expensive equipment are needed. Therefore, numerous nucleic acid amplification techniques have been developed for DNA or RNA amplification under isothermal conditions, where only one temperature is required, and a simple water-bath can be used without the need for expensive thermocyclers. Some of the isothermal methods include loop-mediated isothermal amplification (LAMP), transcription mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), signal mediated amplification of RNA technology, strand displacement amplification (SDA), rolling circle amplification (RCA), isothermal multiple displacement amplification, single primer isothermal amplification, and circular helicase-dependent amplification. Some of these methods have been researched for foodborne application based on RNA detection and are discussed below.

Reverse-Transcriptase Loop-mediated isothermal amplification (RT-LAMP)*:* Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay that is rapid, specific, and relatively simple and easy to perform. First described by Notomi et al. in 2000, this

assay relies on an autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi et al., 2000). It also requires 4 to 6 sequence specific primers that recognize 4 to 6 distinct regions on the target gene that allows for accurate and specific pathogen detection in a buffered solution (Salehi et al., 2005). The assay requires only one temperature (60-65°C) in a simple water-bath, eliminating the need for expensive thermo-cycling equipment. As nucleic acid is amplified, insoluble magnesium pyrophosphate is formed. Therefore, the increase in turbidity can be observed either visually or by a hand-held turbidimeter. Moreover, the incorporation of fluorescence dyes or probes along with a fluorometer may aid in the quantification and ease of detection of this assay. However, the current limitation of LAMP-based assay is that only external positive and negative controls are used to determine the success of the amplification reaction, to eliminate false negatives and false positives, respectively. Ideally, similar to PCR-based methods, the incorporation of an IAC in the reaction mix is recommended. Thus, the development and optimization of an appropriate IAC is warranted.

Similar to PCR, LAMP can be developed into a reverse-transcriptase LAMP (RT-LAMP) assay, targeting RNA, by isolating RNA instead of DNA and using an additional reverse transcription step before amplification. The RT-LAMP assay has been used for detection in pure culture and in food, food processing environment and clinical samples for foodborne viruses (Yoneyama et al., 2007; Fukuda et al., 2006; Fukada et al., 2008; Postel et al., 2010; Lan et al., 2009) and bacteria such as *Salmonella* (Techathuvanan et al., 2010b; Techathuvanan et al., in press b), refer to Table 1.9. Likewise, multiplexing can also be achieved by optimization of LAMP assay detecting two or more targets, such as the simultaneous detection of *V.*

parahaemolyticus and related *Vibrio* species targeting the *tdh*, *trh*1, and *trh*2 genes (Yamazaki et al., 2010), along with using various fluorophores for real-time detection.

Nucleic acid sequence-based amplification (NASBA)*:* Although PCR coupled to initial reverse transcription can be used for the detection of target RNA to provide information on viable cells, nucleic acid sequence-based amplification (NASBA) can be employed as an alternative transcription-based RNA amplification method that is carried out at isothermal conditions, typically 41°C. NASBA was first described by Guatelli et al. in 1990 (Fox et al., 2002), that involves the use of 3 different enzymes, reverse transcriptase, RNase H and T7 RNA polymerase, and 2 primers (one containing the bacteriophage T7 promoter sequence at its 5′ end). It can rapidly amplify target RNA sequences by more than 10^8-fold, in a water-bath within 90 min (Compton, 1991). This assay reportedly can also overcome the drawback of RT-PCR without the interference of carry-over DNA, as NASBA theoretically and typically does not detect any background genomic double stranded DNA due to the absence of a denaturation step. Another advantage is that NASBA does not require a thermo-cycler. NASBA has been optimized for detection of several foodborne bacterial and viral pathogens. For *Salmonella* spp. detection, refer to Table 1.9. The mRNA-based NASBA to detect *Salmonella enterica* targeting the *dnaK* gene (Simpkins et al., 2000), was applied to food samples (D'Souza and Jaykus, 2003), with detection sensitivities of 10^2-10^1 CFU/25 g in fresh meats, poultry, fish, ready-to-eat salads and bakery products after 18 h enrichment. Moreover, NASBA (as well as multiplex NASBA) has been used for the detection of other foodborne bacteria, such as *L. monocytogenes*, *V. cholerae*, *C. jejuni* and *M. avium* (Uyttendaele et al., 1995a and b; Rodríguez-Lázaro et al., 2004; Fykse et al., 2007; Blais et al., 1997), and several foodborne viruses, including hepatitis A virus, noroviruses, rotavirus, enteroviruses (Abd El Galil et al., 2005; Jean et al., 2001; Jean et al.,

2002; Jean et al., 2004; Fukuda et al., 2008; Kou et al., 2006; Lamhoujeb et al., 2008; Rutjes et al., 2005; Rutjes et al., 2006; Landry et al., 2003), and avian influenza virus (Lau et al., 2004).

These isothermal amplification methods have high potential to be powerful tools for foodborne pathogen detection. Although, these assays have been tested for detection of bacterial pathogens in pure culture and in clinical samples (Piersimoni and Scarparo, 2003), their application in food matrices is currently very limited.

Salmonella Detection: Future Perspectives

Each individual detection technique described has its own advantages and limitations. Therefore, the trend of integrating two or more emerging technologies is expanding in order to enhance assay performance with added benefits of overcoming the existing drawbacks. Several rapid foodborne pathogen detection techniques have been developed and some are commercially available as kits and equipments. Although these rapid assays propose several advantages such as high speed, less labor, and high specificity and sensitivity, validation of the assays still remain a challenge. Before detection methods can officially be employed for use, standardization of methods for their accuracy, specificity, reproducibility, and robustness is crucial. This includes the absence of false positive and false negative detection by the assay as it could result in large safety impacts or unnecessary costly recalls. Moreover, cost effective and user-friendly assays, as well as the ability to transfer the technologies to the field or on-site testing/monitoring methods would allow the food industry to easily adopt these new tools for routine use. Thus, development and application of microfluidics and microfabrication fields are significantly important to the on-going field of foodborne pathogen detection. While many challenges are being overcome, there still remains a lot of room for improvement in the field of pathogen

detection, including sample preparation and concentration, in addition to the final detection assay with improved signal amplification and improved assay sensitivity.

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Appendix

Table 1.1. *Salmonella* spp. inactivation by high pressure processing.

Method	Strain	Food/Medium	Condition	Reduction	Reference
HHP	*Salmonella* spp.	UHT Milk	600 MPa for 10 min and 21.5°C	6.5-8.2 log	Chen et al., 2006
HHP	*Salmonella* spp.	Orange juice	600 MPa and 20°C	7.0 log	Bull et al., 2004
HHP	*S.* Enteritidis	TSB	250 MPa	1.0 log	Lee and Kaletunç, 2010
HHP	*S.* Enteritidis	TSB	300 MPa	2.0-4.0 log	Lee and Kaletunç, 2010
HHP	*S.* Enteritidis	TSB	450-500 MPa	8.0 log	Lee and Kaletunç, 2010
HHP	*S.* Enteritidis	TSB	250 MPa and 200 IU/ml nisin	1.0 log	Lee and Kaletunç, 2010
HHP	*S.* Enteritidis	TSB	300 MPa and 200 IU/ml nisin	5.0-6.0 log	Lee and Kaletunç, 2010
HHP	*S.* Enteritidis	TSB	350-400 MPa and 200 IU/ml nisin	8.0 log	Lee and Kaletunç, 2010
HHP	*S.* Typhimurium	TSB	250 MPa and 4.61 min	1.0 log	Erkmen, 2009
HHP	*S.* Typhimurium	TSB	300 MPa and 2.59 min	1.0 log	Erkmen, 2009
HHP	*S.* Typhimurium	TSB	350 MPa and 2.09 min	1.0 log	Erkmen, 2009
HHP	*S.* Typhimurium	TSB	450 MPa and 1.8 mi	1.0 log	Erkmen, 2009
HHP	*S.* Typhimurium	Milk	300 MPa and 1.75 min	1.0 log	Erkmen, 2009
HHP	*S.* Typhimurium	Orange juice	300 MPa and 1.5 min	1.0 log	Erkmen, 2009
HHP	*S.* Enteritidis	Liquid whole egg	300 MPa and 10 min	4.99-5.31 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	200 MPa and 10	4.89 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	300 MPa and 17 min	5.75 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	230 MPa and 5 min	4.91 log	Nemeth et al., 2012

Table 1.1. *Salmonella* spp. inactivation by high pressure processing (Continued).

Method	Strain	Food/Medium	Condition	Reduction	Reference
HHP	*S.* Enteritidis	Liquid whole egg	370 MPa and 5 min	5.96 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	230 MPa and 15 min	5.0 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	400 MPa and 10 min	5.31 log	Nemeth et al., 2012
HHP	*S.* Enteritidis	Liquid whole egg	370 MPa and 15 min	6.11 log	Nemeth et al., 2012
HHP	*S.* Typhimurium	TSB	300 MPa and 5 min	3.56 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	300 MPa and 10 min	3.89 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	200 MPa and 25 min	1.18 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	250 MPa and 25 min	3.76 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	300 MPa and 25 min	5.4 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	400 MPa and 25 min	>7.5 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	350 MPa and 30 min	>7.5 log	Erkmen, 2011
HHP	*S.* Typhimurium	TSB	300 MPa and 50 min	>7.5 log	Erkmen, 2011
HHP	*S.* Typhimurium	Raw milk	400 MPa and 45 min	6.51 log	Erkmen, 2011
HHP	*S.* Typhimurium	Orange juice	400 MPa and 10 min	7.04 log	Erkmen, 2011
HHP	*S.* Enteritidis	0.1% peptone water	60,000 psi, 25°C and 5 min	~7.5-8.0 log	Goodridge et al., 2006
HHP	*S.* Enteritidis	Raw almond	60,000 psi, 50°C and 5 min	0.83 log	Goodridge et al., 2006
HHP	*S.* Enteritidis	Raw almond	60,000 psi, 50°C and 9.78 min	1.0 log	Goodridge et al., 2006
HHP	*S.* Enteritidis	Raw almond	6 cycles of 60,000 psi, 50°C and 20 sec	1.16-1.27 log	Goodridge et al., 2006
HHP	*S. enterica*	Dry green onion	300 MPa and 20°C	0.7 log	Neetoo et al., 2012

Table 1.1. *Salmonella* spp. inactivation by high pressure processing (Continued).

Method	Strain	Food/Medium	Condition	Reduction	Reference
HHP	*S. enterica*	Dry green onion	350 MPa and 20°C	1.8 log	Neetoo et al., 2012
HHP	*S. enterica*	Dry green onion	450 MPa and 20°C	2.5 log	Neetoo et al., 2012
HHP	*S. enterica*	Soaked green onion	300 MPa and 20°C	3.0 log	Neetoo et al., 2012
HHP	*S. enterica*	Soaked green onion	350 MPa and 20°C	>4.4 log	Neetoo et al., 2012
HHP	*S. enterica*	Dry green onion	300 MPa and 40°C	2.5 log	Neetoo et al., 2012
HHP	*S. enterica*	Dry green onion	350 MPa and 40°C	3.3 log	Neetoo et al., 2012
HHP	*S. enterica*	Dry green onion	450 MPa and 40°C	>4.9 log	Neetoo et al., 2012
HHP	*S. enterica*	Soaked green onion	300 MPa and 40°C	3.7 log	Neetoo et al., 2012
HHP	*S. enterica*	Soaked green onion	350 MPa and 40°C	>4.4 log	Neetoo et al., 2012
HHP	*S. enterica*	Culture media	400 MPa, 15°C and 10 min	7.0-8.0 log	Jofré et al., 2010
HHP	*S.* Typhimurium	Phosphate buffer pH 7.0	400 MPa and 10 min	>8.0 log	Ritz et al., 2000
HHP	*S.* Typhimurium	Phosphate buffer pH 7.0	600 MPa and 10 min	>8.0 log	Ritz et al., 2000
HHP	*S.* Typhimurium	Citrate phosphate buffer pH 5.6	350 MPa and 10 min	>6.99 log	Ritz et al., 2000
HHP	*S.* Typhimurium	Citrate phosphate buffer pH 5.6	600 MPa and 10 min	>7.99 log	Ritz et al., 2000
HHP	*S.* Newport	Culture media	250 MPa, 20°C and 120 sec	6.0 log	Maitland et al., 2011
HHP	*S.* Newport	Culture media	450 MPa, 20°C and 120 sec	>8.0 log	Maitland et al., 2011
HHP	*S.* Anatum	Culture media	350 MPa, 20°C and 120 sec	>7.9 log	Maitland et al., 2011
HHP	*S.* Javiana	Culture media	350 MPa, 20°C and 120 sec	5.0 log	Maitland et al., 2011

Table 1.1. *Salmonella* spp. inactivation by high pressure processing (Continued).

Method	Strain	Food/Medium	Condition	Reduction	Reference
HHP	*S.* Javiana	Culture media	450 MPa, 20°C and 120 sec	6.0 log	Maitland et al., 2011
HHP	*S.* Javiana	Culture media	550 MPa, 20°C and 120 sec	>8.0 log	Maitland et al., 2011
HHP	*S.* Braenderup	Culture media	350 MPa, 20°C and 120 sec	4.5 log	Maitland et al., 2011
HHP	*S.* Braenderup	Culture media	450 MPa, 20°C and 120 sec	5.6 log	Maitland et al., 2011
HHP	*S.* Braenderup	Culture media	550 MPa, 20°C and 120 sec	>7.6 log	Maitland et al., 2011
HHP	*S.* Braenderup	Diced tomato	400 MPa, 20°C and 120 sec	5.4 log	Maitland et al., 2011
HHP	*S.* Braenderup	Diced tomato	550 MPa, 20°C and 120 sec	3.6 log	Maitland et al., 2011
HHP	*S.* Braenderup	Whole tomato skin	350 MPa, 20°C and 120 sec	3.5 log	Maitland et al., 2011
HHP	*S.* Braenderup	Whole tomato skin	550 MPa, 20°C and 120 sec	>4.0 log	Maitland et al., 2011
HHP	*S.* Braenderup	Whole tomato pulp	350 MPa, 20°C and 120 sec	1.3 log	Maitland et al., 2011
HHP	*S.* Braenderup	Whole tomato pulp	450 MPa, 20°C and 120 sec	2.7 log	Maitland et al., 2011
HHP	*S.* Braenderup	Whole tomato pulp	550 MPa, 20°C and 120 sec	3.4 log	Maitland et al., 2011
HHP	*S.* Enteritidis, Tennessee, Oranienburg, Anatum, and Montevideo cocktail	0.1% peptone buffer	600 MPa and 18 min	>7.0 log	D'Souza et al., 2012

Table 1.1. *Salmonella* spp. inactivation by high pressure processing (Continued).

Method	Strain	Food/Medium	Condition	Reduction	Reference
HHP	*S.* Enteritidis, Tennessee, Oranienburg, Anatum, and Montevideo cocktail	Creamy peanut butter	400-600 MPa and 4-18 min	1.6-1.9 log	D'Souza et al., 2012
HPH	*S.* Typhimurium	Phosphate buffer	200 MPa and 25°C	2.0 log	Wuytack et al., 2002
HPH	*S.* Typhimurium	Buffer	62.5 MPa	1.0 log	Vannini et al., 2004
HPH	*S.* Typhimurium	Buffer	50 MPa and 1650 U/ml lysozyme	1.0 log	Vannini et al., 2004
HPH	*S.* Enteritidis	Buffer	71.4 MPa	1.0 log	Vannini et al., 2004
HPH	*S.* Enteritidis	Buffer	50 MPa and 1650 U/ml lysozyme	1.0 log	Vannini et al., 2004
HPH	*S.* Enteritidis	Milk	130 MPa	1.4 log	Vannini et al., 2004
HPH	*S.* Senftenberg	Orange juice	200 MPa and 6°C	2.0 log	Velázquez-Estrada et al., 2011
HPH	*S.* Senftenberg	Orange juice	300 MPa and 6°C	5.0 log	Velázquez-Estrada et al., 2011
HPH	*S.* Senftenberg	Orange juice	400 MPa and 6°C	>6.5 log	Velázquez-Estrada et al., 2011
HPH	*S.* Senftenberg	Grape juice	200 MPa and 6°C	~1.5 log	Velázquez-Estrada et al., 2011
HPH	*S.* Senftenberg	Grape juice	300 MPa and 6°C	5.0 log	Velázquez-Estrada et al., 2011
HPH	*S.* Senftenberg	Grape juice	400 MPa and 6°C	>6.5 log	Velázquez-Estrada et al., 2011

HHP = high hydrostatic pressure; HPH = high pressure homogenization; TSB = trypticase soy broth

Table 1.2. Inactivation of *Salmonella* spp. by pulsed electric field.

Strain	Food/Medium	Condition	Reduction	Reference
S. Typhimurium	Orange juice	45°C, 90 kV/cm, 50 pulses, 2 μs pulse width, nisin 100 U/ml + lysozyme 690 U/ml	>5.0 log	Liang et al. 2002
S. Enteritidis	Melon Juice	3.66 mS/cm, pH 5.46, 35 kV/cm, 4 μs, t = 1250 μs, 7541 kJ/L and <40°C	3.75 log	Mosqueda-Melgar et al., 2007
S. Enteritidis	Watermelon Juice	3.66 mS/cm, pH 5.46, 35 kV/cm, 4 μs, t = 2000 μs, 7541 kJ/L and <40°C	4.27 log	Mosqueda-Melgar et al., 2007
S. Enteritidis	Sodium sulphate and glucose solution	8 mS/cm, 30-70 kV/cm, 0.05-3.0 μs, 0-110 kJ/L and <50°C	1.0-5.0 log	Korolczuk et al., 2006
S. Enteritidis	Skim milk	4.5-6.8 mS/cm, pH 6.5, 35-55 kV/cm, 0.25-3.0 μs, t = 2.1-3.5 μs, 30-90 kJ/L and 62°C	1.4 log	Flouryet al., 2006a and b
S. Typhimurium	Citric-phosphate buffer	2 mS/cm, pH 3.0-7.0, 12-25 kV/cm, 2 μs, t = 20-400 μs and < 35°C	1.0-3.0 log	Garcia et al., 2005
S. Senftenberg	Citric-phosphate buffer	2 mS/cm, pH 3.0-7.0, 12-25 kV/cm, 2 μs, t = 20-400 μs and < 35°C	1.0-4.5 log	Garcia et al., 2005
S. Senftenberg	Citric-phosphate buffer	2 mS/cm, pH 4.0/7.0, 25 kV/cm, 50-300 pulses, 300-1800 kJ/L and < 35°C	1.0-4.5 log	Garcia et al., 2005
S. Dublin	Skim milk	15-40 kV/cm, 10-50°C, 12-127 μs	~3.0 log	Sensoy et al. 1997
S. Enteritidis	Liquid whole egg	25 kV/cm, 1.2 ml/s, 200 Hz, 2.12 μs, t= 250 μs	4.3 log	Hermawan et al. 2004
S. Typhimurium	Distilled water; 10 mM	35°C, 26.7 kV/cm, monopolar square	0.050-55%	Reyns et al. 2004

Table 1.2. Inactivation of *Salmonella* spp. by pulsed electric field (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
	HEPES-KOH, pH 7.0; 10 mM Tris-HCl, pH 7.0	pulses, 2 µs, 300 pulses, 5 Hz		
S. Enteritidis	Liquid egg	30 kV/cm, 60 pulses, 120 µs and 10°C	1.8 log	Amiali, 2005
S. Enteritidis	Liquid egg	30 kV/cm, 60 pulses, 120 µs and 20°C	2.6 log	Amiali, 2005
S. Enteritidis	Liquid egg	30 kV/cm, 60 pulses, 120 µs and 30°C	3.7 log	Amiali, 2005
S. Dublin	Milk	63°C, 3.7 V/µm, 36 µs, 40 pulses	4.0 log	Dunn and Pearlman 1987
S. Typhymurium	10 mM HEPES	15-30 kV/cm, 300 pulses of monpolar square wave , 2 µs, 1 Hz	<5.0 log	Wuytack et al. 2003
S. Enteritidis	Egg white	30°C, 35 kV, 900 Hz, monopolar exponential decay pulses	3.5 log	Jeantet et al. 1999
S. Senftenberg	McIlvein buffer	28 kV/cm, square wave, 15 µs, 5 Hz	~6.8 log	Raso et al. 2000
S. Senftenberg	McIlvein buffer	square wave pulses, 2 µs, 2 Hz, 200 pulses, 19 kV/cm	6.0 log	Álvarez et al. 2000
S. Typhimurium	Distillated water	<4°C, 10,000, 20 kV/cm, exponential decay pulses, 50 µs, 30Hz	6.0 log	Russell et al. 2000

Table 1.3. *Salmonella* spp. inactivation by irradiation.

Radiation source	Strain	Food/Medium	Condition	Reduction	Reference
Gamma	*S.* Typhimurium	Blended oyster	0.1 Mrad	1.0 log	Shiflett et al., 1967
Gamma	*S.* Enteritidis	Blended oyster	0.1 Mrad	1.0 log	Shiflett et al., 1967
Gamma	*S.* Typhimurium	Ground beef	0.55 kGy	1.0 log	Tarkowski et al., 1984
Gamma	*S.* Typhimurium	Roast beef	0.569-0.585 kGy	1.0 log	Grant and Patterson, 1992
Gamma	*S.* Typhimurium	Gravy	0.416-0.533 kGy	1.0 log	Grant and Patterson, 1992
Gamma	*S.* Typhimurium	Cauliflower (cooked, crushed)	0.549-0.590 kGy	1.0 log	Grant and Patterson, 1992
Gamma	*S.* Typhimurium	Roast potato	0.462-0.639 kGy	1.0 log	Grant and Patterson, 1992
Gamma	*S.* Typhimurium	Mashed potato	0.464-0.504 kGy	1.0 log	Grant and Patterson, 1992
Gamma	*S.* Typhimurium	Minced chicken	0.436-0.502 kGy	1.0 log	Patterson, 1988
Gamma	*S.* Enteritidis	Shell egg	488 Gy	1.0 log	Al-Bachir and Zeinou, 2006
Gamma	*S.* Enteritidis	Shell egg	1.0 kGy	3.9 log	Tellez et al., 1995
Gamma	*S.* Enteritidis	Shell egg	2.0-3.0 kGy	8.0 log	Tellez et al., 1995
Gamma	*S.* Enteritidis	Natural shell egg	0.39-0.41 kGy	1.0 log	Serrano et al., 1997
Gamma	*S.* Enteritidis	Whole egg powder	1 kGy	2.0-3.0 log	Kohler et al. 1989
Gamma	*S.* Typhimurium	Whole egg	0.26-0.31 kGy	1.0 log	Verde et al., 2004
Gamma	*S.* Enteritidis	Whole egg	0.19-0.20 kGy	1.0 log	Verde et al., 2004
Gamma	*S.* Typhimurium	Cucumber	1.0 kGy	3.0 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Cucumber	2.0 kGy	>4.0 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Cucumber	3.0 kGy	>7.0 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Blanched and seasoned spinach	1.0 kGy	~3.0 log	Lee et al., 2006

Table 1.3. *Salmonella* spp. inactivation by irradiation (Continued).

Radiation source	Strain	Food/Medium	Condition	Reduction	Reference
Gamma	*S.* Typhimurium	Blanched and seasoned spinach	2.0 kGy	>7.0 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Seasoned burdock	1.0 kGy	2.5 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Seasoned burdock	2.0 kGy	>7.0 log	Lee et al., 2006
Gamma	*S.* Typhimurium	Broccoli seeds	0.81 kGy	1.0 log	Waje et al., 2009
Gamma	*S.* Typhimurium	Red radish seeds	0.8 kGy	1.0 log	Waje et al., 2009
Gamma	*S.* Typhimurium	Broccoli sprout	0.13 kGy	1.0 log	Waje et al., 2009
Gamma	*S.* Typhimurium	Red radish sprout	0.14 kGy	1.0 log	Waje et al., 2009
E-beam	*S.* Typhimurium	Sliced Ham	2 kGy	3.78 log	Song et al., 2011
E-beam	*S.* Typhimurium	Salchichon (vacuum-packed ready-to-eat dry fermented sausage)	0.53 kGy	1.0 log	Cabeza et al., 2009
E-beam	*S.* Enteritidis	Chorizo (vacuum-packed ready-to-eat dry fermented sausage)	0.41 kGy	1.0 log	Cabeza et al., 2009
E-beam	*S.* Typhimurium	Salchichon (vacuum-packed ready-to-eat dry fermented sausage)	0.54 kGy	1.0 log	Cabeza et al., 2009
E-beam	*S.* Enteritidis	Chorizo (vacuum-packed ready-to-eat dry fermented sausage)	0.43 kGy	1.0 log	Cabeza et al., 2009
E-beam	*S.* Tennessee	Peanut butter	0.72 kGy	1.0 log	Hvizdzak et al.,

Table 1.3. *Salmonella* spp. inactivation by irradiation (Continued).

Radiation source	Strain	Food/Medium	Condition	Reduction	Reference
					2010
E-beam	*S.* Tennessee	Peanut butter	3.0 kGy	5.0 log	Hvizdzak et al., 2010
E-beam	*S.* Typhimurium	Peanut butter	0.82 kGy	1.0 log	Hvizdzak et al., 2010
E-beam	*S.* Typhimurium	Peanut butter	3.0 kGy	>4.0 log	Hvizdzak et al., 2010
E-beam	*S.* Typhimurium	Broccoli seeds	0.6 kGy	1.0 log	Waje et al., 2009
E-beam	*S.* Typhimurium	Red radish seeds	1.35 kGy	1.0 log	Waje et al., 2009
E-beam	*S.* Typhimurium	Broccoli sprout	0.3 kGy	1.0 log	Waje et al., 2009
E-beam	*S.* Typhimurium	Red radish sprout	0.23 kGy	1.0 log	Waje et al., 2009
E-beam	*S. typhi*	Nutrient broth	1.5 kGy	2.0 log	Martin et al., 2005
E-beam	*S.* Typhimurium	Powdered weaning food	0.98 kGy	1.0 log	Hong et al., 2008
E-beam	*S.* Montevideo	Tomato cubes	0.7-0.95 kGy	1.8-2.2 log	Schmidt et al., 2006
E-beam	*S.* Montevideo	Tomato stem scars	0.7 kGy	2.4 log	Schmidt et al., 2006
E-beam	*S.* Agona	Tomato cubes	0.7-0.95 kGy	1.3-1.5 log	Schmidt et al., 2006
E-beam	*S.* Agona	Tomato stem scars	0.7-0.95 kGy	1.3-2.2 log	Schmidt et al., 2006
E-beam	*S.* Montevideo	Roma tomato puree, pH 4.4	1.07 kGy	1.0 log	James et al., 2010
E-beam	*S.* Montevideo	Roma tomato puree, pH 4.9	1.5 kGy	1.0 log	James et al., 2010
E-beam	*S.* Typhimurium	Beef steak	1.5-3.0 kGy	6.0 log	Chung et al., 2000
E-beam	*S.* Agona, Gaminara, Michigan, Montevideo, Poona, and Typhimurium	Spinach	0.4 kGy	3.4 log	Neal et al., 2008
E-beam	*S.* Agona, Gaminara, Michigan,	Spinach	0.7 kGy	4.0 log	Neal et al., 2008

Table 1.3. *Salmonella* spp. inactivation by irradiation (Continued).

Radiation source	Strain	Food/Medium	Condition	Reduction	Reference
E-beam	Montevideo, Poona, and Typhimurium *S.* Agona, Gaminara, Michigan, Montevideo, Poona, and Typhimurium	Spinach	1.07 kGy	>6.0 log	Neal et al., 2008
X-ray	*S.* Typhimurium, *S.* Montevideo and *S.* Javiana (cocktail)	Banner green onion	0.26 kGy	1.0 log	Murugesan et al., 2011
X-ray	*S.* Typhimurium, *S.* Montevideo and *S.* Javiana (cocktail)	Baja verde green onion	0.32 kGy	1.0 log	Murugesan et al., 2011
X-ray	*S.* Enteritidis	Almond	0.226-0.363 kGy	1.0 log	Jeong et al., 2011
X-ray	*S.* Enteritidis	Walnut	0.474-1.092 kGy	1.0 log	Jeong et al., 2011
X-ray	*S.* Tennessee	Almond	0.256-0.479 kGy	1.0 log	Jeong et al., 2011
X-ray	*S.* Tennessee	Walnut	0.554-1.029 kGy	1.0 log	Jeong et al., 2011
X-ray	*S. enterica*	Ready-to-eat shrimp	0.3 kGy	2.5 log	Mahmoud, 2009b
X-ray	*S. enterica*	Ready-to-eat shrimp	0.75 kGy	4.0 log	Mahmoud, 2009b
X-ray	*S. enterica*	Ready-to-eat shrimp	1.0 kGy	4.5 log	Mahmoud, 2009b
X-ray	*S. enterica*	Ready-to-eat shrimp	2.0 kGy	5.5 log	Mahmoud, 2009b
X-ray	*S. enterica*	Ready-to-eat shrimp	3.0 kGy	>7.0 log	Mahmoud, 2009b
X-ray	*S. enterica*	Shredded iceberg lettuce	1.0 kGy	4.8 log	Mahmoud, 2010
X-ray	*S. enterica*	Shredded iceberg lettuce	2.0 kGy	>5.0 log	Mahmoud, 2010
X-ray	*S. enterica*	Spinach leaves	0.1 kGy	0.6 log	Mahmoud et

Table 1.3. *Salmonella* spp. inactivation by irradiation (Continued).

Radiation source	Strain	Food/Medium	Condition	Reduction	Reference
					al., 2010
X-ray	*S. enterica*	Spinach leaves	1.0 kGy	3.4 log	Mahmoud et al., 2010
X-ray	*S. enterica*	Spinach leaves	2.0 kGy	>5.0 log	Mahmoud et al., 2010

Table 1.4. *Salmonella* spp. inactivation by UV treatment.

Strain	Food/Medium	Condition	Reduction	Reference
S. Typhimurium	Sliced Ham	8000 J/m^2	2.02 log	Chun et al., 2009
Salmonella spp.	Waste water	6 mJ/cm^2	1.0 log	Hijnen et al., 2006
Salmonella spp.	Waste water	12 mJ/cm^2	2.0 log	Hijnen et al., 2006
Salmonella spp.	Waste water	17 mJ/cm^2	3.0 log	Hijnen et al., 2006
Salmonella spp.	Waste water	51 mJ/cm^2	4.0 log	Hijnen et al., 2006
S. typhi	Sterile buffered water	5 mW-sec/cm^2	2.0 log	Chang et al., 1985
S. typhi	Sterile buffered water	10 mW-sec/cm^2	5.0 log	Chang et al., 1985
S. Enteritidis	Liquid egg white	9.22 J/cm^2 and 39 min	5.3 log	de Souza and Fernández, 2011
S. Enteritidis	Liquid egg yolk	9.22 J/cm^2 and 39 min	3.3 log	de Souza and Fernández, 2011
S. Enteritidis	Liquid whole egg	9.22 J/cm^2 and 39 min	3.8 log	de Souza and Fernández, 2011
S. Eastbourne	Peptone water (0.5, 1.0, and 2.0 mm thin film)	17 X 10^5 erg/cm^2s	5.0 log	Lee et al., 1989
S. Eastbourne	Chocolate (0.1 mm)	17 X 10^5 erg/cm^2s and 1.5 min	5.0 log	Lee et al., 1989
S. Eastbourne	Chocolate (0.5 mm)	17 X 10^5 erg/cm^2s and 10 min	0.7 log	Lee et al., 1989
S. Eastbourne	Agar plate	76 X 10^3	99.99%	Lee et al.,

Table 1.4. *Salmonella* spp. inactivation by UV treatment (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
	surface	erg/cm^{2}s and 6 sec		1989
S. Tennessee	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Typhymurium	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Infantis	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Montevideo	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Senftenberg	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Anatum	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Alachua	Agar plate surface	76 X 10^3 erg/cm^{2}s and 6 sec	99.99%	Lee et al., 1989
S. Enteritidis	Shell egg	1,179 mJ/cm^{2}s and 5 sec	3.2 log	Keklik et al., 2010a
S. Enteritidis	Shell egg	1,179 mJ/cm^{2}s and 15 sec	4.0 log	Keklik et al., 2010a
S. Enteritidis	Shell egg	1,179 mJ/cm^{2}s and 20 sec	7.7 log	Keklik et al., 2010a
S. Enteritidis	Shell egg	827 mJ/cm^{2}s and 5 sec	2.7 log	Keklik et al., 2010a

Table 1.4. *Salmonella* spp. inactivation by UV treatment (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
S. Enteritidis	Shell egg	827 mJ/cm^{2}s and 15 sec	3.4 log	Keklik et al., 2010a
S. Enteritidis	Shell egg	827 mJ/cm^{2}s and 20 sec	5.3 log	Keklik et al., 2010a
S. Typhymurium	Unpacked chicken breast	1,117 mJ/cm^{2}s and 5 sec	1.3 log	Keklik et al., 2010b
S. Typhymurium	Unpacked chicken breast	1,117 mJ/cm^{2}s and 60 sec	2.2 log	Keklik et al., 2010b
S. Typhymurium	Unpacked chicken breast	931 mJ/cm^{2}s and 5 sec	1.3 log	Keklik et al., 2010b
S. Typhymurium	Unpacked chicken breast	931 mJ/cm^{2}s and 60 sec	2.2 log	Keklik et al., 2010b
S. Typhymurium	Unpacked chicken breast	581 mJ/cm^{2}s and 5 sec	1.2 log	Keklik et al., 2010b
S. Typhymurium	Unpacked chicken breast	581mJ/cm^{2}s and 60 sec	1.8 log	Keklik et al., 2010b
S. Typhymurium	Vacuum-packed chicken breast	1,117 mJ/cm^{2}s and 5 sec	1.2 log	Keklik et al., 2010b
S. Typhymurium	Vacuum-packed chicken breast	1,117 mJ/cm^{2}s and 60 sec	1.9 log	Keklik et al., 2010b
S. Typhymurium	Vacuum-packed chicken breast	931 mJ/cm^{2}s and 5 sec	1.1 log	Keklik et al., 2010b
S. Typhymurium	Vacuum-packed chicken breast	931 mJ/cm^{2}s and 60 sec	1.9 log	Keklik et al., 2010b
S. Typhymurium	Vacuum-packed chicken breast	581 mJ/cm^{2}s and 5 sec	0.8 log	Keklik et al., 2010b
S.	Vacuum-	581mJ/cm^{2}s and	1.7 log	Keklik et al.,

Table 1.4. *Salmonella* spp. inactivation by UV treatment (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
Typhymurium	packed chicken breast	60 sec		2010b
S. Enteritidis	Phosphate buffer	15 W, 5.0-mm distance and 0.1 min	1.0 log	Gabriel and Nakano, 2009
S. Enteritidis	Apple juice	15 W, 5.0-mm distance and 0.61 min	1.0 log	Gabriel and Nakano, 2009
S. Typhymurium	PBS	15 W, 5.0-mm distance and 0.26 min	1.0 log	Gabriel and Nakano, 2009
S. Typhymurium	Apple juice	15 W, 5.0-mm distance and 0.27 min	1.0 log	Gabriel and Nakano, 2009
S. Typhymurium	LB agar	133 W/cm^2 and 100 pulses	7.0 log	Luksiene et al., 2007
S. Typhymurium	Chicken breast	5.4 J/cm^2 and 1000 pulses	~2.0 log	Paškevičiūtė and Lukšienė, 2009

Table 1.5. *Salmonella* spp. inactivation by high intensity ultrasound.

Strain	Food/Medium	Condition	Reduction	Reference
Salmonella spp.	Broiler drumstick skin	47 kHz, 200 W, 15 min and 25°C	None	Sams and Feria, 1991
Salmonella spp.	Broiler drumstick skin	47 kHz, 200 W, 30 min and 40°C	None	Sams and Feria, 1991
Salmonella spp.	Broiler drumstick skin	47 kHz, 200 W, 15 min and 25°C	None	Sams and Feria, 1991
Salmonella spp.	Broiler drumstick skin	47 kHz, 200 W, 30 min and 40°C	None	Sams and Feria, 1991
Salmonella spp.	Peptone water	160 kHz, 100 W and 10 min	4.0 log	Lee et al., 1989
S. Eastbourne	Peptone water	160 kHz, 100 W, 3 min and 5°C	1.0 log	Lee et al., 1989
S. Anatum	Peptone water	160 kHz, 100 W, 2.1 min and 5°C	1.0 log	Lee et al., 1989
S. Eastbourne	Chocolate	160 kHz, 100 W, 5°C and 10 min	26%	Lee et al., 1989
S. Eastbourne	Chocolate	160 kHz, 100 W, 5°C and 30 min	74%	Lee et al., 1989
S. Typhimurium	Lettuce	32-40 kHz		Seymour et al., 2002
S. Typhimurium	Brain heart infusion broth	20 kHz, 30 min and 20°C	1.0 log	Wrigley and Llorca, 1992
S. Typhimurium	Brain heart infusion broth	20 kHz, 30 min and 40°C	>3.0 log	Wrigley and Llorca, 1992
S. Typhimurium	Skim milk	20 kHz, 30 min and 40°C	2.5 log	Wrigley and Llorca, 1992
S. Typhimurium	Skim milk	20 kHz, 30 min and 50°C	3.0 log	Wrigley and Llorca, 1992
S. Typhimurium	Liquid whole egg	20 kHz, 30 min and 50°C	<1.0 log	Wrigley and Llorca, 1992
S. Typhymurium	Citrate phosphate buffer	117 µm, 200 kPa, 0.78 min and 40°C	1.0 log	Mañas et al., 2000
S. Typhymurium	Citrate phosphate buffer	117 µm, 200 kPa, 0.12 min and 60°C	1.0 log	Mañas et al., 2000
S. Typhymurium	Liquid whole egg	117 µm, 200 kPa, 0.84 min and 40°C	1.0 log	Mañas et al., 2000
S. Typhymurium	Liquid whole egg	117 µm, 200 kPa, 0.2 min and 60°C	1.0 log	Mañas et al., 2000

Table 1.5. *Salmonella* spp. inactivation by high intensity ultrasound (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
S. Enteritidis	Citrate phosphate buffer	117 µm, 200 kPa, 0.73 min and 40°C	1.0 log	Mañas et al., 2000
S. Enteritidis	Citrate phosphate buffer	117 µm, 200 kPa, 0.068 min and 60°C	1.0 log	Mañas et al., 2000
S. Enteritidis	Liquid whole egg	117 µm, 200 kPa, 0.76 min and 40°C	1.0 log	Mañas et al., 2000
S. Enteritidis	Liquid whole egg	117 µm, 200 kPa, 0.12 min and 60°C	1.0 log	Mañas et al., 2000
S. Senftenberg	Citrate phosphate buffer	117 µm, 200 kPa, 0.84 min and 40°C	1.0 log	Mañas et al., 2000
S. Senftenberg	Citrate phosphate buffer	117 µm, 200 kPa, 1.0 min and 60°C	1.0 log	Mañas et al., 2000
S. Senftenberg	Liquid whole egg	117 µm, 200 kPa, 1.4 min and 40°C	1.0 log	Mañas et al., 2000
S. Senftenberg	Liquid whole egg	117 µm, 200 kPa, 5.5 min and 60°C	1.0 log	Mañas et al., 2000
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw >0.99, 0.89 min and 35°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw >0.99, 0.77 min and 50°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw >0.99, 0.02 min and 63°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.98, 0.85 min and 35°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.98, 4.6 min and 50°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.98,	1.0 log	Álvares et al., 2003

Table 1.5. *Salmonella* spp. inactivation by high intensity ultrasound (Continued).

Strain	Food/Medium	Condition	Reduction	Reference
		1.6 min and 60°C		
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.96, 1.37 min and 35°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.96, 0.87 min and 50°C	1.0 log	Álvares et al., 2003
S. Enteritidis	Nutrient broth	20 kHz, 117 µm, 175 kPa, Aw 0.96, 0.25 min and 60°C	1.0 log	Álvares et al., 2003
S. Typhymurium	Broiler breast skin	20 kHz and 30 min	1.0-1.5 log	Lillard, 1993
S. Typhymurium	Broiler breast skin	20 kHz, 30 min and 0.5 ppm chlorine	2.5-4.0 log	Lillard, 1993
S. Typhymurium	Ozonated PBS	40 kHz, 150 W and 0.5 min	~4.0 log	Burleson et al., 1975
S. Typhymurium	Ozonated secondary effluent	40 kHz, 150 W and 1 min	>7.0 log	Burleson et al., 1975
S. Typhymurium	Ozonated PBS	40 kHz, 150 W and 0.5 min	~6.0 log	Burleson et al., 1975
S. Typhymurium	Ozonated secondary effluent	40 kHz, 150 W and 1 min	>7.0 log	Burleson et al., 1975

Table 1.6. Examples of natural antimicrobials and their sources.

Origin	Antimicrobial	Source
Animals	Lactoperoxidase, lactoferrin, lactoferricin B, lactoglobulins	Milk
	Lysozyme, ovotransferrin, ovoglobulin, avidin	Eggs
	Transferrins	Serum
	Myeloperoxidase	Phagosomes
	Antibodies	Immune system
	Attacins, cecropins	Insects
	Defensins	Chickens, mammals
	Chitosan	Crustaceans, arthropods
	Pleurocidin	Winter flounder
Plants	Organic acids	
	Phenolic compounds	
	Flavones	Herbs, spices, and other plants
	Flavonols/flavonoids	
	Alkaloids	
	Glucosides, glycosides, dienes	
	Terpenes	
	Aliphatic alcohols	
	Quinines	
	Lectins	
Microorganisms	Nisin	*Lactococcus lactis*
	Pediocin	*Pediococcus acidilactici* and *P. pentosaceus*
		Lactobacillus reuteri
	Reuterin	Lactic acid bacteria

Table 1.6. Examples of natural antimicrobials and their sources (Continued).

Origin	Antimicrobial	Source
	Other bacteriocins	Other microorganisms
	Pimaricin, subtilin, natamycin, diacetyl	

Table 1.7. Typical addition levels of nisin in foods.

Food	**Target organisms**	**Nisin concentration (mg/kg or mg/l)**
Processed cheese	*Clostridium* spp. and *Bacillus* spp.	5.0-15.0
Milk and milk products	*Clostridium* spp. and *Bacillus* spp.	0.25-10.0
Pasteurized chilled soups	*B. cereus* and *C. pasteurianum*	2.5-6.25
Crumpets	*B. cereus*	4.0-6.25
Canned foods	*C. botulinum* and *C. thermosaccharolyticum*	2.5-5.0
Ricotta cheese	*Listeria monocytogenes*	2.5-5.0
Cooked sausage	LAB, *Brochothrix thermosphacta*, and *L. monocytogenes*	5.0-25.0
Dipping sauces	LAB	1.25-6.25
Salad dressings	LAB	1.25-5.0
Beer: pitching yeast wash	LAB (*Lactobacillus* and *Pediococcoccus*)	25.0-37.5
Beer: post fermentation	LAB (*Lactobacillus* and *Pediococcoccus*)	0.25-1.25

LAB = lactic acid bacteria

Table 1.8. Limitations and advantages of non-thermal processing techniques.

	Advantages	**Limitations**
Irradiation	- Effective for several foods - Many different sources available (Gamma rays, electron beam, X-ray)	- Limited public acceptance - Lipid oxidation of meat products - Require special processing facility
UV radiation	- No chemicals are used - Non-heat related method - Lesser changes in quality attributes of food	- Long term exposure can be harmful to the industry workers
HHP	- Independent of the shape of food - Can be used for both solid and liquid samples	- Changes in quality of food has been observed - Can be used in only batch process
HPH	- Can be used in a continuous process	- Can be used for only liquid samples - Commercial application is expensive
PEF	- Pulse applied for a short period so no generation of heat - Less usage of energy	- Cannot be applied to foods which cannot withstand high fields - Cannot be applied to foods that form bubbles
HIU	- Can be used in a continuous process - No chemicals are used	- Dependent on physical characteristics of foods (e.g. viscosity, size, etc.)
Natural antimicrobials	- Natural "green" preservatives - Have "GRAS" status	- May have a negative effect on the sensory properties of foods - High concentration required for food applications

HHP = high hydrostatic pressure, HPH = high pressure homogenization; PEF = pulse electric field; HIU = high intensity ultrasound; GRAS = generally recognized as safe.

Table 1.9. RNA-based assays for *Salmonella* spp. detection.

Microorganism (Target Gene)	**IAC**	**Matrices**	**Primer and Probe/Sequence (5'-3')/Fluorescence Dye**	**Enrichment Media/Time/Temp**	**Detection Limit**	**Reference**
RT-PCR Assay						
Salmonella spp. (*ompC*)	N	Organic waste samples	S18: ACCGCTAACGCTCGCCTGTAT	None	10^7 CFU/g	Burtscher and Wuertz, 2003
			S19: AGAGGTGGACGGGTTGCTGCCGTT	Peptone water/20 h/37ºC	<10 CFU/g	
Salmonella enterica *(invA)*	Y	Spinach, tomatoes, jalapeno, and serrano peppers	invA_176F: CAACGTTTCCTGCGGTACTGT invA_291R: CCCGAACGTGGCGATAATT invA_Tx_208: TX-CTCTTTCGTCTGGCATTATCG ATCAGTACCA-BHQ2	Lactose broth/24 ± 2 h/35 ±2°C	2 CFU/25 g	Gonzalez-Escalona et al., 2009
Salmonella sp. *(invA)*	N	Soil Chicken manure	F: ACAGTGCTCGTTTACGACC R: ACTGGTACTGATCGATAAT P: BIOTIN-CTGAGGATTCTGTCAATGTAGA ACGACCCCATAAACACCAATAT CGCCAGTACGATATTCAGTGCG AT	None	5×10^4 cells/g None	Jacobsen and Holben, 2007
Salmonella	Y	Pure culture	F: CACGCTCTTTCGTCTGGCA	None	10^2 CFU/ml	D'Souza et al.,

Table 1.9. RNA-based assays for *Salmonella* spp. detection (Continued).

Microorganism (Target Gene)	IAC	Matrices	Primer and Probe/Sequence (5'-3')/Fluorescence Dye	Enrichment Media/Time/Temp	Detection Limit	Reference
enterica *(invA)*			R: TACGGTTCCTTTGACGGTGCGA SYBR Green I			2009; Techathuvanan et al., 2010a
Salmonella enterica *(invA)*	Y	Inoculated pork chop, pork sausage, and pork carcass rinse;	F: CACGCTCTTTCGTCTGGCA R: TACGGTTCCTTTGACGGTGCGA SYBR Green I	None; Tetrathionate broth/10 h/37°C;	10^6 CFU/25 g (pork sample) or 500 ml (pork carcass rinse); 10^0-10^1 CFU/25 g (pork sample) or 500 ml (pork carcass rinse);	Techathuvanan et al., (2010a and b)
		Natural pork carcass rinses, pork carcass swabs, and pork processing surface swabs		Buffered peptone water/4 h/37°C and tetrathionate broth/12 h/37°C	N/A	
Salmonella enterica *(invA)*	Y	Pure culture;	F: CACGCTCTTTCGTCTGGCA R: TACGGTTCCTTTGACGGTGCGA SYBR Green I	None;	10^6 CFU/ml;	Techathuvanan et al., 2010a
		Liquid whole egg		None	10^7 CFU/25 ml	
				Tetrathionate	10^4 CFU/25 ml	

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	IAC	Matrices	Primer and Probe/Sequence (5'-3')/Fluorescence Dye	Enrichment Media/Time/Temp	Detection Limit	Reference
				broth/6 h/37°C		
				Tetrathionate broth/12 h/37°C	10^2 CFU/25 ml	
				Tetrathionate broth/16 h/37°C	10^0-10^1 CFU/25 ml	
Salmonella Typhimurium *(invA)*	Y	Lettuce, tomato, jalapeño and serrano peppers	F: CACGCTCTTTCGTCTGGCA R: TACGGTTCCTTTGACGGTGCGA SYBR Green I	None; Buffered peptone water/6 h/37°C	10^6-10^7 CFU/g (pepper) or 25 g (lettuce) or 100 g (tomato); 10^4 CFU/g (pepper), 25 g (lettuce) or 100 g (tomato)	Miller et al., 2010a and in press
Salmonella Enteritidis *(sefA)*	N	Pure culture; Raw shell eggs	SEFA-F: GGCTTCGGTATCTGGTGGTGTG SEFA-R: GTCATTAATATTGGCTCCCTGAATA SEFA-P: CCACTGTCCCGTTCGTTGATGGA	Tissue culture infection/5 h/37°C	10^1 CFU/ml; 10^1 CFU/ml	Day et al., 2009

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	**IAC**	**Matrices**	**Primer and Probe/Sequence (5'-3')/Fluorescence Dye**	**Enrichment Media/Time/Temp**	**Detection Limit**	**Reference**
			CA			
Salmonella Enteritidis *(orgC)*	N	Pure culture and raw shell eggs	ORGC-F: CTTTATGATGCATTCTACCAACG ACTG ORGC-R: CCGAATCACCACTGTTAGGA ORGC-P: CGCTTCCTGAGTCAGCCTCTTCT GAAACG	Tissue culture infection/5 h/37°C	10^1 CFU/ml	Day et al., 2009
Salmonella Typhimurium (16S rRNA)	N	Pure culture Tap water fishpond water	F: CGGGGAGGAAGGTGTTGTG R: GAGCCCGGGGATTTCACATC	None	10^3 nucleic acid copies/reaction N/A	Fey et al., 2004
Salmonella Typhimurium (invA)	N	Pure culture Tap water fishpond water	F: GATTCTGGTACTAATGGTGATGA TC R: GCCAGGCTATCGCCAATAAC	None	20 nucleicacid copies/reaction N/A	Fey et al., 2004
Salmonella Typhimurium *(kdpA)*	N	Pure culture	F: GGCGCTACTGACGCTCAATC R: AGGCTTGCCAGTTGGTATTGG	N/A	N/A	Balaji et al., 2005

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	**IAC**	**Matrices**	**Primer and Probe/Sequence (5'-3')/Fluorescence Dye**	**Enrichment Media/Time/Temp**	**Detection Limit**	**Reference**
(proV)			F: GGATTATCCGGCTCGGGTAA R: GAGCGCAAATGACTGGAAGAC			
(proP)			F: TGCCTACGCGTTGGGTAAAG R: CCGTATTTATCGCCGAGCAT			
(rpoS)			F: GTTGGACGCGACTCAGCTTT R: TTTTACCACCAGACGCAGGTT			
(otsB)			F: TTAACCGTATCCCCCGAACTC R: CCGCGAGACGGTCTAACAAC			
(ompC)			F: GCGCCGACATCAACGTATTT R: GCCAACAAAGCGCAGAACTT			

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	IAC	Matrices	Primer and Probe/Sequence (5'-3')/Fluorescence Dye	Enrichment Media/Time/Temp	Detection Limit	Reference
(gnd)			F: CAACATCGAAAGCCGTGGTT R: GGCGTTTCGAGGGATTCAA			
(lacZ)			F: CACCAGCAGCAGTTTTTCCA R: ATCCAGTGCAGGAGCTCGT			
(phoA)			F: GCGATGCTGCCTCACTGAAT R: TTGCGGATTTGGCGTACAG			
(16S rRNA)			F: ATTGACGTTACCCGCAGAAGA R: GGGATTTCACATCCGACTTGA SYBR Green I			
RT-LAMP Assay						
Salmonella Typhimurium	N	Pure culture	*invA*/ FIP: GACGACTGGTACTGATCGATAG TTTTTCAACGTTTCCTGCGG	None	10^1 CFU/ml	Techathuvanan et al., 2010b

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	**IAC**	**Matrices**	**Primer and Probe/Sequence (5'-3')/Fluorescence Dye**	**Enrichment Media/Time/Temp**	**Detection Limit**	**Reference**
		Pork chops	BIP: CCGGTGAAATTATCGCCACACA AAACCCACCGCCAGG	Tetrathionate broth (TTB)/10 h/37°C	10^2 CFU/25 g	
		Pork sausage	F3: GGCGATATTGGTGTTTATGGGG	TTB/10 h/37°C	10^2 CFU/25 g	
		Natural pork chop, ground pork, and pork sausage	B3: AACGATAAACTGGACCACGG		N/A	
			FLoop: GACGAAAGAGCGTGGTAATTAA C	BPW/4 h/37°C and tetrathionate broth/12 h/37°C		
			BLoop: GGGCAATTCGTTATTGGCGATA G			
Salmonella enterica	N	Natural pork carcass rinses, pork carcass swabs	*invA*/ FIP: GACGACTGGTACTGATCGATAG	BPW/4 h/37°C and tetrathionate broth/12 h/37°C	N/A	Techathuvanan et al., in press b
			TTTTTCAACGTTTCCTGCGG			
			BIP: CCGGTGAAATTATCGCCACACA AAACCCACCGCCAGG			
			F3: GGCGATATTGGTGTTTATGGGG			
			B3:			

Table 1.9. RNA-based assays for *Salmonella* spp. detection. (Continued).

Microorganism (Target Gene)	IAC	Matrices	Primer and Probe/Sequence (5'-3')/Fluorescence Dye	Enrichment Media/Time/Temp	Detection Limit	Reference
			AACGATAAACTGGACCACGG FLoop: GACGAAAGAGCGTGGTAATTAAC BLoop: GGGCAATTCGTTATTGGCGATAG			
NASBA						
Salmonella Enteritidis	N	Pure culture	*dnaK*/SDnaK1: AATTCTAATACGACTCACTATAGGGAGAGGCAGTCGGTTCGTTGATG SDnaK2: GATGCAAGGTCGCATATGAGCTTGATGTGAAAGGTCAGA	None	10^1 CFU/reaction	D'Souza and Jaykus, 2003
		Cake, chocolate, infant formula, macaroni, non-fat dry milk and red pepper		Lactose broth, brilliant green water or skim milk/8 h/35°C	10^2-10^1 CFU/25 g	
		Liquid whole egg		Buffered peptone water/16 h/37°C	2.8 CFU/25 g	Cook et al., 2002

F = forward; R = reverse; P = probe; MB = molecular beacon; TX = Texas red; R = A or G; Y = C or T; N = any.

FIP consisted of the F1 complementary sequence and the F2 direct sequence; BIP consisted of the B1 direct sequence and the B2 complementary sequence; F1c, sequence complementary to F1; F2c, sequence complementary to F2; B3c, sequence complementary to B3; LFc, sequence complementary to LF.

CHAPTER II

Comparison of RT-PCR, Loop-Mediated Isothermal Amplification, and Culture-Based Assays for *Salmonella* Detection from Pork Processing Environments

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Abstract

Novel rapid *Salmonella* detection assays without the need for sophisticated equipment or labor remain in high-demand. Real-time reverse-transcriptase-PCR (RT-PCR) assays though rapid and sensitive, require expensive thermocyclers, while a novel reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) method requires only a simple waterbath. Our objective was to compare the detection sensitivity of *Salmonella* Typhimurium from the pork processing environment by RT-LAMP, RT-PCR and culture-based assays. Carcass and surface swabs, and carcass rinses were obtained from a local processing plant. Autoclaved carcass rinses (500 ml) were spiked with *S.* Typhimurium and filtered. Filters were placed in stomacher bags containing tetrathionate broth (TTB), and analyzed with or without 10-h enrichment at 37^{o}C. Natural swabs were stomached with buffered peptone water, and natural carcass rinses filtered, pre-enriched and further enriched in TTB. Serially-diluted enriched samples were enumerated by spread plating on XLT4 agar. RNA was extracted from 5-ml of enriched TTB with TRIzol®. RT-LAMP assay using previously described *inv*A primers was conducted at 62^{o}C for 90 min in a waterbath with visual detection and by gel electrophoresis. SYBR Green I-based-real-time-RT-PCR was carried out with *inv*A primers followed by melt temperature analysis. RT-LAMP detection for spiked carcass rinses was comparable to RT-PCR and cultural plating with detection limits of 1-$\log_{10}$CFU/ml, though significantly faster within 24 h including pre-enrichment and enrichment. RT-LAMP showed 4/12 while RT-PCR showed 1/12 positives for rinse samples. For swabs, 6/27 positives by RT-LAMP and 5/27 by RT-PCR were obtained. This 1-day-RT-LAMP assay shows promise for routine *Salmonella* screening by the pork industry.

Introduction

Pork has been implicated as one of the major sources associated with human salmonellosis (Boughton et al., 2004; Delhalle et al., 2009; Murase et al., 2000; Pontello et al., 1998; Vieira-Pinto et al., 2007). *Salmonella* Typhimurium has been reported to be among the most frequently isolated serotype associated with swine (Vieira-Pinto et al., 2006). Pigs can get infected with *Salmonella* at the farm, during transport and especially at the lairage environment of slaughterhouses (Boughton et al., 2007; Vieira-Pinto et al., 2007), and pre-slaughter contamination may lead to cross-contamination of pork carcasses and pork processing surfaces. The increased consumption of pork (the other white meat) in the United States along with the changing dynamics of animal production and consumer exposure has lead to challenges in the prevention and control of this organism (Foley and Lynne, 2008). The need for rapid and sensitive detection methods for routine testing continues to grow in order to prevent outbreaks and recalls caused by *Salmonella* contamination. *Salmonella* culture-based detection methods are highly sensitive but can take up to 5-7 days and are labor intensive (Okamura et al., 2008; USDA/FSIS, 2007). Real-time PCR (rt-PCR) methods allow the detection of increased fluorescence as DNA gets amplified, eliminating the need for gel electrophoresis. The melt temperature (Tm) of amplicons is analyzed in the real-time machine when fluorescent dyes (such as SYBR Green I) are used. However, a thermocycler, which may not be available in small processing facilities, is required for this automated process (Hara-Kudo et al., 2005).

Loop-mediated isothermal amplification (LAMP) is a novel rapid and simple nucleic acid amplification assay, that relies on an autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment and 6 specific target primers (Salehi et al., 2005). The reaction occurs at one temperature (60-65°C), thus only a simple waterbath is needed. The visual

detection is based on the formation of insoluble magnesium pyrophosphate which can be observed by visual turbidity or a simple turbidimeter. This LAMP assay has been successfully applied for the detection of many foodborne bacteria (Goto et al., 2007; Hara-Kudo et al., 2008; Karanis et al., 2007; Misawa et al., 2007; Ohtsuki et al., 2007; Wang et al., 2008; Yamazaki et al., 2008 and 2009; Yano et al., 2007) and viruses (Fukuda et al., 2007; Yoneyama et al., 2007). The LAMP assay has also been used for specific *Salmonella* detection in pure culture (Hara-Kudo et al., 2005; Okamura et al., 2008; Wang et al., 2008) and in food samples (Ohtsuka et al., 2005; Okamura et al., 2008; Techathuvanan et al., 2010a). The specificity of the *inv*A LAMP assay has been tested against various bacterial isolates and has shown no cross-reactivity (Hara-Kudo et al., 2005).

The incorporation of reverse-transcriptase (RT) targeting mRNA instead of DNA (as in PCR and LAMP assays) can allow the potential detection of live cells or recent contamination as mRNA has shorter half-life than DNA (Maurer, 2006). Recently, we successfully developed the RT-LAMP and rt-RT-PCR assays to detect *S.* Typhimurium from artificially contaminated pork products (Techathuvanan et al., 2010a and b). However, besides pork commodities, *Salmonella* is also found to be associated with lairage floors, the pork processing environment, and carcass rinses that can all be sources of contamination (Larsen et al., 2004; Swanenburg et al., 2001). This study was therefore designed to further explore the application of the newly developed RT-LAMP assay for *Salmonella* detection from spiked pork carcass rinses, to be used as a routine *Salmonella* diagnostic tool in the pork processing environment (using natural samples of carcass rinses, carcass swabs and environmental surfaces) and for comparison to culture-based and RT-PCR detection methods.

Materials and Methods

Bacterial strain and preparation of bacterial suspension

Salmonella enterica serovar Typhimurium DT 104 2582 was obtained from the University of Tennessee culture collection, cultured in trypticase soy broth (TSB; Difco Becton Dickinson Microbiology Systems, Sparks, MD) at 37°C for 24 h, and transferred at least twice at 24 h intervals prior to use. Overnight *S.* Typhimurium cultures were used for inoculation and as a positive control. Serial dilutions in phosphate buffered saline (PBS; pH 7.2; Difco), were used for inoculation. Inocula were enumerated on Xylose Lysine Tergitol 4 (XLT4) agar (Difco) after incubation at 37°C for 24 h.

Artificial contamination of pork carcass rinse samples with Salmonella

Twelve pork carcass rinse water samples were collected from the processing plant in sterile containers, immediately placed on ice during transportation and then stored at 4°C until analysis. All analyses were carried out within two weeks. One 500 ml portion of each pork carcass rinse samples was autoclaved to eliminate background bacteria for further use in spiking studies. Another 500 ml portion of carcass rinse water (untreated) was used in the natural sample studies.

For spiking studies, autoclaved rinse water samples (500 ml) were inoculated with 1 ml of 10^8 to 10^1 CFU/ml of *S.* Typhimurium. Spiked rinse water samples were aseptically sequentially filtered through sterile 20-25 µm filter paper (Whatman #4; Whatman, England), 11µm filter paper (Whatman #1; Whatman), and finally a 0.8 µm filtration unit (Nalgene, Nalge Nunc International, NY). Filter papers and filtration unit membranes were collected and placed in sterile stomacher bags containing 224 ml of freshly prepared Tetrathionate broth (TTB;

Difco). Samples were stomached for 2 min and either used directly for assay or incubated at 37°C for 10 h and then assayed. Each experiment was run in duplicate and replicated twice.

For comparison of detection, portions of the TTB were used for enumeration on XLT4 agar in 3 replicates and 2 portions of 5 ml were used for nucleic acid extraction for rt-RT-PCR or RT-LAMP assays. Samples of enriched TTB were serially diluted in PBS and plated on XLT4 agar and incubated at 37°C for 24 h before enumeration. Each experiment was run in duplicate and replicated twice.

Analysis of natural pork carcass and processing environment swabs, and rinse samples

Fourteen pork carcass swabs (from pork carcasses after slaughter) and 13 pork processing surface swabs were obtained from the processing plant. Samples were collected using Speci-Sponge®, a sterile sponge in a sterile Whirl-Pak bag (Nasco Whirl-Pak®, Fort Atkinson, WI). The Speci-Sponge® was pre-moistened with 10 ml PBS prior to wet swabbing 100 cm^2 of pork carcasses, including ham, belly, back, and leg regions of swine, or processing surfaces, including floor, counter top, cutting board and knife. The carcasses were swabbed as hide-on before (4 samples) and after (4 samples) first wash, and hide-off before (3 samples) and after (3 samples) final wash (total of 3 washes). Upon collection, samples were immediately placed on ice during transportation and then stored at 4°C until analysis. These natural samples were processed within 24 h of collection, without autoclaving or spiking.

Twelve carcass rinse water samples were collected from a local pork processing plant during the final wash before going to the trimming process. The rinse water samples were prepared as mentioned above, but without autoclaving or spiking. Serial filtrations were used as

described above for the artificially contaminated samples. Filter papers and membranes from each filtration unit were aseptically collected, stored at 4°C and processed within 2 weeks.

Pork carcass and surface swabs, and filter papers and membranes for carcass rinse water were enriched for culture-based detection using modified USDA-MLG methods (USDA/FSIS, 2007) or detection by RT-PCR or RT-LAMP assays. Each sample was pre-enriched in 225 ml BPW for 4 h. Then 25 ml of pre-enriched media was transferred into 225 ml TTB for further incubation at 37°C for 12 h. Portions of the TTB were used for enumeration on XLT4 and portions were used for nucleic acid extraction and molecular assays. Negative controls included autoclaved swab samples or filters from autoclaved rinse water and distilled deionized water; positive controls were autoclaved swab/rinse water samples inoculated with overnight cultures of *S*. Typhimurium. Serially diluted samples of enriched TTB were made in PBS and plated on XLT4 agar and incubated at 37°C for 24 h before enumeration. Typical black colonies were isolated and confirmed using biochemical tests, including inoculation in Triple Sugar Iron (TSI; Difco) agar and citrate slants (Difco).

Nucleic acid extraction and DNAse I treatment

Nucleic acid was extracted from un-inoculated TTB (negative control), un-inoculated swab or rinse samples (negative control), pre-enriched samples (natural or spiked), and overnight cultures of *S*. Typhimurium (positive control) using the TRIzol® extraction protocol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions as described earlier *(*Techathuvanan et al., 2010a and b*)*. Extracted RNA was passed through the QIAshredder (Qiagen, Valencia, CA) column and resuspended in RNAse-DNAse free water for immediate use or stored at -80°C until use. Each experiment was run in duplicate and replicated twice. A DNAse I treatment (Ambion, Austin, TX) following the manufacturer's instruction was carried out at 37^{o}C for 30 min for

removal of any possible carry-over DNA in the RNA samples. Nucleic acid samples with and without DNAse I treatment were used to compare detection sensitivity by PCR and rt-RT-PCR assays.

Analysis of nucleic acid quality

Absorbance ratios of nucleic acid extracts were measured at A260/A280 and A260/A230 using the NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE).

RT-LAMP assay

A modified LAMP protocol of Hara-Kudo et al. (2005) was used and converted to a reverse-transcriptase-LAMP (RT-LAMP) assay as described before (Techathuvanan et al., 2010a). Previously described 6 specific primers consisting of 2 inner primers, FIP (5'-GACGACTGGTACTGATCGATAGTTTTTCAACGTTTCCTGCGG-3') and BIP (5'-CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG-3'), 2 outer primers, F3 (5'-GGCGATATTGGTGTTTATGGGG-3') and B3 (5'-AACGATAAACTGGACCACGG-3') and 2 loop primers, (FLoop 5'-GACGAAAGAGCGTGGTAATTAAC-3', and BLoop 5'-GGGCAATTCGTTATTGGCGATAG-3'), were used to target the *Salmonella inv*A gene for amplification (Hara-Kudo et al., 2005). The reaction mixtures consisted of 0.04 μM of forward inner primer, 0.08 μM of reverse inner primer, 0.01 μM of each outer primer, 0.02 μM of each loop primer (Sigma-Genosys, St. Louis, MO), 1 mM dNTP, 0.8 M betaine (Sigma, St. Louis, MO), 10 mM $MgSO_4$, 8 U Bst polymerase large fragment (New England Biolabs, MA), 10X Thermopol Buffer (New England Biolabs, MA), and 5 μl of nucleic acid extract (treated or un-treated with DNase I) per 50 μl reaction along with 3.75 U avian myeloblastosis virus (AMV)-RTase (Invitrogen, Carlsbad, CA) (for RT-LAMP assays) as described earlier (Techathuvanan et

al., 2010a). Negative controls including RNAse-DNAse free water and nucleic acid extracts from TTB and autoclaved pork products, swab samples or filters from autoclaved carcass rinse water samples were used to determine any possible cross-reactivity or contamination (false positives). Positive controls included nucleic acid extracts of overnight cultures of *S.* Typhimurium and its serial dilutions, and autoclaved swab/rinse water samples inoculated with *S.* Typhimurium. The reaction mixture was incubated at 62°C for 90 min in a water-bath. All experiments were replicated twice.

Internal amplification control (IAC) for the PCR assay

The rt-RT-PCR reaction contained an IAC to eliminate false negatives as described by D'Souza et al. (2009). The DNAse I treated IAC product of 154 bp was diluted to the optimal determined concentration of 1.9fg/µl prior to use.

Real-time RT-PCR assay

Real-time RT-PCR was performed on the RNA extracts of the spiked pork carcass rinse samples and also natural samples (swabs, rinses) following the previously described procedure of D'Souza et al. (2009) and Techathuvanan et al. (2010b) in 50 µl reaction volumes. Cycling conditions included RT at 50ºC/30 min, denaturation at 95ºC/5 min, followed by 40 cycles at 95°C/30 s, 58°C/30 s, 72°C/30 s, and a final extension at 72ºC/7 min in a BioRad iCycler (BioRad, Hercules, CA). Post-amplification melt temperature (Tm) analysis from 50ºC to 95ºC with 0.5ºC increments was conducted to determine specific *inv*A product (Tm= 87.5^{o}C) and IAC product (Tm= 82^{o}C). The iCycler detection software was used to determine threshold cycle (Ct) and Tm values. Negative and positive controls were used as described in LAMP assay. All experiments were run in duplicate and were replicated twice.

Analysis of RT-LAMP products and rt-RT-PCR products

Ten microliter portions of the amplified products were also analyzed by agarose gel electrophoresis using 2% agarose gels (Promega, WI) in 1X Tris acetate-EDTA buffer (10 mM Tris-Acetate and 1 mM EDTA, Fisher BioReagents, NJ), stained with ethidium bromide (Bio-Rad, CA), and visualized under UV transillumination using the Gel-Doc Camera and Quantity One program (Bio-Rad, CA) as described before (Techathuvanan et al., 2010a). A 100-bp DNA ladder (Promega, Madison, WI) was used as a marker to determine the size of the rt-RT-PCR products and visualize the ladder pattern of the RT-LAMP products.

Results

Nucleic acid quantity and quality

For spiked rinse samples, our results showed A260/A280 ratios to be between 1.54 and 1.66 and A260/A230 ratios to be between 0.40 and 0.72, indicating some carryover protein and salt, respectively. The quantity of nucleic acid was found to be between 298.99 to 776.97 ng/µl for spiked carcass rinses and with lower values for the lower inocula. For natural samples, A260/A280 ratios were between 1.25 and1.89 and A260/A230 ratios were between 0.37 to 1.01. Nucleic acid quantity of 282.42 to 1156.26 ng/µl, 333.66 to 1240.94 ng/µl, and 69.47 to 692.88 ng/µl per 100 cm^2 of carcass swabs, 100 cm^2 of processing surface swabs, and 500 ml of carcass rinses were obtained, respectively.

S. *Typhimurium detection in spiked pork carcass rinse samples*

As expected for the rt-RT-PCR assay, Tm peaks at 87.5ºC for amplified *Salmonella inv*A products were obtained along with IAC products that showed Tm peaks at 82ºC, indicating the absence of false negative reactions. *Salmonella* positive samples showed the target amplified product at 347 bp on agarose gel electrophoresis, and negative samples showed the IAC product at 154 bp. For the RT-LAMP assay, the expected ladder pattern was observed on agarose gels indicating positive samples, where the products were comparable to the *Salmonella* positive (standard) control. The lowest inoculated detection limit of the rt-RT-PCR assay for *S.* Typhimurium was evaluated using Tm curves and confirmed by agarose gel electrophoresis. The rt-RT-PCR, RT-LAMP and culture-based assays gave similar detection limits up to 10^6 CFU/500 ml in carcass rinse water samples using the high inocula levels between 10^8 to 10^6 CFU without enrichment (Table 2.1). However, when the low inocula levels of 10^5 to $l0^1$ CFU were used, DNAse I treated samples did not show any detection (data not shown), without enrichment.

When pork rinse water samples were spiked with low inocula levels and enriched for 10 h in TTB followed by nucleic acid extraction, detection sensitivity was shown to increase. The rt-RT-PCR assay gave improved detection limits of 10^1 CFU/500 ml for spiked carcass rinses (Fig. 2.1). When samples were tested by the RT-LAMP assay, the detection limit was also 10^1 CFU/500 ml of carcass rinse water (Fig. 2.2). This is a significant improvement in detection of *Salmonella* in the pork environment requiring a total assay time of only 24 h that includes enrichment, nucleic acid extraction, and detection. When DNAse I treated nucleic acid extracts from10 h enriched samples were used in the rt-RT-PCR and RT-LAMP assays, detection limits dropped by about 1 to 2 $\log_{10}$ CFU/500 ml sample with low inocula levels (data not shown).

S. Typhimurium detection in natural samples

The results showed that 2/13, 3/14 and 1/12 samples of natural pork processing surface swabs, pork carcass swabs, and pork carcass rinse water samples tested positive by rt-RT-PCR assay, with Tm peaks at 87.5ºC and 347 bp products on agarose gels (Fig. 2.3). An exception is that 1 surface swab that showed positive by Tm analysis, did not show any target product at 347 bp on agarose gels (data not shown). When using RT-LAMP assays, *Salmonella* was detected from 2/13 pork processing surface swabs, 4/14 pork carcass swabs (Techathuvanan et al., 2010a), and 4/12 pork carcass rinse water samples with the same ladder pattern on agarose gels as compared to positive controls (autoclaved samples inoculated with *S.* Typhimurium as well as *Salmonella* pure culture) as shown in Fig. 2.4. As culture-based methods were used for comparison, the results showed that these methods with pre-enrichment and enrichment steps could detect *Salmonella* contamination from 4 pork processing surface swabs, 4 pork carcass swabs, and 4 pork carcass rinse water samples (Table 2.2). Black colonies obtained from XLT4 plates were enumerated, isolated and confirmed positive by TSI and citrate tests.

Screening of 39 natural samples from the local pork processing facility resulted in 12 positives by culture-based methods (30.8%), 6 positives by rt-RT-PCR (15.4%), and 10 positives by RT-LAMP assays (25.6%) with only 2 samples testing positive by all the 3 assays, from the total of 16 positives obtained out of a total of 39 (41.0% positive) tested samples. Comparing the isolates, RT-LAMP assay gave 7 out of 12 matched positives, while rt-RT-PCR gave 3 out of 6, to those found by culture-based methods. Four samples were determined to be positive by both RT-LAMP and rt-RT-PCR assays. For autoclaved samples, no positive results were obtained by rt-RT-PCR, RT-LAMP, or culture-based methods, indicating the absence of any no cross-reactivity (data not shown) from the food matrix (or any background flora).

Discussion

The current study shows that the detection sensitivity of *S*. Typhimurium by the RT-LAMP assay is comparable to rt-RT-PCR and culture-based assays when tested in spiked pork carcass rinses, but faster than these two methods. The RT-LAMP and RT-PCR assays showed no evidence of false negatives or any signs of interference by the tested sample matrices or culture media. In the setup of the filtration process for carcass rinses, the 20-25 µm pore-sized filter was used to remove flesh, fat, and other particles that would clog the filter while still letting bacteria in the permeate go through the filter. Then, the rinse sample was passed through the 11 µm pore-sized filter to screen out smaller particles. Finally, the 0.8 µm pore-sized filter was employed to recover all the target bacteria. All filters were collected and enriched as target bacteria may not only be on the last step filter but may also adhere to any other particles that remain on other filters. It is important to report that the described filtration protocol has some limitations. In some cases, the filter was clogged by the sample if the rinse sample contained high levels of solid particles, slowing down the process. It was possible to speed up the process by removing the clogged filter, replacing with a new fresh filter, and stomaching all filters used in the same 225 ml of BPW. As previously reported by other researchers, this procedure did not interfere with or compromise our results (Wolffs et al., 2006). The results obtained from the DNAse I treated nucleic extracts study are in agreement with our previous work (Techathuvanan et al., 2010a and b) showing decreased detection by at least 1 $\log_{10}$ CFU/sample compared to untreated nucleic acid extracts. By detecting mRNA, the food industry could be benefited for rapid monitoring and

validation of their inactivation processes to ensure proper process functioning and that the finished products contain no viable cells.

Salmonella detection in pork carcass rinses spiked with low inocula levels improved by 1-log using the RT-LAMP assay after enrichment compared to pork chop and pork sausage study (Techathuvanan et al., 2010a). For rt-RT-PCR and culture-based methods, the detection limits were the same for all tested spiked carcass rinses, similarly as reported for spiked pork products (Techathuvanan et al., 2010b). The filtration step used for carcass rinses could possibly contribute to the improved detection in rinses as it can holdup the process resulting in some level of bacterial growth. Also, the rinse water may contain lower content of inhibitors as compared to those in meat products. As is generally known, higher levels of fat, and/or protein or other complex food matrices in samples could result in interference of the molecular amplification reactions, decreasing detection sensitivity (Lampel et al., 2000; Rossen et al., 1992).

In 2006, Wolffs et al. (2006) reported quantification of cell numbers as low as 7.5 $X10^2$ CFU *Salmonella* per 100 ml chicken rinse and spent irrigation water and with occasional detection as low as 2.2 CFU/100 ml using SYBR Green I *inv*A based rt-PCR. Sequential use of decreasing pore-sized filtration units enabled the filtration of 400 ml of *Salmonella* spiked chicken carcass rinses in the study by Hoszowski et al. *(*1996) followed by total 21-h enrichment and then colony blot immunoassay to detect as low as ~10^1 CFU/400 ml, which is comparable to our results. Although, 100 ml of water for 2 to 2½ lb broiler carcasses was reported to be suitable for analysis (Cox et al., 1981), a 500-ml rinse sample was chosen in the present study. As the pathogens may be unevenly distributed on pork carcasses and low levels of contamination may be present, 500 ml rinse sample seems to be reasonable and sufficient sample representation for *Salmonella* detection in pork carcasses.

Several studies have been conducted to determine the prevalence of *Salmonella* contamination in pork and pork products (Banks and Board, 1983; Berends et al., 1998; Boughton et al., 2004), with significant lower prevalence reported in recent studies due to improved good manufacturing practices (GMP) and hazard analysis and critical control point (HACCP) schemes (Boughton et al., 2004; Ropkins and Beck, 2000). Recently, Duffy et al.(2001) revealed that 9.6% of pork and pork products from retail stores in 6 U.S. cities were contaminated with *Salmonella*, with 8.3 and 10.4% contamination of whole-muscle pork and enhanced pork, and 7.3 and 12.5% contamination of store-ground fresh pork and/or pork sausage and prepackaged ground pork and/or pork sausage, respectively. In our previous RT-LAMP study, 12.5% of pork chop and 16.7% of ground pork were found to be *Salmonella* positive (Techathuvanan et al., 2010a). However, not surprisingly, studies showed that natural carcass rinses, carcass swabs, and processing surface swabs were found positive for *Salmonella* at higher prevalence levels, such as in this study (Boughton et al., 2007; Larsen et al., 2004; Vieira-Pinto et al., 2006). Larsen et al. (2004) reported the *Salmonella* contamination levels ranging between 39-59% from 160 pork carcass content, meat, and swab samples, and 88% from the 16 lairage floor swab samples. Swanenburg et al. (2001) reported that 70-90% of samples collected from the lairage environment, including floor and wall surface swabs, and residing fluids on the floor, were contaminated with *Salmonella*. Pigs can become infected with *Salmonella* once they are exposed to contamination at preslaughter (Hurd et al., 2001). A considerably higher number of *Salmonella* positive pork samples from slaughterhouses (40%) were reported compared to 5.3% from farms (Hurd et al., 2002).

The RT-LAMP assay has advantages over RT-PCR assays, and gave the same detection probability as culture-based methods, but was faster. Also, it requires only a simple waterbath to

maintain one needed reaction temperature that can enable some medium and small sized food manufacturers to adopt this technology for routine monitoring of *Salmonella* in food products and processing environments. However, our procedure still requires enrichment to ensure the recovery of stressed/injured cells (Techathuvanan et al., 2001a and b). Future research should include lowering total assay time to improve RNA yield and purification that might result in the possibility of decreasing enrichment time. Moreover, there is a potential for developing this RT-LAMP assay to a real-time format by incorporation of fluorescent dyes and using simple hand-held fluorometers or turbidimeters. To ensure that the absence of false negatives, similar to PCR assay, the LAMP assay lacks an IAC and research is warranted in this area. In this present study, only the external positive and negative controls have been used. Overall, the RT-LAMP assay shows potential to be routinely used for the screening of *Salmonella* in the pork environment.

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Appendix

Table 2.1. Limits of detection of *Salmonella* Typhimurium from spiked pork carcass rinses by culture-based, RT-PCR and RT-LAMP assays.

Type of sample (inocula levels)	**Lowest inoculated detection limit, CFU/500 ml (no. of positive samples/no. tested) in:**		
	Culture-based methods	**RT-PCR assay**	**RT-LAMP assay**
Un-enriched Carcass rinse (10^8 to 10^6)	10^6 (4/4)	10^6 (4/4)	10^6 (4/4)
10-h enriched in TTB Carcass rinse (10^5 to 10^1)	10^1 (4/4)	10^1 (4/4)	10^1 (4/4)

Table 2.2. Comparison of *Salmonella* detection by traditional culture-based, rt-RT-PCR, and RT-LAMP assays among natural swab and rinse samples that tested positive.

Natural Sample	**Result using:**		
	Traditional Culture-Based Methods	**rt-RT-PCR Assay**	**RT-LAMP Assay**
Processing surface swab I	+	+	-
Processing surface swab II	+	-	-
Processing surface swab III	+	-	+
Processing surface swab IV	+	-	+
Processing surface swab V	-	+	-
Pork carcass swab I	+	-	-
Pork carcass swab II	+	+	+
Pork carcass swab III	+	-	-
Pork carcass swab IV	+	-	+
Pork carcass swab V	-	+	+
Pork carcass swab VI	-	+	+
Carcass rinse water I	+	-	-
Carcass rinse water II	-	-	+
Carcass rinse water III	+	+	+
Carcass rinse water IV	+	-	+
Carcass rinse water V	+	-	+
Total no. of positives/total no. of samples	**12/39**	**6/39**	**10/39**

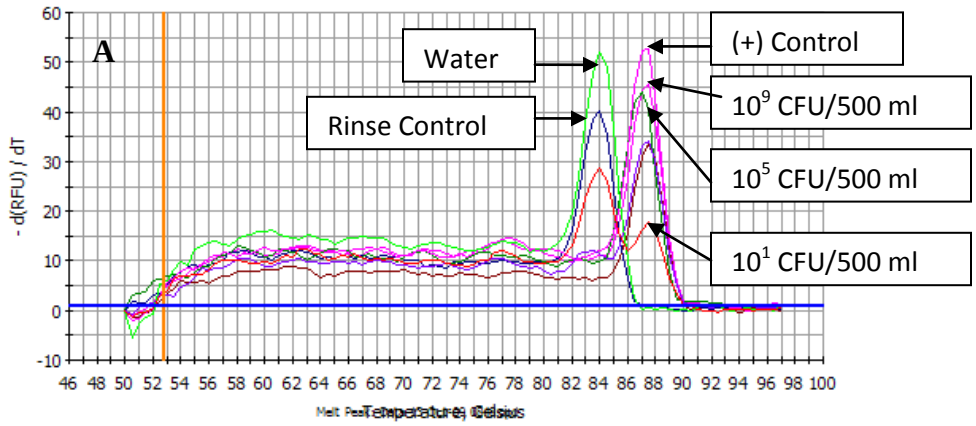

Figure 2.1. *Salmonella* detection in carcass rinse by RT-PCR assay:
(A) Melt temperature curves of the RT-PCR products from carcass rinse samples spiked with *Salmonella* and enriched at 37°C in TTB for 12 h showing specific speaks at 87.5°C. The peaks from the negative samples and the water control at 82°C show the presence of IAC products, depicting the absence of false negatives.

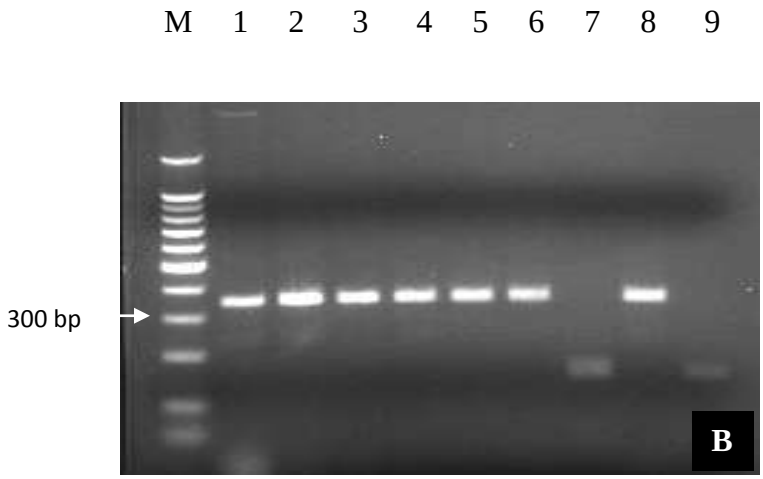

Figure 2.1. *Salmonella* detection in carcass rinse by RT-PCR assay (Continued):
(B) Agarose gel electrophoresis of RT-PCR products (347 bp *inv*A product and 154 bp IAC product) from carcass rinse samples spiked with *Salmonella* and enriched at 37°C in TTB for 12 h. Lane M: 100 bp DNA Marker; Lanes 1-5: 10^1-10^5 CFU/500 ml; Lane 6: 10^9 CFU/500 ml; Lane 7: negative un-inoculated carcass rinse control; Lane 8: Positive *Salmonella* control; Lane 9: negative water control.

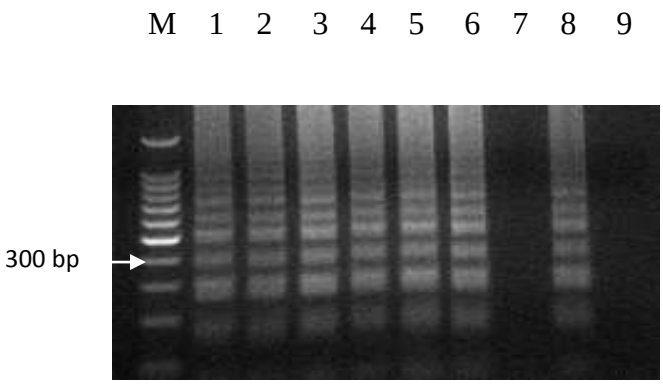

Figure 2.2. *Salmonella* detection in carcass rinse by RT-LAMP assay. Agarose gel electrophoresis of RT-LAMP products indicating *Salmonella* detection from spiked carcass rinse samples that were enriched at 37^{o}C in TTB for 12 h. Lane M: 100 bp DNA Marker; Lanes 1-5: 10^{1}-10^{5} CFU/500 ml; Lane 6: 10^{9} CFU/500 ml; Lane 7: negative un-inoculated carcass rinse control; Lane 8: Positive *Salmonella* control; Lane 9: negative water control.

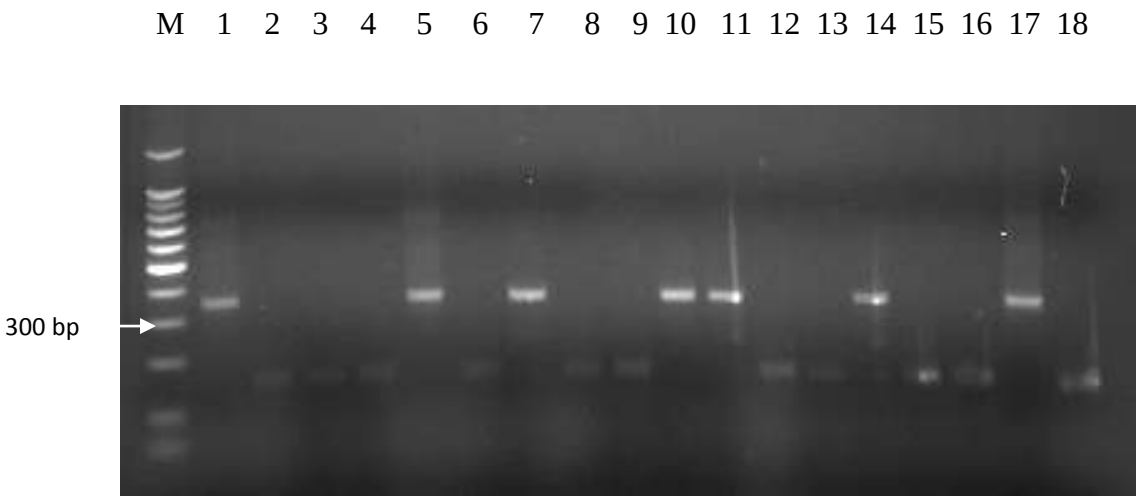

Figure 2.3. Agarose gel electrophoresis of RT-PCR products (347 bp *inv*A product and 154 bp IAC product) from natural samples obtained from the pork processing environment after enrichment in BPW for 4-h and in TTB for 12-h at 37^{o}C. Lane M: 100 bp DNA Marker, Lanes 1-5: pork processing surface swab I to V; Lanes 6-11: pork carcass swab I to VI; Lanes 12-16: pork carcass rinse water I to V; Lane 17: Positive *Salmonella* control; Lane 18: Water control.

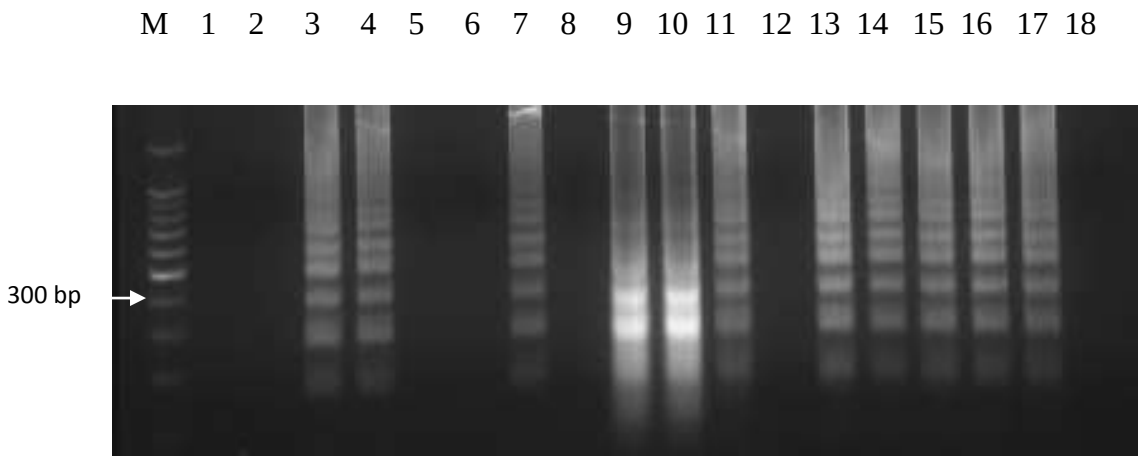

Figure 2.4. Agarose gel electrophoresis of RT-LAMP products from natural samples obtained from the pork processing environment after enrichment in BPW for 4-h and in TTB for 12-h at 37^{o}C. Lane M: 100 bp DNA Marker, Lanes 1-5: pork processing surface swab I to V; Lanes 6-11: pork carcass swab I to VI; Lanes 12-16: pork carcass rinse water I to V; Lane 17: Positive *Salmonella* control; Lane 18: Water control.

CHAPTER III

Optimization of Rapid *Salmonella* Enteritidis Detection in Liquid Whole Eggs by SYBR Green I-Based Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Reproduced with permission from the Foodborne Pathogens and Disease: "Techathuvanan C, D'Souza DH. 2011. Optimization of rapid *Salmonella* Enteritidis detection in liquid whole eggs by SYBR Green I-based real-time reverse-transcriptase polymerase chain reaction. Foodborne Path Dis. 8:527-534."

Abstract

Eggs and egg products have a high risk of *Salmonella* Enteritidis (*S*. Enteritidis) contamination causing gastroenteritis outbreaks in humans. Thus, a rapid screening tool for viable *S.* Enteritidis cells in the egg industry is needed. Our objective was to rapidly and sensitively detect viable *S.* Enteritidis from liquid whole eggs (LWE) within 24 h using SYBR green I-based real-time RT-PCR targeting the *Salmonella* specific *inv*A gene along with an internal amplification control in a Bio-Rad iCycler. LWE was inoculated with *S*. Enteritidis, mixed with tetrathionate broth and 100 µl of serially diluted portions in phosphate buffered saline were plated on Xylose Lysine Tergitol 4 agar or 5-ml were used for RNA extraction by the TRIzol method immediately or after enrichment of 6, 12, or 16 h at 37^{o}C. The real-time RT-PCR assay was carried out using previously described *Salmonella inv*A gene primers. Melt temperature analysis of the PCR product was included to determine *inv*A specific amplification. Without enrichment, the assay detection limit was 10^7 CFU/25 ml LWE. After enrichment for 6 and 12 h, *S.* Enteritidis could be detected from LWE up to 10^4 and 10^2 CFU/25 ml, respectively. Improved *S.* Enteritidis detection up to 10^0 CFU/25 ml was obtained after 16-h enrichment. Even with 16-h enrichment, the results could be obtained within 24-h, which is much faster than by traditional cultural detection that takes several days. Therefore, this assay appears suitable for routine detection of *S.* Enteritidis contamination by the egg industry to help prevent the transmission of egg associated *S.* Enteritidis outbreaks and timely recall of contaminated products.

Introduction

The prevalent nature and potential severity of salmonellae infection caused by contaminated food and water consumption has raised the awareness of the importance of detection and inactivation techniques among researchers and the food industry. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is reported to be the most frequent *Salmonella* strain associated with eggs and poultry which is capable of causing human disease (Betancor et al., 2010; Clavijo et al., 2006; FSA, 2004; FSA, 2007). *Salmonella* associated outbreaks have been reported to be the leading cause of foodborne outbreaks caused by bacteria (CDC, 2009). In the U.S. alone, the *Salmonella* related outbreaks cost more than $2.5 billion annually (ERS/USDA, 2008). Approximately 14% of all eggs in the U.S. are reportedly contaminated with *S.* Enteritidis, and their consumption can lead to infection (Ebel and Schlosser, 2000). The Centers for Disease Control and Prevention (CDC, 2005) reported that current epidemics of egg-associated *Salmonella* outbreaks are found to be related to intact and disinfected grade A eggs, where the contamination caused by infection of hen's ovary passes on to the eggs before the shells are formed. They report that approximately 1 of 10,000 eggs may be naturally internally contaminated by this organism in the northeastern U.S. Moreover, cross-contamination and post-processing contamination have also been implicated as a transmission route of *S.* Enteritidis. The UK Food Standards Agency reported that *S.* Enteritidis cases in the UK have been on the rise since mid-August 2009, with an increase of more than 30% since 2008 (from 137 to 443 cases) (FSA, 2009).

Though the detection speed and sensitivity can be improved by PCR methods, viable and dead cells cannot be distinguished by traditional PCR or real-time PCR assays which is based on

the amplification and detection of DNA. Reverse-transcriptase PCR (RT-PCR) assay is one potential technique that has been developed to overcome this drawback. The RT-PCR assay targets detection of mRNA that has a shorter half-life than DNA which typically represents presence of viable organisms or recent contamination (Maurer, 2006). RT-PCR could benefit the food industry to determine the efficacy of pathogen inactivation by control measures used during food manufacturing and could potentially allow for rapid detection of recent contamination with faster results than traditional culture based assays that can take several days.

Recently, our laboratory successfully demonstrated the application of a SYBR Green I real-time RT-PCR assay using newly described *inv*A gene primers for *S.* Typhimurium detection in pure cultures (D'Souza et al., 2009), as well as on produce items such as peppers (Miller et al., 2010a), lettuce and tomatoes (Miller et al., 2010b), and pork products (Techathuvanan et al., 2010). The goal of this research was to optimize and apply this molecular based real-time RT-PCR method for *S.* Enteritidis detection in LWE with increased speed and detection sensitivity.

Materials and Methods

Bacterial strain and preparation of bacterial suspension

S. Enteritidis strain H4267 from the University of Tennessee culture collection was cultured at 37°C for 24 h into trypticase soy broth (TSB; Difco Becton Dickinson Microbiology Systems, Sparks, MD). Cultures were transferred a minimum of two times after overnight intervals prior to use. For the study involving the ability of the assay to detect viable cells alone, and not dead cells, cultures were autoclaved at 121.1°C for 15 min and allowed to cool down at room temperature, before use. For determining the ability of the assay to detect cold-stressed

cells, overnight *S.* Enteritidis cultures were stored at 4ºC for 24 h before use. These various preparations of *S.* Enteritidis cultures were serially diluted in phosphate buffered saline (PBS; pH 7.2; Difco), and enumerated after spread plating 100 µl of each preparation on Xylose Lysine Tergitol 4 (XLT4) agar (Difco) and incubating at 37°C for 24 h to 48 h.

LWE preparation

LWE was prepared using commercialized large shell eggs with expiration dates > 2 weeks. Shell eggs were decontaminated by dipping in 5% trisodium phosphate (Difco) for 1 min and washing in sterile deionized water, air dried under UV light for 10 min, aseptically cracked under a BSL-2 hood, and stomached for 1 min in sterile stomacher bags. The pH of LWE was measured to be 7.5 to 8.0. LWE was either used immediately or stored at -20°C until use. Twenty-five ml of prepared LWE samples were enriched separately in 225 ml of buffered peptone water (BPW; Difco Becton Dickinson Microbiology Systems, Sparks, MD) or in Tetrathionate broth (TTB; Difco) for 16 h at 37°C before diluting in 1X PBS and 100 µl were plated on trypticase soy agar (Difco) and XLT4 agar and incubated at 37°C for 24 to 48 h to determine the initial bacterial load or any *Salmonella* contamination.

Artificial contamination of LWE

LWE samples were thawed at 4ºC prior to use. Twenty-five ml of LWE samples were inoculated with 1 ml of overnight *S.* Enteritidis inocula ranging from 10^9 to 10^0 CFU/ml. Samples were then stomached in sterile stomacher bags containing 224 ml freshly prepared TTB for 2 min. Inoculated LWE samples in TTB were then either immediately assayed or incubated for enrichment at 37°C for 6, 12, or 16 h and then assayed for *Salmonella* detection.

Similarly for overnight cold stressed *S*. Enteritidis, 1 ml of 10^4 to 10^0 CFU/ml of were inoculated into 25-ml LWE samples. The inoculated samples were then mixed with 224 ml of BPW or TTB prior to incubation at 37°C for 16 h and assayed. Also, 25-ml portions of inoculated LWE in BPW after 3 h incubation were transferred into 225 ml of TTB and then further incubated at 37°C for 16 h and then assayed.

Additionally, 1 ml of autoclaved *S*. Enteritidis cells ranging from 10^9 to 10^0 CFU/ml were inoculated in 25 ml LWE samples and enriched as described above for stressed cells and assayed. All experiments were run in duplicate and repeated at least twice.

Nucleic acid extraction

Nucleic acid was extracted from 1 ml of *S*. Enteritidis pure culture or 5 ml each of un-inoculated TTB (negative control), un-inoculated LWE (negative control), inoculated LWE samples, and overnight cultures of *S*. Enteritidis (positive control) using the TRIzol® extraction protocol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The RNA extracts were passed through the QIAshredder column (Qiagen, Valencia, CA) to improve the quality of nucleic acid and possibly remove any residual inhibitors of the RT-PCR assay. Purified RNA samples were either used immediately or stored at -80°C until use. Each experiment was run in duplicate and replicated twice.

DNAse I treatment

A DNAse I treatment (Ambion, Austin, TX) was carried out at 37°C for 30 min to degrade any possible carry-over DNA in RNA samples by following the manufacturer's instructions. RNA samples before and after DNAse I treatment were used to compare the detection sensitivity by real-time RT-PCR and traditional PCR assays.

Analysis of nucleic acid quantity and quality

Quantity and quality of nucleic acid were determined by using the NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE). Quantity of nucleic acid in ng/µl was determined using absorbance at 260. Absorbance ratios of nucleic acid extracts were measured at A260/A280 and A260/A230 to determine potential protein contamination or salt/organic carry-over, respectively. RNA samples with absorbance ratios >1.8 are typically considered as optimal.

Preparation of the internal amplification control

The internal amplification control (IAC) was included in the real-time RT-PCR reaction as previously reported (D'Souza et al., 2009). Briefly, the *stx1* primer set was designed from the shiga toxin region of *E. coli* O157:H7 DNA using the Beacon Designer Software (BioRad, Hercules, CA) to obtain a product of 109 bp. The forward (containing a T7 RNA promoter sequence) and reverse *Salmonella inv*A primers were coupled to the *stx1* forward and reverse primers, respectively to amplify a 182 bp product. RNA was amplified using the MEGAscript T7 Transcription Kit (Ambion). The amplified DNAse I treated RNA product of 154 bp was diluted to the optimal concentration (1.9fg/µl) prior to use as an IAC in the real-time RT-PCR assay.

Real-time RT-PCR assay, PCR assay and traditional cultural detection

Real-time RT-PCR (rt-RT-PCR) was performed on the RNA extracts of *S.* Enteritidis pure culture, un-inoculated TTB, un-inoculated LWE, and inoculated LWE samples. Fifty microliter reactions containing 5 µl RNA extracts in RNAse-DNAse free water, SYBR Green I Superscript III (SSIII) one-step RT-PCR kit reagents (Invitrogen), 0.02 µM of each *inv*A primer (previously described Forward primer: 5'-CACGCTCTTTCGTCTGGCA-3'; Reverse primer: 5'-TACGGTTCCTTTGACGGTGCGA-3' (D'Souza et al., 2009), bovine serum albumin (BSA;

0.06 µg/µl) and IAC (1.9 fg/µl) were used. Cycling condition included RT at 50ºC/30 min, denaturation at 95ºC/5 min, followed by 40 cycles at 95°C/30 s, 58°C/30 s, 72°C/30 s, and a final extension at 72ºC/7 min in a BioRad iCycler (BioRad). Post-amplification melt temperature (Tm) analysis from 50ºC to 95ºC with 0.5ºC increments was conducted to determine specific *inv*A product (Tm= **87.5°C**) and IAC product (Tm= 82°C) as previously described (Miller et al., 2010a, 2010b; Techathuvanan et al., 2010). The iCycler detection software was used to determine threshold cycle (Ct) and Tm values. Negative controls included RNAse-DNAse free water and nucleic acid extracts from un-inoculated TTB and un-inoculated LWE samples to determine any possible cross-reactivity or contamination (false positives). The positive control included nucleic acid extract from overnight cultures of *S.* Enteritidis. The IAC was used in the reaction to determine any reaction failure and to eliminate false negatives. Samples were analyzed for detection of the lowest inoculated level of *S.* Enteritidis. For detection comparison, samples were also analyzed by traditional PCR and traditional cultural methods. In the 50 µl PCR reaction, 5-µl portions of RNA samples were added into the reaction mix containing 0.03 µM of the same set of *inv*A primers used in the rt-RT-PCR assay, and Platinum® PCR SuperMix (Invitrogen). The PCR reactions were conducted as described in RT-PCR reaction, but without RT and Tm analysis steps in a Mastercycler® gradient thermocycler (Eppendorf, Hamburg, Germany). For detection by direct cultural plating, *S.* Enteritidis pure culture and portions of unenriched or enriched TTB with inoculated LWE were also used for enumeration on XLT4 agar. *S.* Enteritidis pure culture in TSB or inoculated LWE samples in TTB were serially diluted in PBS, spread plated on XLT4 agar and incubated at 37°C for 24 to 48 h before enumeration. All experiments were run in duplicate and were replicated twice.

Analysis of real-time RT-PCR and PCR products

The amplified real-time RT-PCR and PCR products were analyzed by agarose gel electrophoresis on 2% agarose gels (Promega, Madison, WI) in 1X Tris acetate-EDTA buffer (10 mM Tris-Acetate and 1 mM EDTA, Fisher BioReagents, NJ), followed by staining with ethidium bromide (Bio-Rad, Hercules, CA). Products on gels were observed by the Gel-Doc Camera and Quantity One program (Bio-Rad, Hercules, CA) under UV transillumination. A 100 bp DNA ladder (Promega, Madison, WI) was used as a marker for product size comparison. The results were also reported as a lowest inoculated detection level of each sample.

Results

Nucleic acid quality and quantity

The quantity of nucleic acids, and A260/A280 and A260/A230 ratios of samples from *S.* Enteritidis pure culture in TSB, unenriched inoculated LWE samples and enriched inoculated LWE samples with and without the DNAse I treatment are shown in Table 3.1. Our results showed that increasing the enrichment time to 16 h gave the highest yield of nucleic acid corresponding to the inocula levels. The amount of RNA extracted was directly proportional to the inocula level. Also, as the inocula level increased, the purity of nucleic acids extracted seemed to increase.

Specificity and sensitivity of real-time RT-PCR assay

Melt temperature (Tm) analysis was used for specificity determination. Our results revealed that *S.* Enteritidis positive samples showed the specific Tm peaks at 87.5ºC, while the

Tm peaks at 82ºC were obtained as expected indicating IAC amplification that depicts the absence of PCR inhibition (Fig. 3.1A). Detection of each sample was also determined by agarose gel electrophoresis for confirmation and research purposes only, though not required for real-time assays and when applied in real-world scenarios. RT-PCR products from *S.* Enteritidis positive LWE samples (*inv*A gene amplicons) and the IAC product showed bands at 347 and 154 bp, respectively as expected by agarose gel electrophoresis (Fig. 3.1B). This confirmed the detection sensitivity at 10^6 CFU/ml for pure overnight culture *S.* Enteritidis similar to that as determined by the iCycler software. All negative controls used in the study, including un-inoculated TTB, un-inoculated LWE, and water did show only the IAC product with the Tm peak at 82ºC and the 154 bp product by gel electrophoresis, without any Tm peak at 87.5ºC Tm or 347 bp product on agarose gels, as expected.

After the pure culture samples were treated with DNAse I, the RT-PCR assay showed detection at 10^9 CFU/ml (Fig. 3.1C), while the DNA-based PCR assay showed no detection of *S.* Enteritidis (Fig. 3.1D), indicating the absence of any carry-over DNA in the RNA extracts.

Dead (autoclaved) cell detection by real-time RT-PCR and PCR assays

Our results showed that the autoclaved *S.* Enteritidis pure cultures tested positive by traditional PCR assays using the *invA* gene primers, but negative by the real-time RT-PCR assay. However, un-autoclaved live cells showed positive detection by both PCR and RT-PCR assays, as expected (data not shown) as well as cultural plating. The results obtained from unenriched LWE inoculated with autoclaved and unautoclaved *S.* Enteritidis were in agreement with the results obtained using pure cultures (data not shown).

Salmonella ***detection in unenriched LWE***

The detection limit of inoculated LWE products with 10^8 to 10^5 CFU/25 ml without enrichment was 10^7 CFU/25 ml by the real-time RT-PCR assay, while traditional cultural methods could detect up to 10^5 CFU/ 25 ml (Table 3.2).

Salmonella ***detection in enriched LWE***

Salmonella detection limits of inoculated LWE samples improved to 10^4, 10^2 and 10^1 to 10^0 CFU/25 ml of LWE after 6, 12 (Table 3.2) and 16-h enrichment (Fig. 3.2A and B), respectively. The traditional plating method showed the lowest inoculated detection level at 10^3, 10^1 and 10^0 CFU/25 ml after inoculated LWE samples were enriched for 6, 12 and 16 h, respectively (Table 3.2).

The detection limits were also determined once nucleic acids were treated with DNAse I treatment. The real-time RT-PCR assay showed detection of *S*. Enteritidis at 10^2 CFU/25 ml after 16-h enrichment, while the traditional PCR assay resulted in the detection limit of 10^3 CFU/25 ml (Fig. 3.2C and D).

Cold stressed* S. *Enteritidis detection in inoculated LWE

After enrichment at 37°C for 16 h in BPW, overnight cold stressed *S*. Enteritidis could be detected at 10^2 CFU/25 ml (Table 3.3). Improved detection of the stressed cells was obtained by enrichment in TTB at 37°C for 16 h or in BPW for 3 h followed by TTB for 16 h at 37°C, where the real-time RT-PCR assay showed the detection limit at 10^0 CFU/25 ml (Table 3.3).

Discussion

In order to improve the detection speed and sensitivity of *S*. Enteritidis in LWE, optimization of RNA extraction, RNA purification, and optimized RT-PCR conditions along with determination of optimal time for sample enrichment were carried out. The TRIzol method was used for RNA extraction from LWE, as LWE is high in protein and lipid content (AEB/CL, 2006), The TRIzol method was found to be suitable for RNA extraction from LWE as it contains phenol and chloroform that help in protein and lipid removal for better RNA quality. However, our results during optimization suggested that the RNA extracts contained high amounts of salt/organic carry-over. Our final step utilized the QIAshredder column to help obtain better results. However, further refinements and improvements in the RNA extraction process are necessary as evident from our quality of nucleic acids based on absorption ratios at 260/280 and 260/230. The optimized RT-PCR conditions as reported from our previous research (D'Souza et al., 2009; Miller et al., 2010a and b; Techathuvanan et al., 2010) were also suitable for this assay without any further modifications.

Our results showed that LWE constituents or enrichment culture media (TTB) did not have inhibitory effects or interfere with the real-time RT-PCR assay. Melt temperature (Tm) peaks and amplified products on agarose gels were clearly obtained as expected. Our previous study showed that this real-time RT-PCR assay did not have cross-reactivity against several foodborne bacterial pathogens (Techathuvanan et al., 2010). Although the real-time RT-PCR assay can provide rapid results with simultaneous confirmation by Tm analysis, agarose gel electrophoresis was used for confirmation purposes in this study. On some occasions, *S*. Enteritidis detection from inoculated LWE determined by agarose gel electrophoresis was 1 order of magnitude (1 $\log_{10}$ CFU) lower in sensitivity as compared with the results obtained by fluorescence detection in the real-time thermocycler. This confirmed the advantage of fluorescence detection over

agarose gel electrophoresis, and chemical or colorimetric reactions as suggested by Gao et al. (2009).

Enrichment time of inoculated LWE samples is another variable that was investigated. Enrichment times of 6, 12 and 16 h were analyzed for the ability to increase detection sensitivity of the assay in comparison to unenriched samples. When enrichment was not included, *S.* Enteritidis could be detected up to 10^7 CFU/25 ml of LWE compared to the detection limit of 10^6 CFU/25 g *S.* Typhimurium from pork samples obtained from our previous study (Techathuvanan et al., 2010), thus showing only about 1-$\log_{10}$ CFU/ml difference between the two products. When compared with the study of Miller et al. (2010a) for *S.* Typhimurium detection from jalapeño and serrano peppers by real-time RT-PCR, similar detection limits at 10^7 CFU/25 g sample without enrichment were obtained. As shown in the results, the real-time RT-PCR assay gave improved detection sensitivity after increasing the enrichment time. Comparable detection sensitivity to traditional cultural methods at 10^0 to 10^1 CFU/25 ml could be obtained after 16-h enrichment using the real-time RT-PCR assay. Although an extensive 16 h enrichment period was required, the entire assay could be completed within 24 h (16 h for enrichment, 2 h for nucleic acid extraction, and 4 h for real-time RT-PCR reaction). This is considered to be much faster than traditional cultural detection techniques that could take up to 7 days (US FDA, 2007; Tomas et al., 2009). The enrichment process will also ensure the detection of injured cells (Gurtler, 2009) when present in inadequately pasteurized LWE and undercooked eggs and egg products or when eggs become contaminated with heat resistant strains and/or heavy bacterial loads. In addition, *S.* Enteritidis cells in contaminated LWE can become stressed during cold storage and the product can be result being tested as a false negative when assayed without enrichment. This is an important fact because at room temperature, these stressed bacteria can

recover and multiply within eggs and egg products and reach high levels of contamination (Lublin and Sela, 2008). Our results showed that as low as 10^0 CFU/25 ml (based on replicate assays and estimated plate counts) of the 4°C stressed *S*. Enteritidis could be detected by the real-time RT-PCR assay after 16-h enrichment in TTB.

When compared to several previous studies for *Salmonella* detection in food matrices that used similar enrichment times, our results showed comparable or even better detection. Recently, Miller et al. (2010a) showed that the real-time RT-PCR assay based on the *inv*A gene could detect the 10^4 CFU/25 g of jalapeño and serrano peppers spiked with *S*. Typhimurium after 6-h enrichment in BPW, which is in agreement with our results with the same enrichment time. Mercanoglu et al. (2009) have demonstrated a combined immunomagnetic separation-polymerase chain reaction assay to detect *S*. Enteritidis in milk, showing similar detection limits at 10^0 -10^1 CFU/ml after 16-h enrichment; however, an additional step of magnetic separation, requiring at least an additional 1 h or more is required. Rijpens et al. (1999) reported a PCR assay that requires enrichment at 37°C for at least 16 h to detect 47 CFU/25 g spiked ice-cream, cheese, milk powder, egg yolk powder, and pasteurized egg yolk. De Medici et al. (2003) also used a PCR assay for *S.* Enteritidis detection in poultry with SYBR Green I using *sef*A gene primers with incubation at 37°C for 18-20 h; however, the detection limit was not determined. In 2004, Malorny et al. (2004) demonstrated that real-time PCR could successfully detect *Salmonella* in fish fillets, carcass rinses and chicken, minced meat, and raw milk after 20-h pre-enrichment. Our study with shorter enrichment times showed comparable detection to others that had longer assay times, such as an *inv*A gene based PCR assay requiring an overnight enrichment for *Salmonella* detection in chicken carcass rinses, ground beef, ground pork and raw milk which showed detection limits of 3 CFU/25 g or 25 ml (Chen et al., 1997). However,

recently, Löfström et al. (2009) have shown to successfully reduce the enrichment time for *Salmonella* detection using a DNA-based real-time PCR assay with a the total analysis time of 14 h for meat samples and 16 h for carcass swab samples with the detection limit of 1-10 CFU/25 g.

Several alternative rapid detection approaches for *Salmonella* detection in eggs have also been developed and evaluated. Fluorescence polarization and lateral flow immunodiffusion assays can both provide results within 15 min; however, the detection sensitivity is quite low. Gast et al. (2003) revealed that these assays require 72-h enrichment in order to detect 10 CFU/ml of *S*. Enteritidis in LWE. Recently, a novel technique using a mouse macrophage cell line to isolate and enrich, coupled to PCR to detect *S.* Enteritidis in shell eggs was successfully demonstrated, showing a detection limit of10 CFU/ml after a10-h intracellular multiplication of *Salmonella* (Day et al., 2009). In the case of screening, the presence or absence of *S*. Enteritidis in chickens or eggs could be determined by another alternative, a piezoelectric quartz crystal based sensor (Su et al., 2001). Although it is a 15-min response, only a positive or negative result can be provided.

To conclusively show that the detection by RT-PCR assay is based on the detection of RNA, the DNAse I treatment was conducted to remove any possible DNA carry-over in RNA samples. In pure culture, the detection limit of *S*. Enteritidis decreased by 3 $\log_{10}$ CFU/ml using real-time RT-PCR assay with DNAse-treated RNA, with no observed detection by the DNA-based PCR assay. Although DNA carry-over in pure culture was shown to be removed by this treatment, only partial DNA carry-over from LWE samples could be eliminated. When RNA samples from inoculated LWE were treated with DNAse I, the detection dropped by 1 to 2 $\log_{10}$ CFU/25 ml using the real-time RT-PCR assay. However, the detection by traditional PCR after

DNAse I treatment in inoculated LWE samples showed that the DNA carry-over from the samples could not be entirely removed, as one would expect. The detection by the PCR assay was 1 $\log_{10}$ CFU lower than by real-time RT-PCR assay with the same DNAse I treated samples. These results suggested that the food matrices may have some interfering or inhibitory effect on the process which may result in incomplete elimination of DNA. Some cations, such as manganese, have been found to inhibit or retard DNAse enzyme activity (Shukla et al., 1976). As reported by the American Egg Board, LWE contains 9.7% of lipid content and several cations (AEB/CL, 2006) which may affect the activity of the DNAse I enzyme. Though the detection sensitivity is lowered, the food industry could still benefit from this assay as killed/inactivated cells which still contain DNA can lead to the false positives and misinterpretation of inactivation protocols. Through the detection of mRNA from viable cells in finished products, the inactivation processes can be validated to ensure implementation of proper control strategies.

Although this SYBR Green I-based RT-PCR assay is less expensive based on cost per analysis compared to real-time RT-PCR using TaqMan probes (Miller et al., 2010a), it still involves a high cost for the initial set-up involving thermocycling equipment. This might limit some small-scale producers from employing this technology in their safety and quality assurance systems.

Other novel detection methods now being researched that show promise for routine surveillance include isothermal amplification assays that do not require expensive thermal cyclers. However, they have their own drawbacks and need further investigation before they can be deployed in field testing or for routine analysis. For e.g., the nucleic acid sequence-based amplification (NASBA) protocol, not only requires three expensive enzymes, compared to two enzymes used in the real-time RT-PCR step, and expertise to perform the essay, but it also

requires long enrichment periods for detection of low level of *Salmonella* in foods (Simpkins et al., 2000; D'Souza and Jaykus, 2003). Another novel isothermal method, reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) assay is also gaining popularity for pathogen detection. Even though the DNA-based LAMP assay has been explored for *Salmonella* detection in LWE (Ohtsuka et al., 2005), the RT-LAMP needs to be explored for the application of viable *Salmonella* detection in LWE and is one of our current research goals/projects. Yet, the need for an IAC still exists to eliminate the possibility of false negatives, as currently only external controls are used. The nucleic acid dyes for viability measurement, such as ethidium monoazide and propidium monoazide, coupled with nucleic acid amplification assays (PCR assays) have as well gained a lot of interest in the recent years (Chang et al., 2009; Josefsen et al., 2010; Wagner et al., 2008). These dyes can be used for differentiation of dead and viable cells due to their ability to enter the cytoplasm of dead cells and cleave the DNA by photoactivation (Soejima et al., 2007). Consequently, the DNA from dead cells cannot be amplified by nucleic acid amplification assays (such as PCR), and thus will not interfere with the detection of viable cells and will not result in false positive results from the presence of dead or inactivated cells. The incorporation of these nucleic acid dyes into PCR or LAMP assays for detection comparison are part of our future goals.

Overall, our results are in agreement with previous research which shows that 16-h enrichment is still required to obtain higher detection sensitivity. Therefore, future research needs to focus on decreased enrichment time and improvement of RNA yield and purity that can potentially result in faster detection within two 8-h working shifts.

Conclusions

In conclusion, a robust real-time RT-PCR assay was optimized for the detection of viable *S*. Enteritidis from LWE within 24 h. The detection of 10^1 to 10^0 CFU/ 25 ml *S*. Enteritidis in LWE within 24 h includes the time for enrichment that shows potential for routine monitoring of contamination by the egg industry. Therefore, this rapid assay can be used as a powerful tool to help prevent and curb outbreaks and recalls associated with *S.* Enteritidis contamination in eggs and the egg environment. However, this assay cannot differentiate between the *Salmonella* serovars or identify the serovar present; it can only detect the presence of *Salmonella enterica*. Serotyping or other assays remain necessary to further identify the exact serovar, if needed.

Acknowledgements

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Appendix

Table 3.1. Nucleic acid quantity and quality determined by NanoDrop Spectrophotometer.

Sample/Enrichment	Inocula level* (CFU/ml or CFU/25 ml)	Nucleic acid quantity (ng/µl)	A260/A280	A260/A230
Without DNAse I treatment				
SE/No Enrichment	10^0-10^9	21.89 – 75.90	1.56 – 1.66	0.60 – 0.93
LWE+SE/No Enrichment	10^5-10^9	22.07 – 349.43	1.20 – 2.28	0.38 – 1.29
LWE+SE/6 to 16 h in TTB	10^0-10^7	5.82 – 1957.18	1.46– 2.04	0.25 – 1.65
LWE+Stressed SE/16 h in BPW	10^0-10^4	108.75 – 267.51	1.04 – 1.66	0.29 – 0.54
LWE+Stressed SE/16 h in TTB	10^0-10^4	163.02 – 1033.85	0.87 – 2.06	0.36 – 2.58
LWE+Stressed SE/3 h in BPW and 16 h in TTB	10^0-10^4	251.54 – 575.26	1.63 – 1.96	0.40 – 1.02
With DNAse I treatment				
SE/No Enrichment	10^0-10^9	1.06 – 27.28	1.00 – 2.26	0.18 – 0.48
LWE+SE/6 to 16 h in TTB	10^0-10^7	7.38 – 215.66	1.38 – 2.10	0.22 – 1.86

*CFU/ml for *S.* Enteritidis pure culture samples, and CFU/25 ml for LWE samples

SE denotes *S.* Enteritidis

Table 3.2. Detection limits of *S.* Enteritidis from pure culture and overnight *S.* Enteritidis spiked LWE samples by traditional cultural plating and by real-time RT-PCR assays, with and without enrichment in TTB.

Sample	**Inocula Levels (CFU/ml)**	**Lowest Inoculated Detection Limit (CFU/ml of pure culture or CFU/25 ml of LWE)**	
		Plating Method	**real-time RT-PCR Assay**
S. Enteritidis pure culture	10^9-10^0	10^0	10^6
LWE without enrichment	10^9-10^5	10^5	10^7
LWE with 6-h enrichment	10^7-10^3	10^3	10^4
LWE with 12-h enrichment	10^5-10^1	10^1	10^2
LWE with 16-h enrichment	10^4-10^0	10^0	10^1-10^0

Table 3.3. Detection of overnight cold stressed *S.* Enteritidis in LWE by real-time RT-PCR assay after enrichment at 37°C in BPW, TTB, and BPW and TTB.

Inocula level (CFU/25 ml)	**Enrichment**		
	16 h in BPW	**16 h in TTB**	**3 h in BPW and 16 h in TTB**
10^4	+	+	+
10^3	+	+	+
10^2	+	+	+
10^1	-	+	+
10^0	-	+	+

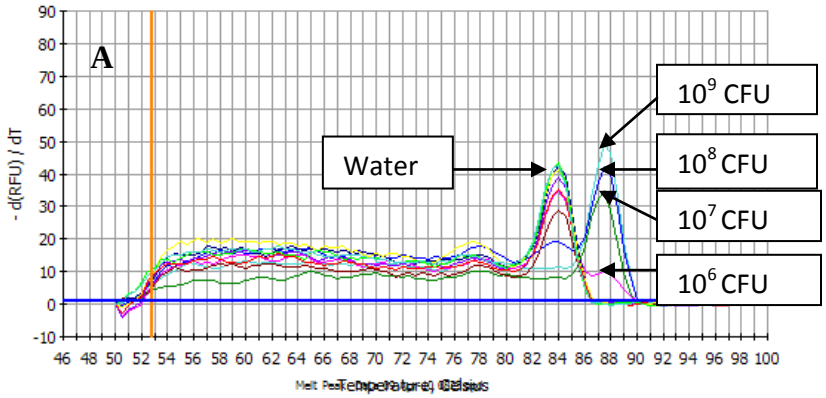

Figure 3.1. *Salmonella* Enteritidis detection in pure culture by RT-PCR assay.
(A) Melt temperature curves of the RT-PCR products from 1-ml overnight pure culture of *S.* Enteritidis showing specific speaks at 87.5^{o}C. The peaks from the negative samples and the water control at 82^{o}C shows the presence of IAC products. Peaks correspond to the amount of fluorescence detected.

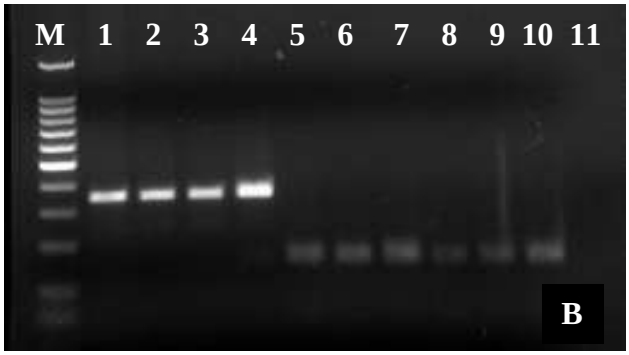

Figure 3.1. *Salmonella* Enteritidis detection in pure culture by RT-PCR assay (Continued).
(B) Agarose gel electrophoresis of the RT-PCR products from 1-ml overnight pure culture of *S.* Enteritidis showing 347 bp *inv*A product and 154 bp IAC product. M: 100 bp Marker, Lanes 1-10: 10^{9}- 10^{0} CFU/ml; Lane 11: Negative water control.

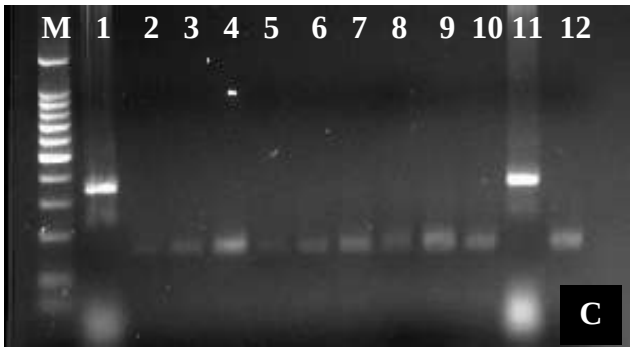

Figure 3.1. *Salmonella* Enteritidis detection in pure culture by RT-PCR assay (Continued). (C) Agarose gel electrophoresis of the RT-PCR products from 1-ml overnight pure culture of *S.* Enteritidis with DNAse I treatment showing 347 bp *inv*A product and 154 bp IAC product. M: 100 bp Marker, Lanes 1-10: 10^9- 10^0 CFU/ml; Lane 11: overnight *S.* Enteritidis positive control; Lane 12: Negative water control.

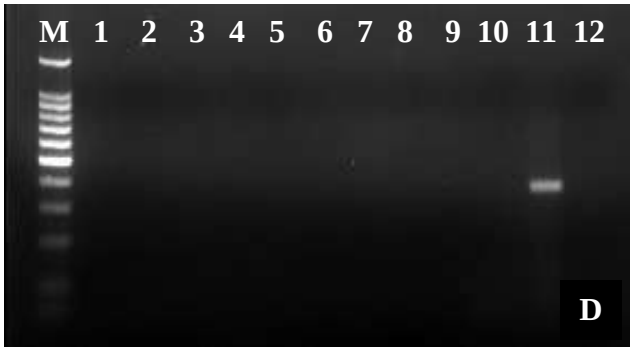

Figure 3.1. *Salmonella* Enteritidis detection in pure culture by RT-PCR assay (Continued). (D) Agarose gel electrophoresis of the PCR products from 1-ml overnight pure culture of *S.* Enteritidis with DNAse I treatment showing 347 bp *inv*A product. M: 100 bp Marker, Lanes 1-10: 10^9- 10^0 CFU/ml; Lane 11: overnight *S.* Enteritidis positive control; Lane 12: Negative water control.

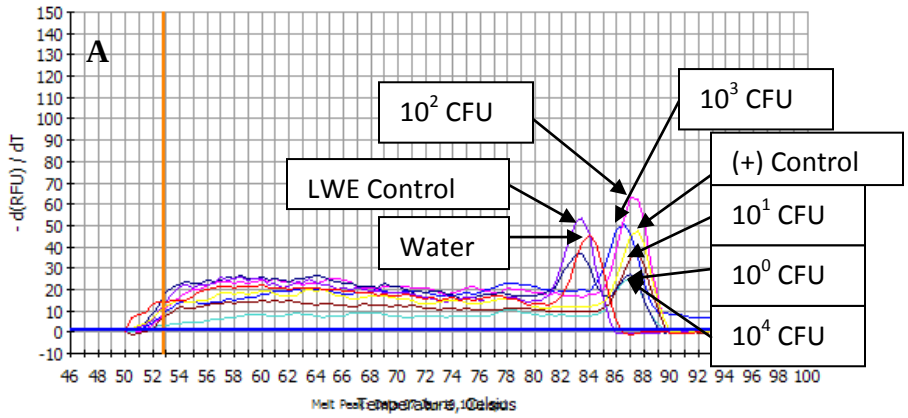

Figure 3.2. *Salmonella* Enteritidis detection in LWE by RT-PCR assay.

(A) Melt temperature curves of the RT-PCR products from 16-h enriched LWE spiked with *S.* Enteritidis at 37°C in TTB showing specific speaks at 87.5°C. The peaks from the negative samples, the un-inoculated LWE control and the water control at 82°C shows the presence of IAC products.

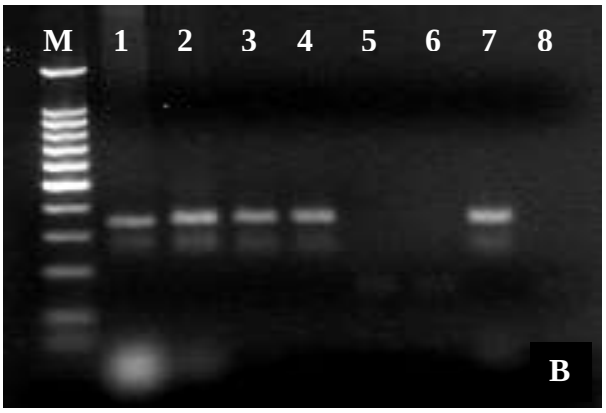

Figure 3.2. *Salmonella* Enteritidis detection in LWE by RT-PCR assay (Continued).

(B) Agarose gel electrophoresis of the RT-PCR products from nucleic acid extracts of LWE spiked with *S.* Enteritidis in TTB after 16-h enrichment at 37ºC showing 347 bp *inv*A product and 154 bp IAC product. M: 100 bp Marker, Lanes 1-5: 10^4-10^0 CFU/ml; Lane 6: un-inoculated LWE control; Lane 7: Positive *Salmonella* control; Lane 8: Negative water control.

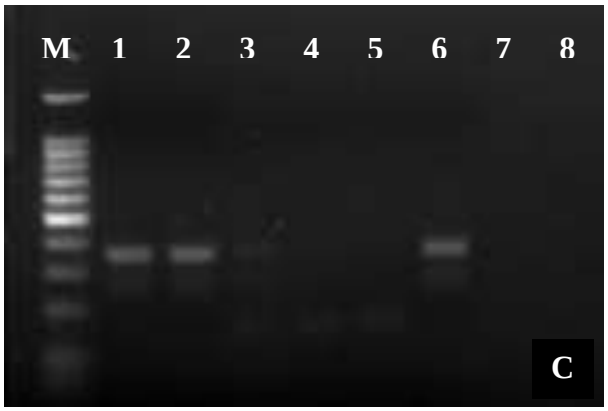

Figure 3.2. *Salmonella* Enteritidis detection in LWE by RT-PCR assay (Continued).
(C) Agarose gel electrophoresis of the RT-PCR products from nucleic acid extracts with DNAse I treatment of LWE spiked with *S.* Enteritidis in TTB after 16-h enrichment at 37ºC showing 347 bp *inv*A product and 154 bp IAC product. M: 100 bp Marker, Lanes 1-5: 10^4-10^0 CFU/ml; Lane 6: Positive *Salmonella* control; Lane 7: un-inoculated LWE control; Lane 8: Negative water control.

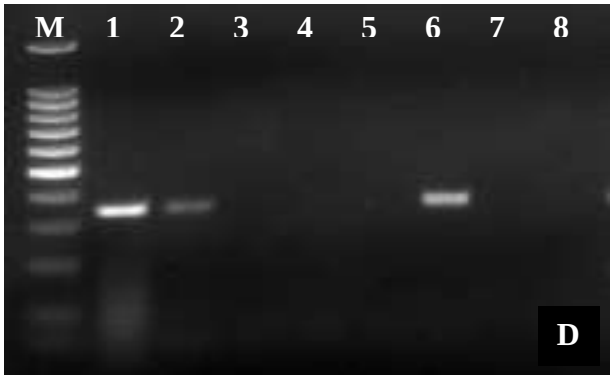

Figure 3.2. *Salmonella* Enteritidis detection in LWE by RT-PCR assay (Continued).
(D) Agarose gel electrophoresis of the PCR products from nucleic acid extracts with DNAse I treatment of LWE spiked with *S.* Enteritidis in TTB after 16-h enrichment at 37ºC showing 347 bp *inv*A product. M: 100 bp Marker, Lanes 1-5: 10^4-10^0 CFU/ml; Lane 6: Positive *Salmonella* control; Lane 7: un-inoculated LWE control; Lane 8: Negative water control.

CHAPTER IV

Reverse-Transcriptase Loop-Mediated Isothermal Amplification as a Rapid Screening/Monitoring Tool for *Salmonella enterica* Detection in Liquid Whole Eggs

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Abstract

Reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) is a novel molecular detection method that is specific, fast, and simple. It is based on reverse transcription followed by DNA amplification using the *Bst* DNA polymerase large fragment requiring one temperature and a simple waterbath, without the need for any expensive equipment. Detection is by turbidity or agarose gel electrophoresis. Our objective was to apply this LAMP-based technology to rapidly and sensitively detect *Salmonella enterica* serovar Enteritidis in liquid whole eggs (LWEs) within 1 day. Inoculated LWE were inoculated with *S*. Enteritidis and blended in tetrathionate (TT) broth, and spread-plated on xylose lysine tergitol 4 agar either immediately or after 6, 12 or 16-h enrichment. RNA was extracted from 5-ml TT broth and the RT-LAMP assay was carried out using *inv*A primers. After 16 and 12-h enrichment, improved *Salmonella* detection up to 10^0 to 10^1 and10^4 CFU/25 ml LWE, respectively was obtained. Without enrichment, *Salmonella* could be detected at 10^7 CFU/25 ml; however, after 6-h enrichment a 1-log improvement to 10^6 CFU/25 ml was obtained. This RT-LAMP assay appears to be suitable as a potential screening/monitoring tool for *Salmonella enterica* from LWE products in routine settings with results obtainable within 24-h, which is significantly faster than traditional cultural assays.

Introduction

Salmonella enterica serovar Enteritidis is one of the most frequent *Salmonella* strains typically associated with salmonellosis outbreaks related to eggs, poultry, and their products (Betancor et al., 2010). As assessed by a risk assessment program using an egg production module, approximately 1 out of 20,000 table eggs are reportedly found contaminated with *S.* Enteritidis (Hope et al., 2002). , with the same estimates reported by the U.S. egg industry and the American Egg Board (Lakins et al., 2008). This results in a prediction of 3.2 million *S.* Enteritidis contaminated eggs produced annually in the U.S. (Ebel and Schlosser, 2000). The consumption of egg contaminated products can lead to infection, especially in susceptible individuals (FDA, 2010b).

Unavoidably, eggs can be contaminated due to infection of the hen's ovary that passes on to the eggs before the shells are formed (CDC, 2005). Moreover, eggs can also be cross-contaminated during processing and handling. These contamination issues affect not only public health but also the food industry due to costly recalls and ill-repute of product brand name. *S.* Enteritidis cases are reported to be on the rise since mid-August 2009, increasing by more than 30% since 2008 in the UK (FSA, 2009). In the U.S., PulseNet declared that the number of *S.* Enteritidis infection cases had increased 4-fold just from the beginning of the year until May 2010 (CDC, 2010). Recently, approximately 1,939 people across the U.S. were reported to be sickened from egg consumption due to *S.* Enteritidis contamination (CDC, 2010) and a recall had been announced for at least 380 million eggs (FDA, 2010a).

Annually in the U.S. approximately 1.7 billion lbs of liquid whole egg (LWE) are produced from 24 billion eggs for both household consumption and industrial use (USDA NASS,

2008). The safety of eggs and egg products remains a very significant concern. Several programs have been implemented to enhance existing safety plans and to prevent and curb the occurrence of outbreaks and recalls in the egg industry, including Good Agricultural Practices such as flock-based *S.* Enteritidis control programs, Good Manufacturing Practices, and HACCP plans that require routine microbiological testing (FDA, 2010b; Patterson et al., 1997). Although, microbiological testing by standard culture-based methods results in high detection sensitivity, it is labor intensive and time-consuming as it requires approximately 5-7 days (Okamura et al., 2008). Thus, improved *Salmonella* detection techniques for rapid *Salmonella* diagnosis in eggs are vital.

Detection assays continue to be researched for increased speed, specificity, and sensitivity. One of the most popular methods is the polymerase chain reaction (PCR)-based detection assay; however, the initial cost of the PCR machine and skilled labor makes it a limitation for routine use by small processing facilities (Hara-Kudo et al., 2005). In the past few years, loop-mediated isothermal amplification (LAMP) has gained interest from researchers as it is rapid, specific, and requires only a simple waterbath. Amplified LAMP products can easily be detected visually or by agarose gel electrophoresis. LAMP-based assays have been investigated for foodborne *Salmonella* detection in pure culture and in food samples (Hara-Kudo et al., 2005 and 2008; Okamura et al., 2008 and 2009; Wang et al., 2008; Francois et al., 2011; Yang et al., 2010; Li et al., 2009; Zhang et al., (in press)), including *Salmonella* in eggshells (Ye et al., 2011) and in LWE (Ohtsuka et al., 2005). However, this reported DNA-based LAMP assay for *Salmonella* detection in LWE cannot differentiate between live and dead cells. Recently, we successfully demonstrated the detection of *Salmonella* in pork and the pork processing environment by converting the described DNA-based LAMP assay to a reverse-transcriptase

LAMP (RT-LAMP) RNA-based assay which primarily targets detection of live cells based on the short half-life of mRNA (Techathuvanan et al., 2010). This study aimed to optimize the detection of *S.* Enteritidis in LWE using this previously described RT-LAMP assay for rapid routine screening within one day.

Materials and Methods

Bacterial strain and preparation of bacterial suspension

Salmonella enterica serovar Enteritidis strain H4267 (isolated from an outbreak associated with eggs, Chantarapanont et al., 2000) was obtained from the University of Tennessee culture collection and cultured in trypticase soy broth (TSB; Difco Becton Dickinson Microbiology Systems, Sparks, MD) at 37°C for 24 h and then transferred in TSB at least twice at 24-h intervals prior to use. Overnight *S.* Enteritidis cultures were enumerated after serially diluting in phosphate buffered saline (1X PBS, pH 7.2; Difco), followed by spread plating on Xylose lysine tergitol 4 (XLT4) agar (Difco) and incubating at 37°C for 24 h to 48 h. Varying titers of 0 log CFU/ml to 9 log CFU/ml were used for pure culture detection and artificial contamination/spiking studies.

Artificially contaminated LWE

Commercial large shell eggs purchased from local grocery stores with expiration dates >2 weeks were used to prepare LWE. Shell eggs were decontaminated by dipping in 5% trisodium phosphate (Difco) for 1 min and washing in sterile deionized water, followed by air drying under UV light for 10 min. The eggs were then aseptically cracked under a BSL-2 hood, and

stomached for 1 min in sterile stomacher bags. The pH of LWE was measured to be 7.5 to 8.0. LWE was stored at -20°C until use. In order to determine the initial bacterial load or any possible *Salmonella* contamination in the prepared LWE, LWE samples were spread plated on trypticase soy agar (Difco) and XLT4 agar, respectively and incubated at 37°C for 24 to 48 h.

Either 25-ml of freshly prepared LWE samples or samples stored at -20°C that were thawed at 4ºC prior to use were inoculated with 1 ml of overnight *S.* Enteritidis inocula ranging from 10^0 to 10^9 CFU/ml. Sterile stomacher bags containing 224 ml freshly prepared Tetrathionate broth (TTB; Difco) were used to stomach the inoculated LWE samples for 2 min. Inoculated LWE samples in TTB were then either immediately assayed or enriched at 37°C for 6, 12, or 16 h and then assayed for the presence of *Salmonella*.

Natural LWE

Natural LWE samples using commercialized large, large brown, medium, organic large, organic large brown, and organic extra large shell eggs from 4 different local grocery stores with expiration dates >2 weeks (total of 17 samples) were prepared by the process described above for artificially contaminated LWE preparation, except without any shell surface decontamination step. Immediately after preparation, LWE samples were screened for *S. enterica* contamination using modified USDA MLG procedures (USDA-FSIS, 2008). Twenty-five milliliters of LWE were non-selectively pre-enriched in 225-ml buffered peptone water (BPW; Difco) for 4 h at 37°C followed by selective enrichment of 25 ml of pre-enriched BPW in 225 ml of TTB and incubation at 37°C for 16 h prior to nucleic acid extraction.

Nucleic acid extraction

The TRIzol® extraction protocol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions was used to extract nucleic acid from 1 ml of *S.* Enteritidis pure culture or 5 ml each of un-inoculated TTB (negative control), un-inoculated LWE (negative control), inoculated LWE samples, and overnight cultures of *S.* Enteritidis (positive control) or natural LWE samples. The RNA extracts were then passed through the QIAshredder column (Qiagen, Valencia, CA). Purified nucleic acid samples were either used immediately or stored at -80°C until use. All experiments were run in duplicate and replicated twice.

DNase I treatment

Nucleic acid extracts from *S.* Enteritidis pure culture, TTB, uninoculated LWE, natural LWE samples, and enriched inoculated LWE samples were treated with DNase I (Ambion, Austin, TX) according to the manufacturer's protocol. Nucleic acid samples before and after DNase I treatment were used to compare the detection sensitivity by the RT-LAMP assay.

RT-LAMP assay

A LAMP protocol of Hara-Kudo et al. (2005) was modified to a reverse-transcriptase-LAMP (RT-LAMP) assay using 3 sets of previously described primers specifically targeting the *Salmonella inv*A gene. The assay reaction mixtures in this study were prepared according to the earlier described protocol (Techathuvanan et al., 2010) with 5 µl of nucleic acid extracts (treated or un-treated with DNase I) per 50 µl reactions and carried out at 62°C for 90 min in a water-bath. Positive controls included nucleic acid extracts from overnight cultures of *S.* Enteritidis. Negative controls included RNase-DNase free water and nucleic acid extracts from un-

inoculated TTB and un-inoculated LWE samples to determine any possible cross-reactivity or contamination (false positives). All experiments were replicated twice.

Analysis of RT-LAMP products

The amplified RT-LAMP products were analyzed by gel electrophoresis on 2% agarose gels (Promega, Madison, WI) with a 100 bp DNA ladder (Promega) for product size comparison in 1X Tris acetate-EDTA buffer (10 mM Tris-Acetate and 1 mM EDTA, Fisher BioReagents, NJ), and stained with ethidium bromide (Bio-Rad, Hercules, CA). Products were analyzed using the Gel-Doc Camera and Quantity One program (Bio-Rad) under UV transillumination and reported as the lowest inoculated detection level of each LWE sample.

Culture-based detection

For detection comparison between cultural based and molecular assays, *Salmonella* pure culture, portions of artificially inoculated un-enriched TTB; or artificially inoculated and enriched TTB were also used for enumeration on XLT4 agar. *S.* Enteritidis pure culture in TSB or artificially inoculated LWE samples in TTB were serially diluted in 1X PBS (pH 7.2), and enumerated by spread plating on XLT4 agar and incubating at 37°C for 24 to 48 h. Appropriate negative controls such as uninoculated TTB and LWE were also used.

Results

Specificity and sensitivity of RT-LAMP assay

Our results revealed that the RT-LAMP assay using previously described *inv*A primers showed no cross-reactivity with either the enrichment broth (TTB) or the un-inoculated LWE

samples as amplified products were not obtained by agarose gel electrophoresis or by visual detection of turbidity. As expected, RNA extracts from overnight *S.* Enteritidis showed the ladder pattern of bands after amplification. For overnight *S.* Enteritidis pure culture samples, the RT-LAMP assay showed detection limits at 10^7 CFU/ml (Table 4.1). Even after the RNA samples from pure culture were DNase I treated to remove any possible DNA carry-over, the detection limit obtained still remained at 10^7 CFU/ml using the RT-LAMP assay (Table 4.1). The detection limit of *Salmonella* pure culture by traditional cultural methods was also determined for comparison purposes. When traditional cultural methods were used, the detection limit found was 10^0 CFU/ml of *S.* Enteritidis pure culture (Table 4.1). This showed that the RT-LAMP assay was not as sensitive compared to traditional methods when using pure culture.

Salmonella *detection in un-enriched LWE*

The detection limit of LWE samples artificially inoculated with 10^9 to 10^5 CFU/25 ml was 10^8 CFU/25 ml by the RT-LAMP assay without any enrichment (Table 4.1). When traditional cultural detection methods were used, the *S.* Enteritidis detection limit of 10^5 CFU/25 ml was obtained (Table 4.1). This showed that a 3-log lower detection was obtained using the RT-LAMP assay within 24 h without enrichment compared to traditional methods for LWE that take at least 5 days.

Salmonella *detection in enriched LWE*

Table 4.1 shows that when artificially inoculated LWE samples were enriched at 37ºC for 6 and 12 h, the detection limit by the RT-LAMP assay improved to 10^6 and 10^4 CFU/25 ml, respectively. With 16-h enrichment, further improvement in *S.* Enteritidis detection of 10^0 CFU/25 ml was obtained. Detection limits by traditional cultural detection assays were at 10^3,

10^1, and 10^0 CFU/25 ml after 6, 12, and 16-h enrichment, respectively. Only after 16-h enrichment were similar results using the RT-LAMP and traditional cultural assays obtained.

When 16-h enriched LWE samples were treated with DNase I, the detection limit by RT-LAMP assay increased by 1-$\log_{10}$ CFU/25 ml, from 10^0 CFU/25 ml before the treatment (Table 4.1) to 10^1 CFU/25 ml after the DNase I treatment (Fig. 4.1).

Salmonella *detection in natural LWE*

Natural LWE prepared from large, large brown, medium, organic large, organic large brown, and organic extra large shell eggs were screened for *S. enterica* contamination. All 17 samples tested were shown to be *Salmonella* negative by traditional culture based methods; however, 1 sample of organic extra large LWE tested as *Salmonella* positive by the RT-LAMP assay (data not shown).

Discussion

The previously described *Salmonella* LAMP assay (Hara-Kudo et al., 2005) has recently been successfully converted to an RT-LAMP format and used for *S.* Typhimurium detection in pork products and pork environmental samples (Techathuvanan et al., 2010). This study has applied the RT-LAMP assay for *S.* Enteritidis detection from LWE. No cross-reactivity associated with either the LWE samples or the TTB was obtained and only RNA extracts from LWE spiked with *S.* Enteritidis were amplified using the RT-LAMP assay. The detection limit of overnight *S.* Enteritidis pure culture by RT-LAMP assay was found to be 10^7 CFU/ml, which is not as sensitive when compared to that reported for *S.* Typhimurium (Techathuvanan et al.,

2010). For spiked LWE samples, the assay could detect *Salmonella* at 10^8 CFU/25 ml, prior to enrichment. Typically, $\geq 10^5$ *Salmonella* cells can cause infection in humans (Kothary and Babu, 2001), and as few as 15-20 organisms are capable of causing salmonellosis in highly susceptible hosts (FDA. 2009). Thus, the enrichment was required to improve the detection sensitivity of the assay.

Although enrichment increases only the number of live microorganisms and DNA can be detected from those cells, it is important to use the RNA-based method as background DNA from inactivated/dead cells can still be present in the enriched sample and can be amplified simultaneously with DNA from live cells during the LAMP-based detection assay. Our results show that improved detection was obtained with increased enrichment time, where the RT-LAMP assay after 16-h enrichment could detect up to 10^0 CFU/25 ml of *S*. Enteritidis (comparable detection to culture-based assays). Although, 16-h enrichment is required, the RT-LAMP assay can still yield results within 24 h (16 h for enrichment, 1.5 h for nucleic acid extraction, 1.5 h for RT-LAMP assay, and 1 h for agarose gel electrophoresis). When comparing the detection of *S*. Enteritidis from LWE to previously obtained findings with raw pork chop and sausage inoculated with *S.* Typhimurium, longer enrichment is generally required for LWE to obtain similar detection, suggesting that LWE possibly contains a higher content of inhibitors that interfere with the detection sensitivity of the assay than found in pork. This strongly suggests that optimization of the RNA extraction and detection assay is crucially needed, when applying the assay to different food matrices.

To further evaluate the application of the RT-LAMP assay in real-world scenarios, naturally contaminated LWEs were analyzed. The RT-LAMP assay showed a positive test in 1 out of 17 samples, demonstrating the promise/potential of this assay as a screening tool.

However, to ensure the recovery of *Salmonella*, 4-h pre-enrichment and 16-h enrichment steps are recommended. Research has previously shown that pre-enrichment with non-selective culture media is necessary for the recovery of stressed/injured cells. Techathuvanan et al. (2010) have shown that 4-h pre-enrichment could be adequate for simulated cold and freeze-stressed *S. enterica* recovery yielding similar detection limit to non-stressed (optimal growth) *Salmonella*. This is important for field testing in the food industry as *Salmonella* is likely to persist in stressed states in non-host environments (Gonzalez-Escalona et al., 2009) as well as it can be stressed/injured due to the processing measures used.

The RT-LAMP assay depends on the detection of mRNA which highly correlates with live (infectious) cells or recent contamination. As inactivation processes such as thermal pasteurization may be used during LWE production (Ohtsuka et al., 2005), the detection of inactivated microorganisms can lead to misinterpretation (in terms of failure) of the inactivation process. To verify that the obtained results are actually based on the detection of RNA, DNase I digestion was used to remove any possible genomic DNA carried over in the RNA samples. In pure culture, similar detection limits from RNA samples with and without the DNase I treatment, at 10^7 CFU/ml, was obtained. However, when DNase I treatment was used with 16-h enriched LWEs, the detection limit dropped by 1 $\log_{10}$ CFU/25 ml when compared to samples without the treatment. Although the detection sensitivity decreases in some cases, these results suggested that the RNA extraction procedure used in this study primarily isolates RNA. As reported earlier, caution must be used to ensure that the nucleic acid is devoid of any DNA to avoid DNA amplification (Miller et al., 2010a and b; Techathuvanan et al., 2010). As a control, direct PCR without addition of reverse-transcriptase did not result in any amplification, indicating the absence of any carry-over DNA in the RNA extracts.

The detection sensitivity of the DNA-based LAMP assay for *Salmonella* using pure culture and *in situ* studies using artificially and naturally contaminated egg and poultry related samples has been previously investigated (Hara-Kudo et al., 2005; Okamura et al., 2008; Ohtsuka et al., 2005; Wang et al., 2008). The LAMP assay was shown to detect *Salmonella* in inoculated LWEs at ~5.6 x 10^1 CFU/ml (Hara-Kudo et al., 2005), and <1 CFU/g in naturally contaminated LWE after 20-h enrichment at 37°C in BPW (Ohtsuka et al., 2005). The detection limit of 1 CFU/cm^2 of *Salmonella* was reported when the LAMP assay was applied to artificially contaminated eggshells after 4-h enrichment (Ye et al., 2011). The LAMP assay yielded a detection limit of 6.1x10^1 CFU/g after 1-day enrichment for *Salmonella* from chicken cecal droppings (Okamura et al., 2008). Although these studies showed comparable or slightly better detection than this study, the assay needed longer enrichment times and also, only the RNA-based RT-LAMP assay can potentially detect live cells.

Over the past years, many rapid detection assays have been developed and optimized for *S.* Enteritidis detection in egg and poultry-related samples (D'Souza and Jaykus, 2003; Ko and Grant, 2005; Malorny et al., 2007; Yang et al., 2009). Among those, PCR-based detection has continuously gained interest and has been constantly studied. In 2003, De Medici et al. (2003) demonstrated 10^7 CFU/ml *S.* Enteritidis detection in poultry by PCR after 18-20 h incubation, with assay detection sensitivity of <10^3 CFU/ml for pure culture. *Salmonella* in carcass rinses and chicken have been successfully detected by real-time PCR assay after pre-enrichment for 20 h (Malorny et al., 2004). A10-h intracellular multiplication enrichment, followed by PCR could detect 10 CFU/ml of *S.* Enteritidis in shell eggs (Day et al., 2009). Immunomagnetic separation-PCR assay was shown to detect 47 CFU/25 g of *Salmonella* in inoculated ice-cream, cheese, milk powder, egg yolk powder, and pasteurized egg yolk after at least 16-h enrichment (Rijpens

et al., 1999) with 1-5 CFU/25 g detection in egg melange, egg melange with sugar, and dried eggs after similar enrichment (Jeníková et al., 2000). More recently, Mercanoglu et al. (2009) used this same assay for *S*. Enteritidis detection in milk with detection at 10^0-10^1 CFU/ml after 16-h enrichment. Fluorescence polarization and lateral flow immunodiffusion assays could detect 10^8 CFU/ml of *Salmonella* in LWE within 15 min; however, 3-day enrichment is needed for improved detection up to 10 CFU/ml (Gast et al., 2003). While taking the enrichment time into account, similar or better detection limits were obtained by the RT-LAMP assay reported in this manuscript when compared with the findings of the previous studies described above.

Recently, a novel approach using nucleic acid dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA), coupling nucleic acid amplification assays for viable cell detection have become more popular. DNA-based EMA- and PMA-LAMP assays have also been researched for live *Salmonella* detection (Lu and others 2009; Chen and others 2011). Chen and others (2011) successfully applied the PMA-LAMP assay for *Salmonella* detection based on the *inv*A gene in inoculated produce with detection limits between 6.1×10^3 and 6.1×10^4 CFU/g. Incorporation of PMA for an optimized PMA-LAMP assay for *S*. Enteritidis detection in LWE remains an attractive option for future exploration.

Since the amplified LAMP product involves the formation of insoluble magnesium pyrophosphate the detection can also be monitored by the increase in turbidity. Thus, faster detection can be obtained by incorporation of a portable turbidimeter; this will not only eliminate the need for gel electrophoresis, it would also minimize the possibility of post-amplified cross-contamination as the reaction tube does not need to be opened. Besides, with proper optimization, this could allow monitoring and quantification of contamination levels in a real-time format. Another alternative is the use of a hand-held fluorometer. Fluorescence detection is

known to be more sensitive than detection by agarose gel electrophoresis or other chemical or colorimetric reactions (Gao et al., 2009; Techathuvanan et al., 2010). Therefore, it might even be beneficial and may increase the detection sensitivity. Further work in this area needs to be undertaken.

However, some current limitations of the assay need to be considered, such as the inability to distinguish between the various serovars of *S. enterica* as the assay is based on the detection of the *inv*A gene. Like other nucleic acid amplification techniques, RT-LAMP reaction can fail and give false negatives. Thus, incorporation of an internal amplification control (IAC) into the reaction is significant for more reliable results. However, in this study, only the external controls have been used. Further research on developing an IAC is needed. Other areas that still need attention include (1) improvements in RNA quality and yield by further optimization of RNA extraction procedures or use of different RNA isolation approaches such as magnetic beads or silica, (2) acceleration of the enrichment process by determining different enrichment conditions and/or media and (3) improvement in amplification efficiency, perhaps by using recombinant DNA polymerase large fragments with higher efficiency or DNA polymerase from different sources such as phi 29 DNA polymerase.

Conclusions

This developed *Salmonella* RT-LAMP assay, including enrichment and detection, can be completed within 24 h with a detection limit of 10^1 to 10^0 CFU/25 ml LWE. This assay has potential for use as a routine screening tool for *S. enterica* contamination (since this assay cannot distinguish between *S. enterica* serovars) in LWE and other egg related products and the egg environment. By incorporation of a fluorescence dye and fluorometer or turbidimeter, the assay

time can be further shortened, as well as be converted to a real-time format. This will provide a platform for convenient and feasible testing for regular screening purposes in diagnostic laboratories or for deployment in field-testing.

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Appendix

Table 4.1. Detection limits of *Salmonella* Enteritidis from pure culture and artificially inoculated LWE samples by traditional cultural plating and by RT-LAMP assays, with and without enrichment.

Sample	Inocula Levels	Lowest Inoculated Detection Limit (CFU/ml of pure culture or CFU/25 ml of LWE)		
		Traditional Method	**RT-LAMP Assay without DNase I Treatment**	**RT-LAMP Assay with DNase I Treatment**
S. Enteritidis pure culture	10^9-10^0 CFU/ml	10^0	10^7	10^7
LWE without enrichment	10^9-10^5 CFU/25 ml	10^5	10^8	ND
LWE with 6-h enrichment	10^7-10^3 CFU/25 ml	10^3	10^6	ND
LWE with 12-h enrichment	10^5-10^1 CFU/25 ml	10^1	10^4	ND
LWE with 16-h enrichment	10^4-10^0 CFU/25 ml	10^0	10^0	10^1

ND = Not Determined

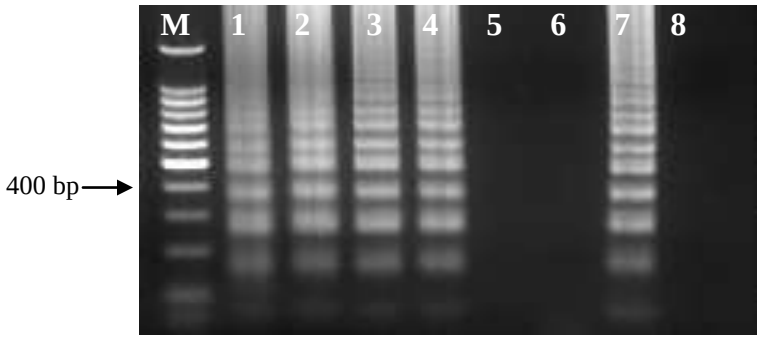

Figure 4.1. Agarose gel electrophoresis of the RT-LAMP products from nucleic acid extracts after DNase I treatment of LWE artificially inoculated with *S*. Enteritidis in TTB after 16-h enrichment at 37ºC. M: 100 bp Marker, Lanes 1-5: 10^4-10^0 CFU/25 ml; Lane 6: un-inoculated LWE control; Lane 7: Positive *Salmonella* control; Lane 8: Negative water control.

CHAPTER V

High Intensity Ultrasound in Combination with Nisin for *Salmonella* Enteritidis Inactivation in Pure Culture and Liquid Whole Eggs

Abstract

High intensity ultrasound (HIU) continues to be studied as a non-thermal inactivation technology that is appealing to food manufacturers. The advantages of HIU include maintenance of product quality, freshness, product homogenization, along with the simultaneous inactivation of pathogens. Besides, it is simple, relatively inexpensive, and easily adaptable to most processing environments. As HIU can cause alterations in bacterial structure, it could be used in combination with nisin (typically affects only Gram-positive bacteria) for *Salmonella* inactivation. The objective of this study was to determine the effect of HIU and HIU in combination with nisin and nisin-EDTA on inactivation of *S.* Enteritidis in pure culture and liquid whole eggs (LWEs). Overnight *S.* Enteritidis cultures and spiked LWE (both at 8 log CFU/ml) were treated with 20-kHz HIU for 0, 1, 5, 10, and 30 min in a temperature-controlled system, not to exceed 20ºC, and replicated thrice. At each time point, surviving *Salmonella* were enumerated on XLT4 agar and TSA and the morphology of *Salmonella* cells was analyzed using scanning electron microscopy. Our results revealed 3.6 log CFU/ml and 2.3 log CFU/25 ml reduction of *S.* Enteritidis after HIU treatment of 10 min in pure culture and 30 min in LWE, respectively ($P<0.05$). After 5 and 10-min HIU treatment, significant reduction of 1.4 log CFU/25 ml *S.* Enteritidis in LWE was obtained ($P<0.05$). Even at 1-min exposure time, HIU showed a significant reduction of 1.9 log CFU/ml in pure culture ($P<0.05$); however, no log-reduction was observed in LWE after 1 min. Scanning electron micrographs showed higher levels of damaged cell structure using longer HIU exposure. Nisin (100 and 1000 IU/ml), EDTA (50 mM), and their combinations were tested for anti-salmonellae activity with and without HIU treatment (0, 5, and 10 min). Addition of nisin alone or in combination with EDTA at selected concentration did not show any additional or synergistic effect to HIU treatment against *S.*

Enteritidis pure culture when tested up to 7 d incubation at 4°C. As to color changes, lower redness and yellowness of LWE were observed visually and instrumentally after 5-min HIU treatment ($P<0.05$). The rheological properties of LWE were measured at 0-200 sec^{-1} shear rate. Shear stress of HIU-treated LWEs decreased after 5-min HIU exposure, but increased after 30-min treatment. This study demonstrated that HIU shows promise for rapid *Salmonella* control in LWE and potentially other liquid foods, as an alternative inactivation method. For use in hurdle approaches with other antimicrobial compounds, research is still needed.

Introduction

Salmonellosis is a major worldwide foodborne disease with common manifestations of mild to moderate gastroenteritis, consisting of diarrhea, abdominal cramps, vomiting, and fever (NIAD, 2007). According to the U.S. Centers for Disease Control and Prevention (CDC, 2008), there are approximately 40,000 cases of salmonellosis in the United States annually. Within the U.S., *Salmonella* associated outbreaks cost more than $2.5 billion annually (ERS/USDA, 2008). The illness is most often linked to consumption of contaminated poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce (Foley and Lynne, 2008). As approximately 1 out of 20,000 eggs are reported to be contaminated with *S.* Enteritidis, ~3.2 million eggs produced annually in the U.S. are accordingly contaminated with *S.* Enteritidis (Lakins et al., 2008; Ebel and Schlosser, 2000). Therefore, it is imperative to minimize and/or eliminate contamination of this foodborne pathogen in at-risk foods.

Thermal inactivation has been commonly used for pasteurization and sterilization of food products due to its effectiveness against a wide range of spoilage and pathogenic organisms. However, thermal processing can alter food components and may cause undesirable sensory changes, lowering their functional properties and nutritional values. Due to the high consumer demand of fresh and high quality products, the food industry is interested in non-thermal pasteurization methods which have minimal to no impact on food sensory and nutritional quality (Ukuku et al., 2009). Many non-thermal microbial inactivation processes, such as high-pressure, pulsed electric field processing, irradiation and ultrasound technology are being studied to reach the goal of maintaining product quality and safety (Barbosa-Cánovas et al., 1999; Piyasena et al., 2003; (Raso and Barbosa-Cánovas, 2003; Ross et al., 2003).

Ultrasound is among one of the novel techniques that has attracted the attention of the food industry. While low intensity ultrasound has been employed for biomedical purposes as a therapeutic, operative, and diagnostic tool (Rubin et al., 2001), high intensity ultrasound which has more destructive power is typically used in cleaning systems and liquid degassing processes. High intensity ultrasound (HIU) treatment (10-1000 $W \cdot cm^{-2}$ and 20-100 kHz in frequency range) seems to be an attractive option for microbial inactivation in liquid foods due to its low cost and feasibility for industrial use, that maintains the sensory and nutritional attributes of food for consumer acceptability (McClements, 1995; Mason, 1998).

HIU has been used to inactivate pathogens, such as *Salmonella* Typhimurium in skim milk and liquid whole egg (LWE) at 20, 40 and 50°C for 15 and 30 min (Wrigley and Llorca, 1992), *Escherichia coli* O157:H7 and *Listeria monocytogenes* in milk and apple cider (D'Amico et al., 2006), *Listeria* and *E. coli* in LWE (Lee et al., 2003), *Salmonella* in broiler skin (Lillard et al., 1994), spoilage microorganisms in juices (Cheng et al., 2007; Yuan et al., 2009), and human enteric viruses (Su and D'Souza, 2010). The mechanism of microbial killing is reported to be mainly due to localized changes in pressure and temperature caused by cavitation that is generated by HIU, which is a phenomenon of extremely rapid creation and collapse of bubbles in a liquid medium (Earnshaw, 1998). This cavitation effect results in cell membrane disruption and thinning, shear-induced breakdown of cell walls, and DNA damage via production of free radicals in bacterial cells (Butz and Tauscher, 2002; Fellows, 2000; Seymour et al., 2002; Earnshaw et al., 1995; Lillard, 1994; Sala et al., 1995).

Nisin is a bacteriocin naturally produced by *Lactococcus lactis* (Thomas and Delves-Broughton, 2005). It is an appealing antimicrobial substance as it is considered as GRAS by the US FDA and has a broad spectrum of antimicrobial activities (Millette et al., 2007; Holzapfel et

al., 1995). Nisin has an antimicrobial effect against Gram-positive bacteria, but not Gram-negative bacteria, including *Salmonella*, under normal conditions due to the barrier of Gram-negative bacterial outer membrane, which contains lipopolysaccharides (Helander et al., 1997). However, after alteration or disruption of Gram-negative bacterial outer structures, nisin could exhibit antibacterial effects against Gram-negative bacteria (Stevens et al., 1991). Ethylenediaminetetraacetic acid (EDTA) is a metal ion chelator used in foods to prevent lipid oxidation, with known antimicrobial activity (Nielsen et al., 2004; Branen and Davidson, 2004). As EDTA can alter bacterial cell membranes, improved antimicrobial effects of nisin could be achieved with the addition of EDTA (Stevens et al., 1991). Branen and Davidson (2004) reported that nisin could effectively inhibit the tested Gram-negative bacteria including *E. coli* O157:H7, *E. coli* O104:H21, *P. fluorescens* 13525, and *S.* Enteritidis.

This study aimed to determine the effect of HIU at 20 kHz alone or in combination with nisin, EDTA or nisin-EDTA in a temperature controlled system (not exceeding 20^{o}C) on *Salmonella enterica* serovar Enteritidis inactivation in pure culture and on artificially contaminated LWEs. Further characterization of physical characteristics (color and rheological properties) of the HIU treated and untreated LWE samples were carried out to determine suitability of using HIU as a control measure for LWE.

Materials and Methods

Bacterial strain and preparation of bacterial suspension

Salmonella enterica serovar Enteritidis strain H4267 (human isolate from an egg-associated outbreak) was obtained from the University of Tennessee culture collection, cultured

into trypticase soy broth (TSB; Difco Becton Dickinson Microbiology Systems, Sparks, MD) at 37°C for 24 h, and transferred a minimum of 2 times at 24-h intervals prior to use. Twenty-five millilitres of overnight *S.* Enteritidis cultures were centrifuged at 8,000 x g for 10 min. Then, pellets were washed by phosphate buffered saline (PBS; pH 7.2; Difco Becton Dickinson Microbiology Systems, Sparks, MD) twice and resuspended in 25-ml PBS for investigation of the inactivation effects of HIU treatment or used for LWE inoculation.

Serial dilutions in PBS were also enumerated on Xylose Lysine Tergitol 4 (XLT4) agar and TSA (Difco Becton Dickinson Microbiology Systems, Sparks, MD) after incubation at 37°C for 24 h.

Preparation of nisin

Powdered nisin (10^6 IU/g; 2.5% actual nisin) 0.1g (Sigma-Aldrich,St. Louis, MO) was mixed with 10 ml of 20 mM HCl (10,000 IU/ml), immersed in boiling water for 4 min, cooled at room temperature, and used immediately or refrigerated for no more than 6 days before use.

Nisin, EDTA, and nisin-EDTA treatment

Nisin stock solution was added into *S.* Enteritidis culture (in PBS) to achieve final concentrations of 10 and 100 IU/ml. EDTA (0.5 M, pH 8.0; Cellgro®, Mediatech, Inc., Herndon, VA) was added to *S.* Enteritidis culture, alone or with nisin, at 50 mM final concentration. Then, cultures with treatments were mixed well by vortex, and sampled or incubated at 4°C up to 7 days. Samples were taken, neutralized with TSB + 3% beef extract, and diluted in 1X PBS for further enumeration.

Preparation and artificial contamination of LWE

Commercialized large shell eggs with expiration dates > 2 weeks were purchased from a local grocery store for LWE preparation. Shell eggs were decontaminated by 5% trisodium phosphate (Difco) and rinsed by sterile deionized water. Then, eggs were air dried under UV light for 10 min, aseptically cracked under BSL-2 hood into sterile stomacher bags, and stomached for 1 min. The pH was measured to be 7.5 to 8.0. Portions of prepared LWE were plated on TSA and XLT4 agar to determine the initial bacterial load. LWE samples were stored at -20ºC until use.

Frozen LWE samples were thawed at 4ºC prior to use. Twenty-five milliliters of LWE was inoculated with 1 ml of 10^9 CFU/ml of *S.* Enteritidis pure culture in PBS. Then, samples were mixed using a vortex prior to use for HIU treatments.

Ultrasound treatment

High intensity ultrasound (HIU) treatment was carried out using a VCX 750 Vibracell™ High Intensity Ultrasonic Liquid Processors (Sonics & Materials, Inc., Newtown, Connecticut, USA) with a 13-mm probe. Twenty-five milliliters of *S.* Enteritidis resuspended in PBS at ~10^9 CFU/ml or artificially contaminated LWE were transferred to a 30-ml sterilized glass beaker which was pre-cooled in ice water. The HIU treatment was performed by immersing the disinfected-probe in bacterial suspensions (with or without nisin-EDTA) or LWE samples and sonicating at 20 kHz and 80% amplitude for durations of 1, 5, 10, and 30 minutes with 30-s on and 30-s off pulsed (in ice water) under the BSL-2 biosafety cabinet. Temperature of experimental unit was controlled and monitored to not exceed 20°C throughout the experiment. All experiments were repeated three times.

Enumeration of bacterial survivors

Immediately after nisin, EDTA, nisin-EDTA, or HIU treatment or at sampling storage time, treated bacterial culture and LWE samples were serially diluted in 1X PBS and directly plated on TSA and XLT4 agar plates. Following 24 to 48-h aerobic incubation at 37°C, survivors were enumerated in duplicate from three samplings.

Color measurements of HIU treated LWEs

Twenty-five milliliters of LWE samples that were treated with HIU at 0, 5, and 30 min were instrumentally analyzed for color (L*, a*, and b*, illuminant A) using a HunterLab MiniScan XE Plus Spectrophotometer (model 45/0 LAV, 2.54-cm diam. aperture, 10° standard observer, Hunter Associates Laboratory Inc., Reston, VA). The color was measured 5 times for each sample, and each experiment was conducted in triplicates. Color photographs were also taken to compare any visual color changes by different times of HIU treatment.

Rheological measurements of HIU treated LWEs

HIU treated or untreated LWE samples (0.4 ml) were used for rheological measurements in a controlled stress AR 2000 rheometer (TA Instruments, New Castle, DE) using a Rheology Advantage Data Analysis Program software (TA Instruments). Cone angle plate geometry (40 mm cone diameter, 30 μm truncation; TA Instruments) was used for measurement. The experimental temperature was controlled at 20°C with equilibration for 30 sec prior to 2-cycle shear changes from 0 to 120 sec^{-1} in 1 min and back to 0 sec^{-1} in next 1 min. Sixty-point data of rheological parameters were collected for each shear cycle by the software. Three-sampling measurements were carried out for each LWE sample after HIU treatment within 1 day.

Statistical analysis

Duplicate data of each replicate treatment were statistically analyzed using analysis of variance (ANOVA) with SAS software (version 9.2, SAS Institute, Cary, NC, USA) and student's t-distribution using 95% confidence intervals on a completely randomized design.

Results

HIU treatment for pure culture Salmonella *Enteritidis inactivation*

As shown in Figure 5.1, when overnight pure culture *Salmonella* was treated with HIU, the number of bacterial survivors decreased when compared to untreated cells. After 1-min HIU treatment, *S.* Enteritidis counts were significantly decreased by 1.9 log CFU/ml, from 7.6 to 5.7 log CFU/ml on XLT4 agar ($P<0.05$). This result coincided with the damage observed under SEM (See Figure 2), even though pre-enrichment or plating on non-selective agar (such as TSA) was not carried out to recover any sub-lethally injured cells. Increased reduction of cells by 2.2 log CFU/ml was observed in *S.* Enteritidis pure culture after 5-min HIU exposure ($P<0.05$). Reduction of 3.6 log CFU/ml was achieved after HIU treatment for both 10 and 30 min, with the level of *Salmonella* survivors being below 4.0 log CFU/ml on XLT4 agar ($P<0.05$).

Figure 5.2 shows the morphological changes of *S.* Enteritidis cells before and after HIU treatment, when observed under the SEM. In the untreated control, *S.* Enteritidis showed the typical structure with flagella on the cell surface (Fig. 5.2A). The SEM micrograph of HIU-treated cells suggest that the structural damage of *S.* Enteritidis cells increases with longer HIU exposure time. After 5-min HIU treatment, even though the cell integrity was still maintained in some cells (with the absence of flagella), some deformation of bacterial cell wall was observed

(Fig. 5.2B). The 30-min HIU treatment resulted in extensive damage of *S.* Enteritidis cells as shown in Fig. 5.2C.

Effect of nisin and nisin-EDTA on Salmonella *Enteritidis inactivation*

S. Enteritidis counts after treatment with nisin at 100 and 1000 IU/ml, EDTA at 50 mM, and nisin-EDTA combination for 0 h, 6 h, 1 d, 2 d, and 7d are shown in Figure 5.3A when enumerated on TSA and Figure 5.3B when enumerated on XLT4 agar. When TSA was used, no significant difference in bacterial survivors was observed in any treatment immediately after cultures were treated (~8.5 to 8.8 log CFU/ml counts). Similar results in bacterial recovery within each time point were obtained when *S.* Enteritidis was treated with EDTA, and both levels of nisin incombination with EDTA after 6-h, 1 d, 2 d, and 7 d incubation ($P<0.05$) with the counts of ~8.0, 6.8-7.3, 6.2-6.8, and 5.6-6.3 log CFU/ml, respectively.

Once *S.* Enteritidis was enumerated on XLT4 agar, similar trends in results were obtained as those observed on TSA. However, approximately 1 to 1.5 log lower bacterial numbers were observed in all treatments with 0-h, 6-h and 1 d incubation times, and in control and both concentrations of nisin treatment alone. Up to 4 to 4.5 logs lower counts were obtained on XLT4 agar than TSA in EDTA and nisin-EDTA treatments after incubation for 2 to 7 days. Approximately ≥ 7.0 log CFU/ml of *S.* Enteritidis was observed in untreated control and nisin alone treatments throughout 7 d storage, as well as in all treatments at 0 h. Significant reduction of bacteria to ca. 6.5 to 7.0 log CFU/ml was obtained in samples treated with EDTA and nisin-EDTA (both nisin levels) after 6-h incubation ($P<0.05$). After 1-d incubation, when compared to 0-h samples, *S.* Enteritidis population significantly decreased in EDTA and nisin-EDTA treated samples to 5.9 to 6.2 log CFU/ml ($P<0.05$). While number of *S.* Enteritidis treated with EDTA after 2 d incubation was 4.6 log CFU/ml, the counts of cultures treated with nisin-EDTA with

nisin concentration at 100 IU/ml was lower at 3.5 log CFU/ml. After 7 d incubation, the counts of bacterial samples treated with EDTA and both levels of nisin-EDTA were ~2.0 and ~1.5 log CFU/ml, respectively.

Effect of HIU, HIU-nisin, and HIU-nisin-EDTA on Salmonella *Enteritidis inactivation*

As the results from nisin and nisin-EDTA treatments without HIU suggested that increase in nisin concentration from 100 to 1000 IU/ml did not show a significant difference on *S.* Enteritidis inactivation when used in combination with EDTA at 50 mM, nisin at 100 IU/ml was selected for use in combination with 50 mM EDTA in the experiments.

The results showed no difference between the counts obtained by plating on TSA and XLT4 media as depicted in Figure 5.4A and B. However, after 7 d incubation at 4°C, cell recovery when treated with EDTA and nisin-EDTA (with and without HIU) on selective media (XLT4) was lower than those observed on non-selective TSA plates. On day 0, no significant difference in bacterial counts was obtained in any treatments (with ~8.0-9.0 log CFU/ml on TSA and ~7.0-8.8 log CFU/ml on XLT4), except the sample treated with EDTA and HIU for 10 min ($P<0.05$). Similar trends of results was obtained with both TSA and XLT4 media, showing ~1.0 and 2.0 log reductions on TSA and XLT4 agar, respectively, in EDTA and 10-min HIU treated samples compared to 0-min HIU control. Approximately 0.5 log increase in reduction from those obtained in EDTA with 10-min HIU on day 0 for both TSA and XLT4 media was found after 1 d incubation. After 7 d incubation at 4°C, no difference in bacterial populations was shown by any treatment when plated on non-selective TSA ($P<0.05$) although longer HIU treatment times seemed to result in lower counts of bacteria. When enumerated on XLT4 agar, EDTA and nisin-EDTA treatments (without HIU) resulted in significant reduction of *S.* Enteritidis when compared to untreated control after incubation for 7 days ($P<0.05$). However, only EDTA

treated samples showed significantly decreased *S.* Enteritidis counts when combined with 10-min HIU treatment, compared to non-EDTA treated control with 10-min HIU treatment, on XLT4 agar after 7 d storage ($P<0.05$).

SEM micrographs of *S.* Enteritidis untreated control, treatment with EDTA at 50 mM, and nisin-EDTA with EDTA concentration at 50 mM and nisin concentrations at 100 IU/ml, with and without HIU for 0, 5, and 10 min, at day 0 are shown in Figure 5.5. SEM micrographs of the same samples after incubation at 4°C for 7 days are shown in Figure 5.6. Damage of *S.* Enteritidis cells can obviously be seen in samples with 5 and 10-min HIU treatments; however, no evidence of further cell structural damage was observed in EDTA and nisin-EDTA treated samples compared to non-EDTA and nisin added controls.

HIU treatment for Salmonella *Enteritidis inactivation in artificially contaminated LWE*

HIU treatment was also tested for its effectiveness on *S.* Enteritidis inactivation in artificially contaminated LWEs. *S.* Enteritidis counts were not found to decrease on XLT4 agar after the spiked LWE was treated with 1-min HIU as shown in Figure 5.7. However, longer HIU exposure time of 5 min showed 1.4 log CFU/25 ml reduction, while 10 min and 30 min showed similar reduction at ~2.3 log CFU/25 ml ($P<0.05$).

Effect of HIU treatment on LWE colors

HIU treated and untreated LWE samples were instrumentally measured for color parameters, L*, a*, and b*. L* defines as +L = Light and -L = black; a* defines as +a = red; -a = green; b* defines as +b = yellow; -b = blue (as described by Hunter Associates Laboratory Inc., Reston, VA). Instrumental color analysis results of LWE are shown in Table 5.1. LWE after longer HIU exposure treatment time seemed to have higher +L-value; however, the difference

was statistically insignificant or negligible ($P>0.05$). The +a-value was found to be significantly decreased for LWE that were treated for 5 and 10-min HIU ($P<0.05$), indicating that the redness of LWE decreased. Similar to +a-value, the +b-value of LWE was significantly lower once samples were exposed to 5 and 10-min HIU treatments ($P<0.05$). This indicates that the yellow color was lowered or diminished as a result of HIU treatment.

Figure 5.8 shows the visual appearance of non-treated and HIU-treated LWE. Lighter color was observed for LWE that had longer HIU treatment/exposure time. In addition, foam was also observed on the surface of the sample treated with 30-min HIU.

Effect of HIU treatment on LWE rheological properties

The rheological properties of LWE were measured at 0-200 sec^{-1} shear rate. Shear stress measurements of LWE with 5 and 30-min HIU treatments were compared to non-treated LWE and shown in Figure 5.9. Shear stress of HIU-treated LWE decreased after 5-min HIU exposure, but increased after 30-min treatment.

Discussion

In this present study, *S.* Enteritidis inactivation by HIU treatment was investigated in bacterial pure culture and in artificially contaminated LWE samples. The number of bacterial survivors after timed-treatment was determined (0, 1, 5, 10, and 30 min). The bacterial cultures were grown overnight and washed twice and resuspended in PBS to minimize the carry-over culture media which may have protective effect against the treatments. The ultrasound experiment was carried out with a temperature controlled system and the temperature of samples

was monitored so as not to exceed 20ºC throughout the experiment. Therefore, the inactivation effect observed should be mainly attributed to the HIU effects and not due to heat.

Based on the SEM results reported, it was found that direct plating on XLT4 without pre-enrichment coincided with the SEM results (though pre-enrichment or plating on non-selective agar is typically done/recommended to recover sub-lethally injured cells) when no incubation time is involved. Therefore, enumeration of *S.* Enteritidis in pure culture and LWE after HIU treatment without incubation (plated immediately after treatment was completed) was done by XLT4 agar. However, antimicrobial effects of HIU in combination with other antimicrobials (nisin, EDTA and nisin-EDTA) with storage at 4°C upto 7 days were determined by both selective (XLT4) and non-selective (TSA) media.

Increased exposure time of HIU was found to exhibit higher levels of *S.* Enteritidis inactivation. Significant bacterial reduction (by 2.0 log CFU/ml) was observed after 1-min treatment for pure culture. Additional reduction of 1.5 log CFU/ml was achieved with 10-min HIU treatment. Similar trends were found in artificially contaminated LWE samples as increased/longer HIU exposure showed greater bacterial inactivation. However, less bacterial reduction was obtained with LWE when compared to the pure culture by ~0.5 to 2.0 logs. This could be due to the protective effect of food components in the LWE samples. The sonoprotective phenomena of foods (such as milk) on bacteria in comparison to buffers have been previously reported (Wrigley and Llorca, 1992; Zenker et al., 2003; Gera and Doores, 2011). Fat content present in milk was earlier shown to reduce bacterial inactivation efficacy of ultrasound compared to fat-free milk (Bermudez-Aguirre and Barbosa-Cánovas, 2008). Similarly, orange juice with pulp was reported to prevent the inactivation of microorganisms after treatment (Valero et al., 2007). HIU treatment has also been used for the inactivation of

many other foodborne pathogens in LWE samples. Wrigley and Llorca (1992) demonstrated that indirect HIU treatment of 1-ml *S.* Typhimurium inoculated LWE resulted in 1 to 3 log reduction of these bacteria at 50°C after 30-min treatment. In 2003, Lee et al. showed that 1 to 2 log reduction of *E. coli* was obtained after 5-min HIU treatment of 10-ml spiked LWE samples at 5°C with 20-kHz HIU. *S.* Enteritidis inactivation by ultrasound was also previously researched in LWE samples (Huang et al., 2006). *S.* Enteritidis reduction of 0.65 log was reportedly obtained after LWE was treated with 40-W ultrasound for 5 min at 55°C. When compared to the results found in this study, ~0.7 log greater reduction was achieved in spiked LWE with the same sample volume (25 ml) using 20-kHz HIU for 5 min without the combined effect of heat, since the HIU experiments were conducted at ≤ 20°C. However, a limitation of this study is that a *Salmonella* selective XLT4 media was used for enumeration. Thus, the recovery of injured cells after HIU treatment might be compromised, though the results obtained tend to correlate with the SEM results.

The mechanisms of action of HIU on bacterial inactivation have been previously explored (Earnshaw et al., 1995; Lillard, 1994; Sala et al., 1995). Cavitation is suggested to cause physical stress to microbial cells resulting in a killing effect (Earnshaw et al., 1995; Sala et al., 1995; Su and D'Souza, 2010). Membrane disruption and cell wall damage can be induced by this physical stress as evidenced by the SEM micrographs. With longer HIU exposure, higher degree of *S.* Enteritidis structural cell damage was observed in this study. After 5-min HIU exposure, *S.* Enteritidis flagella were found to be separated/detached from the cells and a noticeable level of structural damage was observed. This damaging effect was found to be more severe and pronounced on bacterial cells after 30-min HIU treatment. The structural alterations

of cells are responsible for the release of essential cellular contents and dysfunction of organelles that can ultimately inactivate bacterial cells.

When color of HIU treated LWE was analyzed visually, lighter color LWE was observed compared to non-HIU treated samples. Less redness and yellowness of LWE samples were also observed by both visual and instrumental detection. This would be beneficial to the baking and food industry as lighter color egg enables improved color of baked goods. Similar trend of declined yellowness of the products was also shown after thermo-sonication treatment was applied to fat-free, 1%, 2%, and whole milk for 30 min at 63°C (Bermúdez-Aguirre and Barbosa-Cánovas, 2008). Another possible benefit of ultrasound on food color is that it can prevent enzymatic browning due to enzyme inactivation. A study of ultrasound treatment on color of apple cider showed that a slightly less dark color was obtained with after the treatment at both 40°C and 60°C, suggesting the effect on polyphenol oxidase enzyme inactivation and suspended particle separation in the product (Ugarte-Romero et al., 2006). This could be beneficial for potential HIU treatment application in other liquid foods.

Shear stress of LWEs was measured with increase shear rate range of 0-200 sec^{-1} for rheological properties characterization. Once shear rate increased, LWE with 5-min HIU treatment showed decreased shear stress when compared to non-treated LWE control. At this level of HIU exposure, protein structure of LWE could be broken down causing a decrease in shear stress. Ahmed et al. (2003) also reported a similar phenomenon where decreased shear stress in LWE treated with 300 MPa pressure was observed. However, after 30-min HIU treatment, shear stress of LWE was found to increase in this study. As HIU treatment causes cavitation in the treated media, it affected LWE mechanically. With longer HIU exposure time in addition to deformation of protein structure, denaturation of egg proteins could potentially occur.

Huang et al. (2006) reported that when LWE was held at 20°C, coagulation of samples was not observed. As the HIU system used in this study was in a temperature controlled setting (≤ 20°C), observed protein coagulation can be mainly attributed to the effect of HIU treatment.

Our results showed that nisin alone at 100 and 1000 IU/ml did not exhibit any antimicrobial effect against *S*. Enteritidis in pure culture, which is in agreement with other previous investigations (Helander et al., 1997; Branen and Davidson, 2004). EDTA at 50 mM alone and in combination with nisin at 100 and 1000 IU/ml showed significant antibacterial effects against *S*. Enteritidis after 6 h incubation at 4°C. However, addition of nisin did not increase antimicrobial activity obtained by EDTA alone, and the effect obtained was independent of nisin levels at the selected concentrations. Although no additional anti-salmonellae effect of nisin when combined with EDTA at the selected concentrations was observed, the nisin-EDTA combination (at 100 IU/ml nisin) was used for further investigation with HIU treatment. Since HIU treatment could alter bacterial structures, nisin and nisin-EDTA addition might possibly result in additional/synergistic effect when combined with HIU. As HIU treatment for 30 min may result in denaturation of egg proteins, only 0, 5, and 10-min HIU treatment levels were selected for this study. Results showed that no additional or synergistic anti-salmonellae effect was obtained when nisin-EDTA was used in combination with HIU, compared to EDTA with HIU treatment, at all tested HIU levels and storage times.

In summary, this study showed that HIU was found to be effective in reducing the levels of *S*. Enteritidis contamination in LWE, albeit not completely. Visual and instrumental color analysis revealed changes in color and properties that may be suitable for application in foods such as bakery products. However, for greater or complete reduction of *S*. Enteritidis, hurdle technologies using HIU along with other processing measures such as mild heat or pressure

treatment (Mañas et al., 2000; Raso et al., 1998) or natural antimicrobials may be necessary. Future research will focus on combinations of HIU with other natural or GRAS antimicrobial compounds to determine inactivation of *S*. Enteritidis in pure culture and LWE.

Conclusions

This study demonstrated that HIU shows promise for the rapid control of *S*. Enteritidis contamination in LWE. Five-min HIU treatment was found to effectively inactivate 1.4 log CFU/ml of *S*. Enteritidis in LWE without any evidence of egg protein coagulation. This technology could potentially be used for bacterial control in other liquid foods as an alternative pasteurization method or for use in hurdle approaches. However, HIU treatment did not show a synergistic anti-salmonellae effect when used in combination with nisin or nisin-EDTA.

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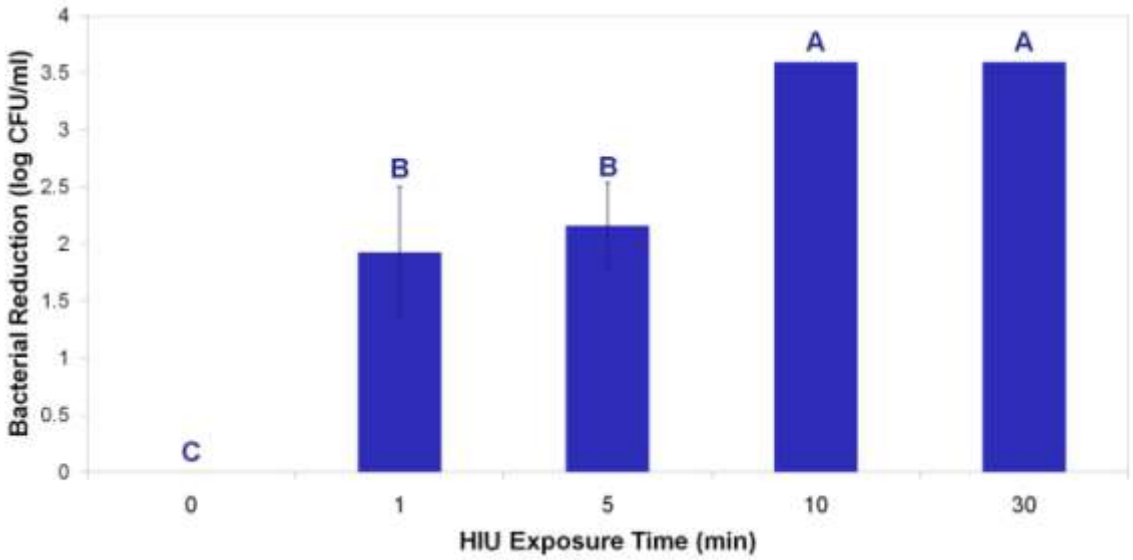
Appendix

Table 5.1. Instrumental color comparison of HIU-treated and untreated LWE.

HIU Treatment	Color Parameters		
	L*	**a***	**b***
0 min	72.95 ± 0.41^{A}	15.47 ± 0.82^{A}	36.41 ± 2.68^{A}
5 min	75.11 ± 2.57^{A}	13.47 ± 1.31^{AB}	30.50 ± 2.90^{AB}
30 min	77.16 ± 4.31^{A}	11.14 ± 0.72^{B}	27.53 ± 1.07^{B}

+L = Light; -L = black; +a = red; -a = green; +b = yellow; -b = blue.

Different letters denote significant differences within each color parameter ($p<0.05$) using data from 3 replicates.



Different letters denote significant differences in reduction ($P<0.05$) using data from 3 replicates.

Figure 5.1. Reduction of pure overnight culture of *S.* Enteritidis after HIU treatment on XLT4 agar.

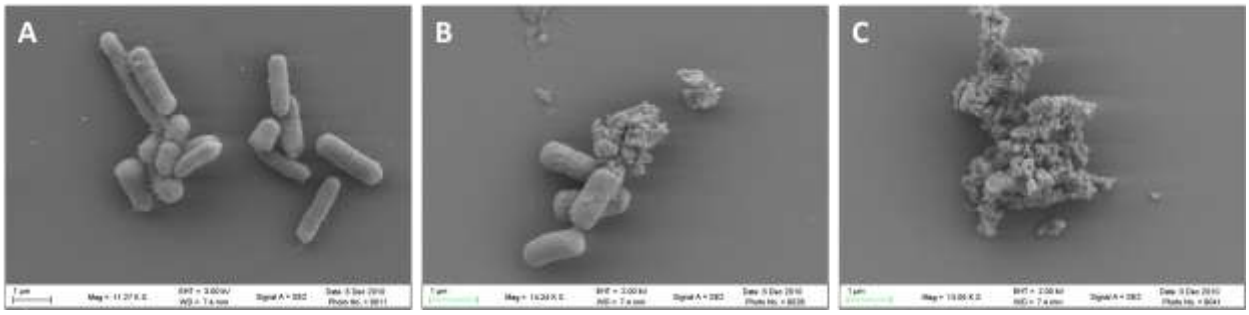
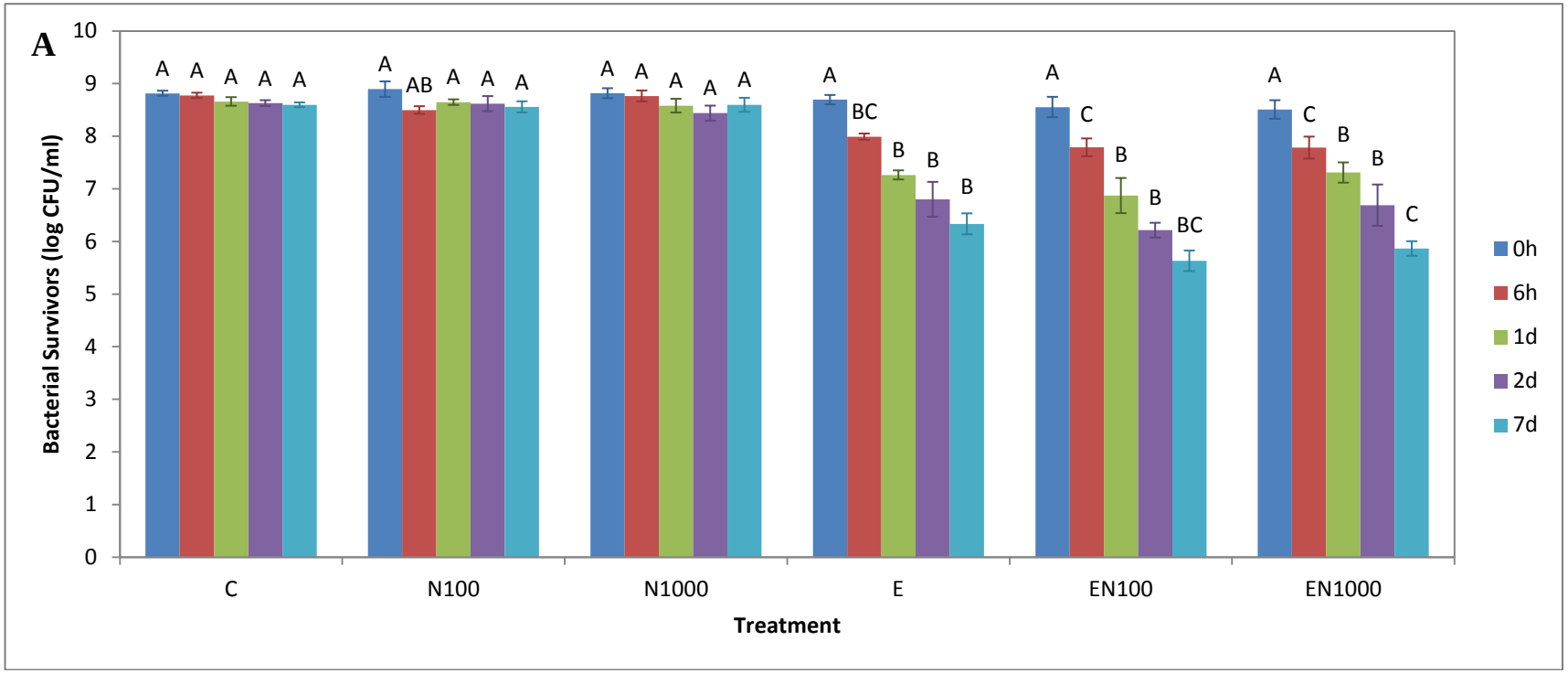


Figure 5.2. SEM micrograph of pure overnight culture of *S*. Enteritidis (A) untreated control, (B) after 5-min HIU treatment, and (C) after 30-min HIU treatment.

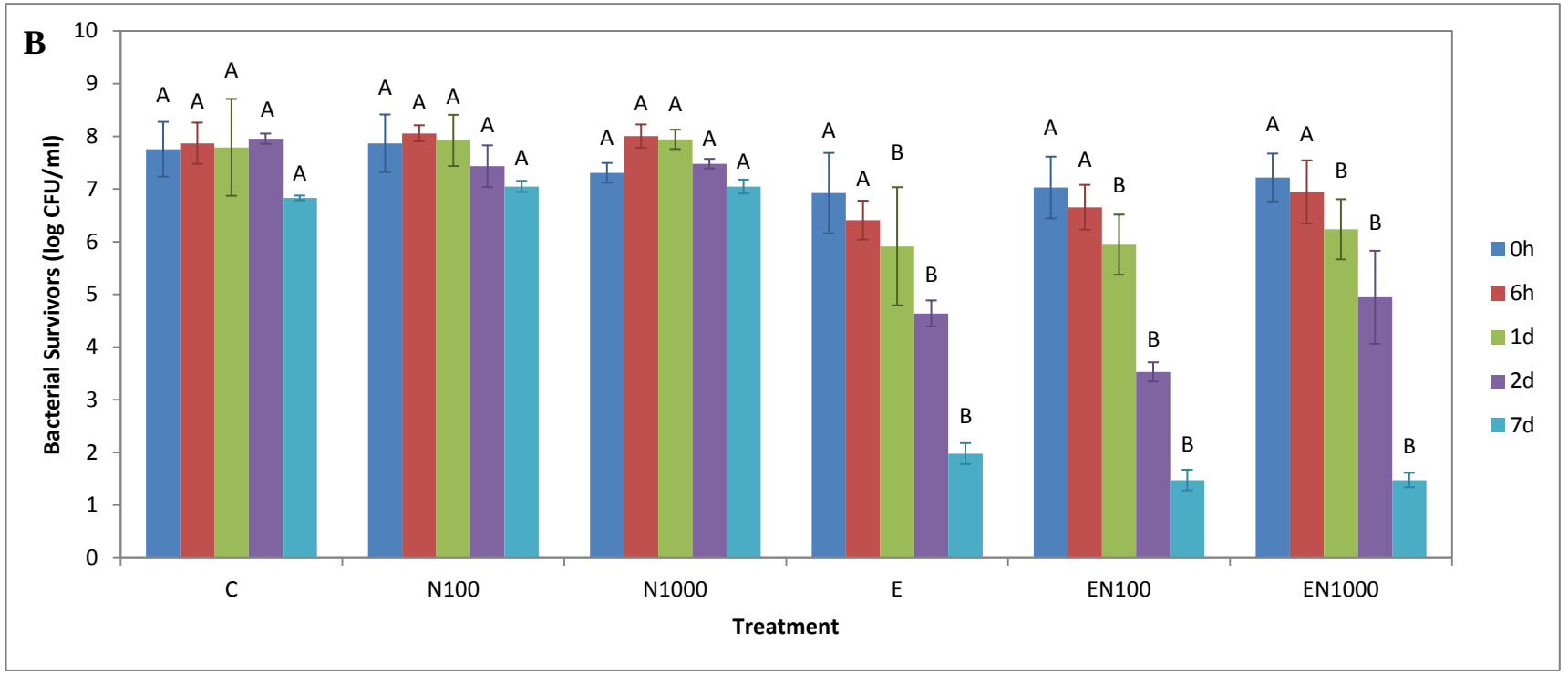


Different letters denote significant differences in bacterial numbers ($P<0.05$) within the same incubation period using data from 2 replicates.

C = Untreated control; N100 = 100 IU/ml Nisin; N1000 = 1000 IU/ml Nisin; E = 50 mM EDTA; EN100 = 50 mM EDTA + 100 IU/ml Nisin; EN1000 = 50 mM EDTA + 1000 IU/ml Nisin

Figure 5.3. *S.* Enteritidis survivors after nisin, EDTA, and nisin-EDTA treatment:

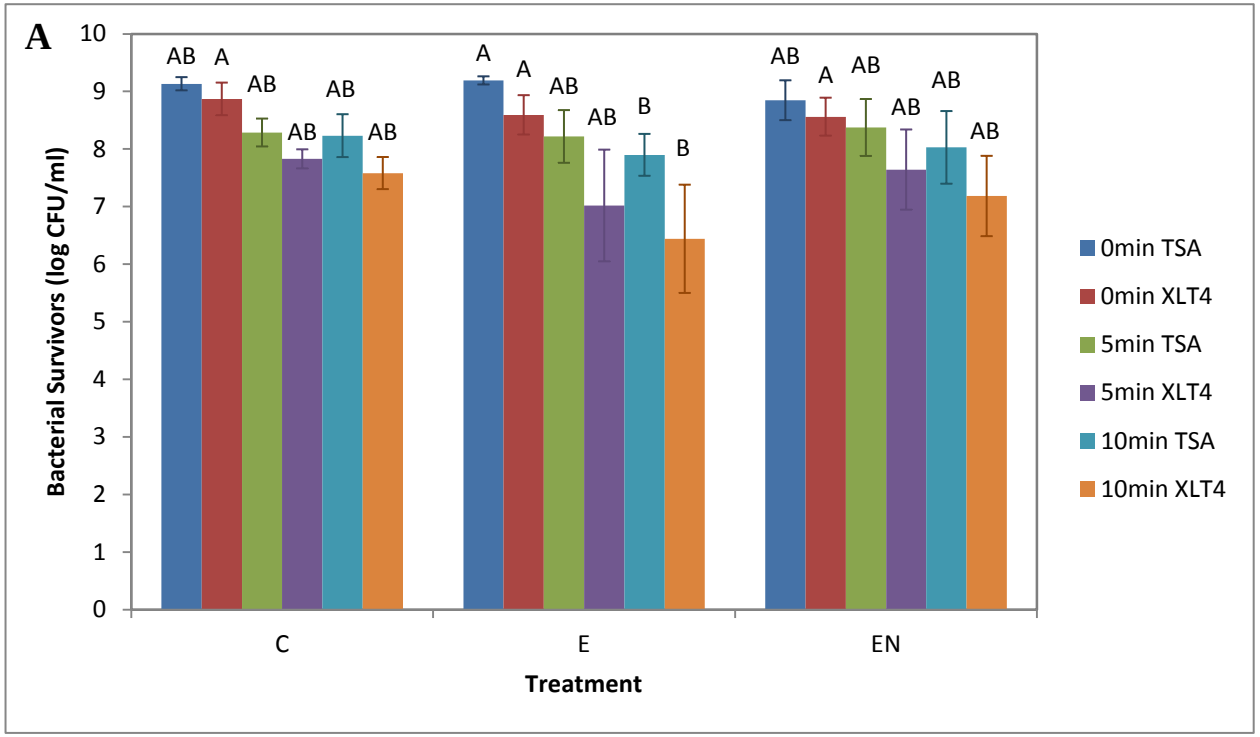
(A) when enumerated on TSA.



Different letters denote significant differences in bacterial numbers ($P<0.05$) within the same incubation period using data from 2 replicates.

C = Untreated control; N100 = 100 IU/ml Nisin; N1000 = 1000 IU/ml Nisin; E = 50 mM EDTA; EN100 = 50 mM EDTA + 100 IU/ml Nisin; EN1000 = 50 mM EDTA + 1000 IU/ml Nisin

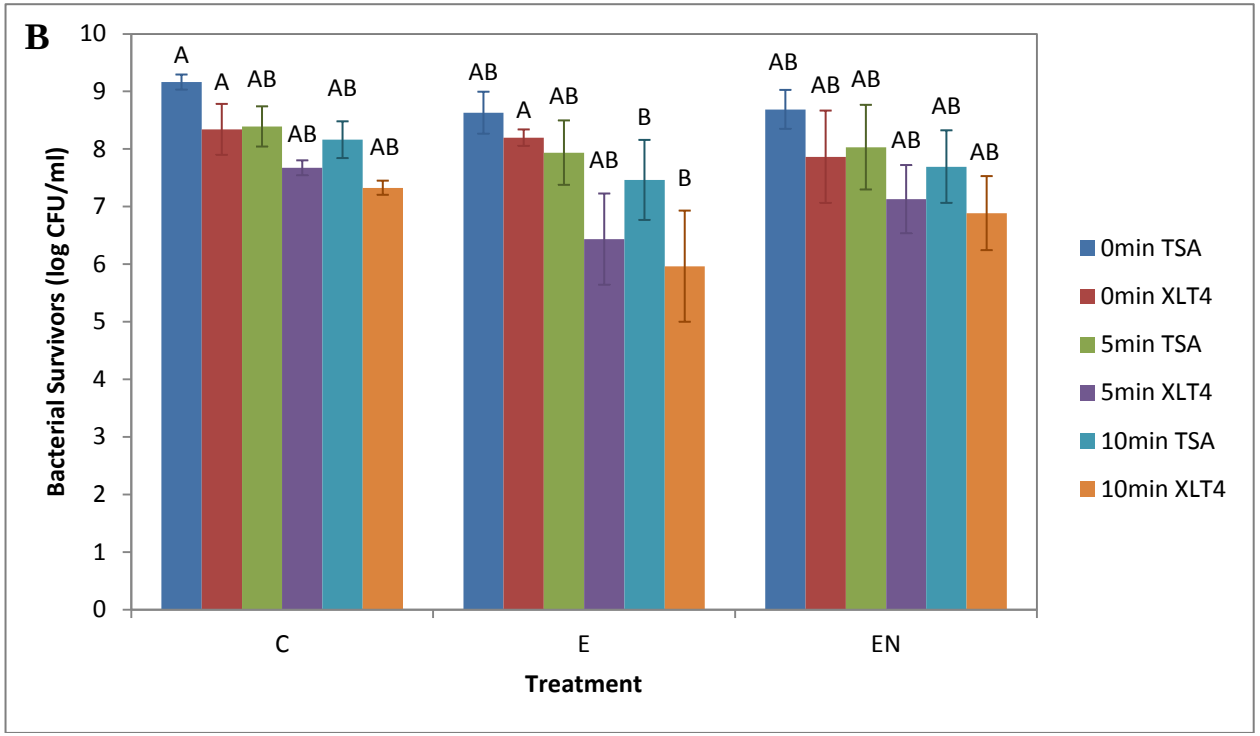
Figure 5.3. *S.* Enteritidis survivors after nisin, EDTA, and nisin-EDTA treatment (Continued):
(B) when enumerated on XLT4 agar.



Different letters denote significant differences in bacterial numbers ($P<0.05$) within the same HIU treatment time and on the same culture media using data from 3 replicates.

C = Untreated control; E = 50 mM EDTA; EN = 50 mM EDTA + 100 IU/ml Nisin

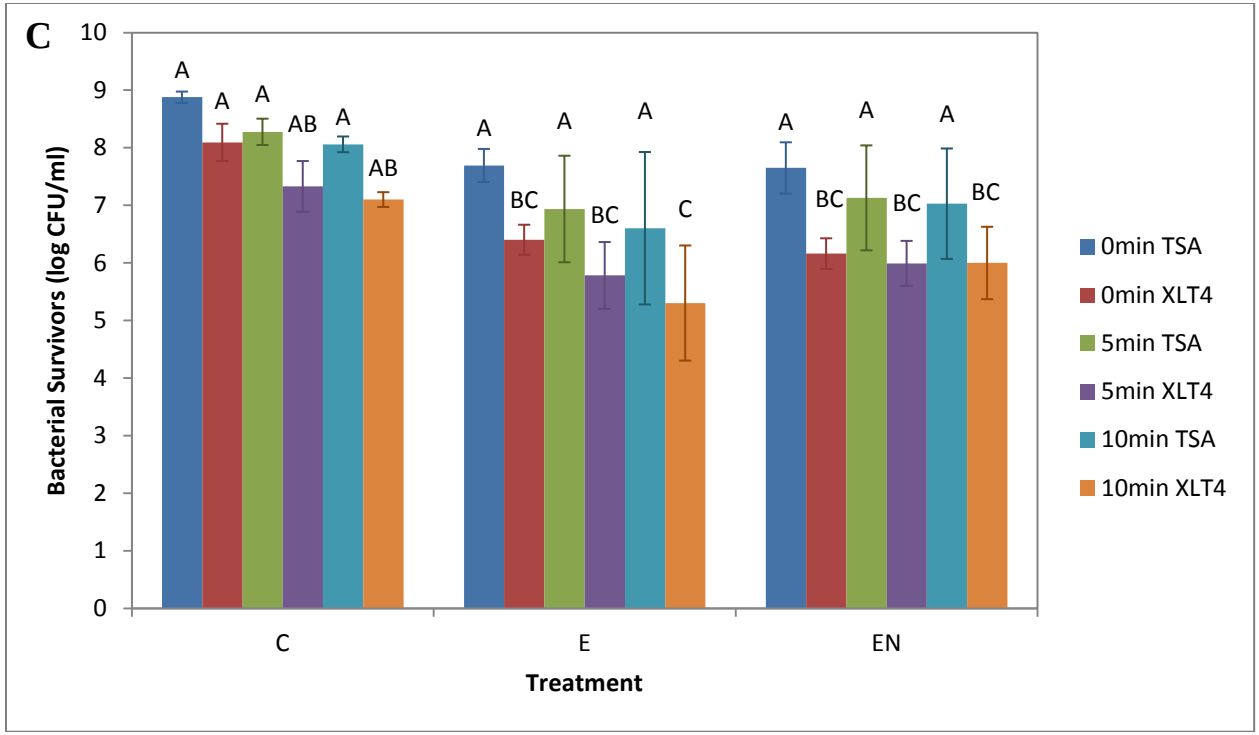
Figure 5.4. *S.* Enteritidis survivors after HIU treatment in combination with nisin, EDTA, and nisin-EDTA:
(A) on Day 0.



Different letters denote significant differences in bacterial numbers ($P<0.05$) within the same HIU treatment time and on the same culture media using data from 3 replicates.

C = Untreated control; E = 50 mM EDTA; EN = 50 mM EDTA + 100 IU/ml Nisin

Figure 5.4. *S.* Enteritidis survivors after HIU treatment in combination with nisin, EDTA, and nisin-EDTA (Continued): (B) on Day 1.



Different letters denote significant differences in bacterial numbers ($P<0.05$) within the same HIU treatment time and on the same culture media using data from 3 replicates.

C = Untreated control; E = 50 mM EDTA; EN = 50 mM EDTA + 100 IU/ml Nisin

Figure 5.4. *S.* Enteritidis survivors after HIU treatment in combination with nisin, EDTA, and nisin-EDTA (Continued): (C) on Day 7.

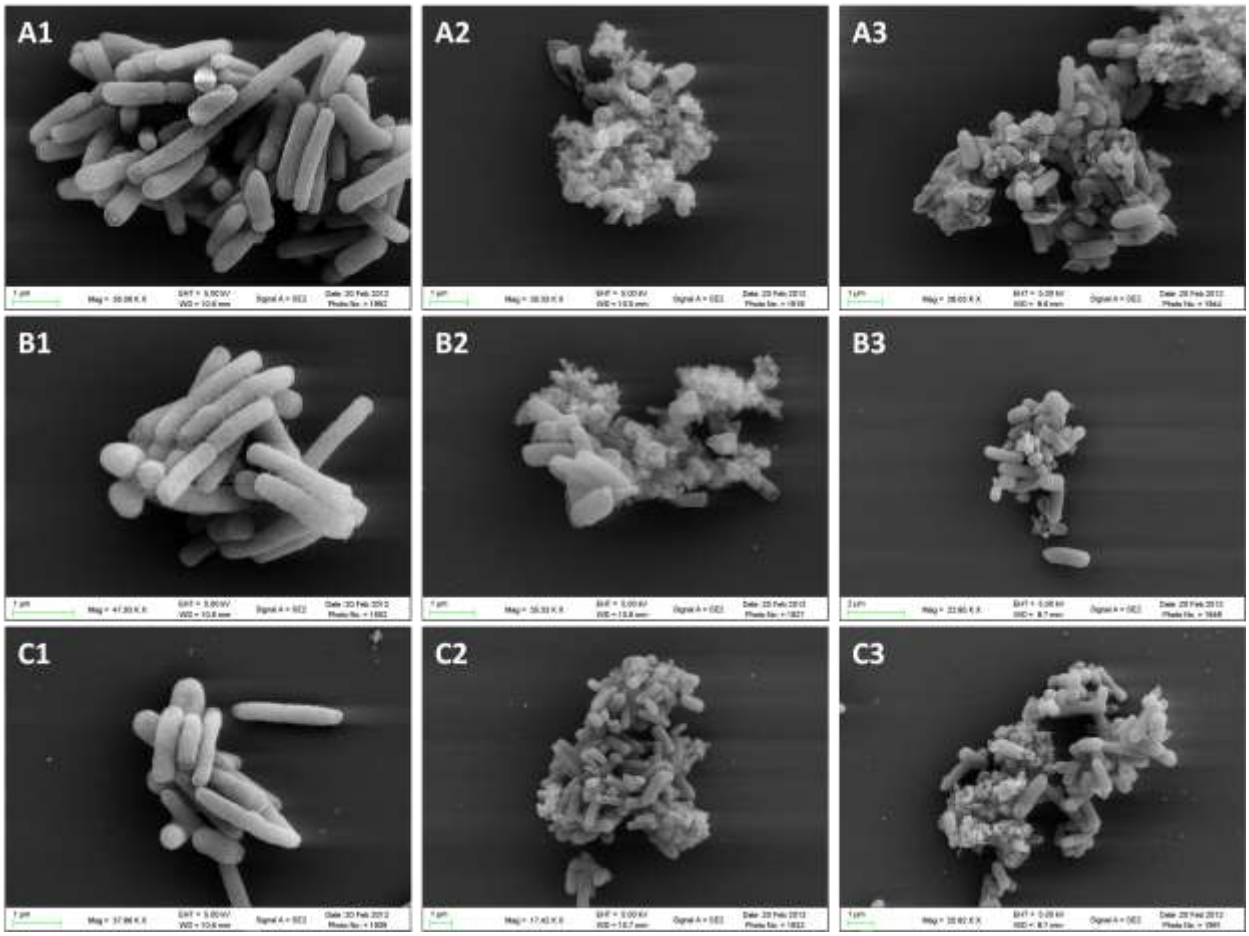


Figure 5.5. SEM micrograph of HIU-treated *S.* Enteritidis with and without nisin, EDTA, and nisin-EDTA treatment: (A1) untreated control, (A2) 5-min HIU treatment, (A3) 10-min HIU treatment, (B1) 50 mM EDTA treatment, (B2) 50 mM EDTA and 5-min HIU treatment, (B3) 50 mM EDTA and 10-min HIU treatment, (C1) 100 IU/ml nisin treatment, (C2) 100 IU/ml nisin and 5-min HIU treatment, and (C3) 100 IU/ml nisin and 10-min HIU treatment.

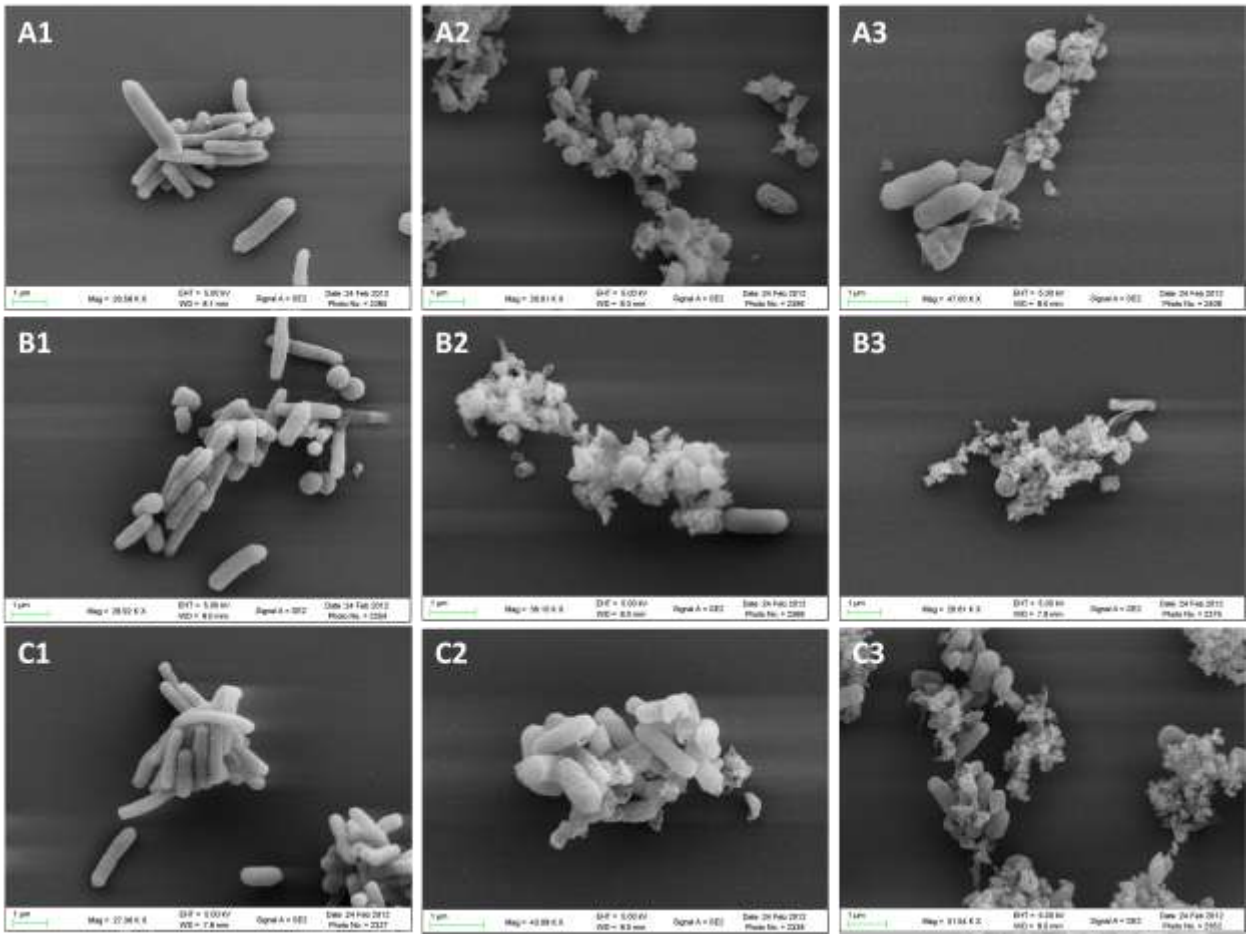
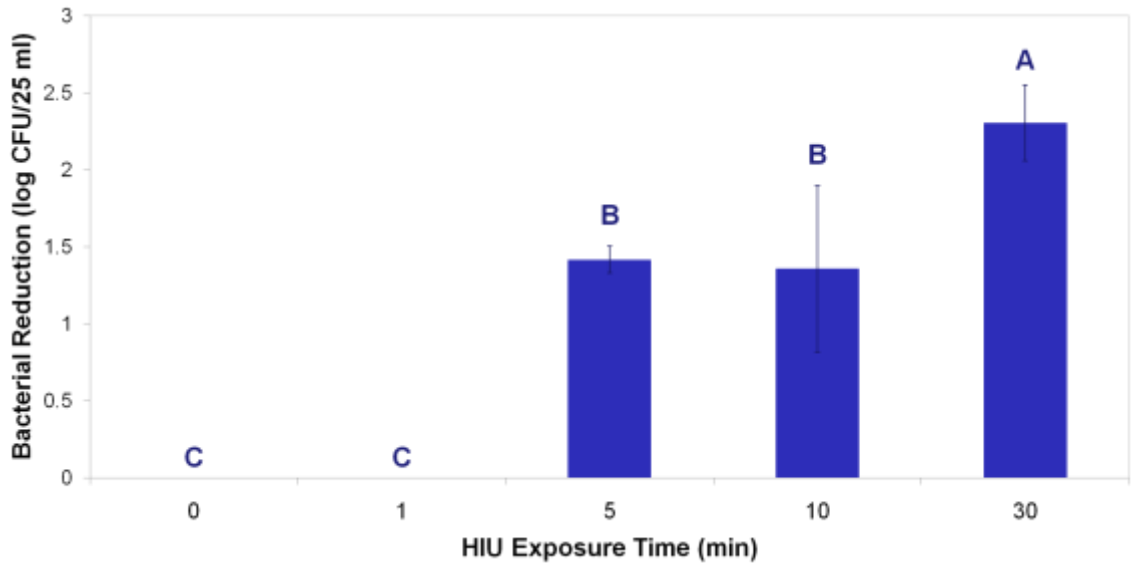


Figure 5.6. SEM micrograph of HIU-treated *S.* Enteritidis with and without nisin, EDTA, and nisin-EDTA treatment after 4°C storage for 7 days: (A1) untreated control, (A2) 5-min HIU treatment, (A3) 10-min HIU treatment, (B1) 50 mM EDTA treatment, (B2) 50 mM EDTA and 5-min HIU treatment, (B3) 50 mM EDTA and 10-min HIU treatment, (C1) 100 IU/ml nisin treatment, (C2) 100 IU/ml nisin and 5-min HIU treatment, and (C3) 100 IU/ml nisin and 10-min HIU treatment.



Different letters denote significant differences in reduction ($P<0.05$) using data from 3 replicates.

Figure 5.7. Reduction of *S.* Enteritidis in artificially contaminated LWE after HIU treatment on XLT4 agar.

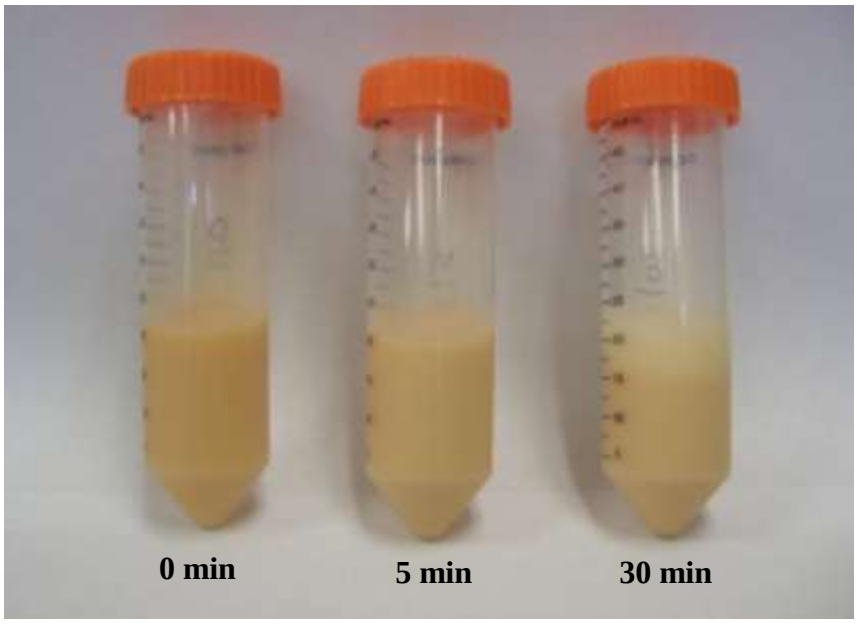


Figure 5.8. Visual comparison of HIU-treated and untreated LWE.

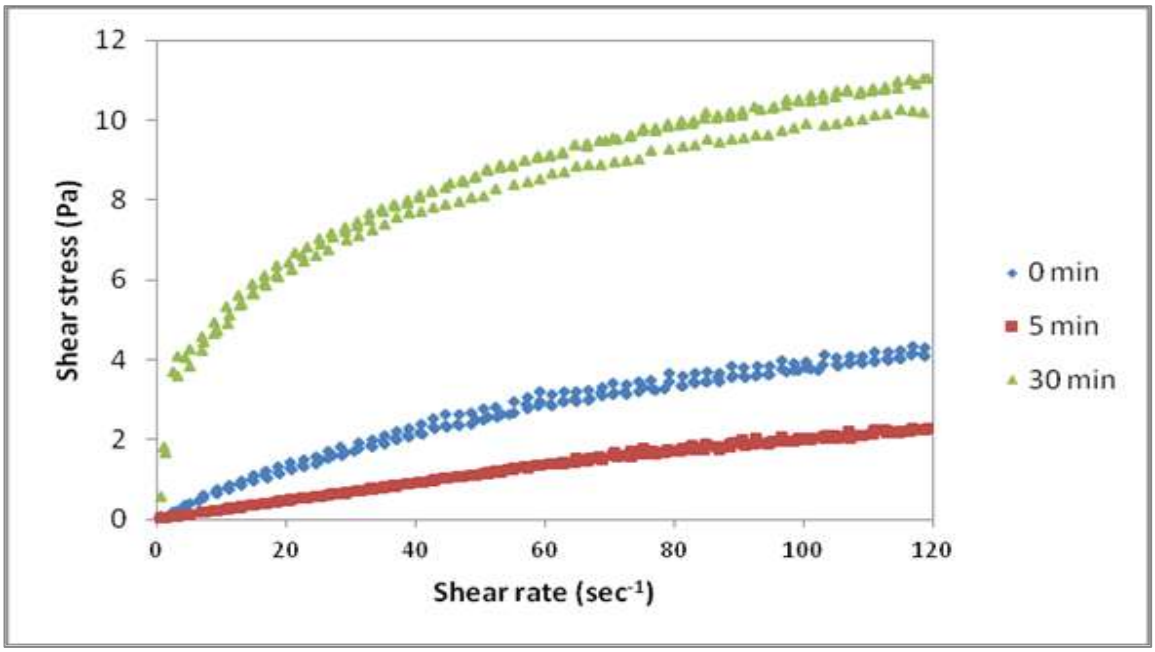


Figure 5.9. Shear stress of HIU-treated and non-treated LWE with increased shear rate.

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